DESIGN AND DEVELOPMENT OF NOVEL DNA TOPOISOMERASE INHIBITORS

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Abstract of Thesis [Parts A and B]

DNA topoisomerase (topo) inhibitors are amongst the most widely used and effective anticancer drugs that target either type I or type II enzymes, however, their clinical application is severely restricted by dose-limiting side effects and the development of drug resistance, commonly the result of diminished expression of the target enzyme and evolution of multidrug resistance (MDR) phenomena.

The motivation for the present study was the hypothesis that **dual** inhibitors that target type I and type II proteins may offer the prospect of circumventing acquired altered-topoisomerase resistance associated with downregulation of a single protein, with consequential improvements in therapeutic index.

A fully characterised [principally by nmr and mass spectrometry], extensive library of >100 novel, spacer-linked anthraquinone-amino acid conjugates [code-named NU:UB] was synthesised as putative dual inhibitors of topo I and II. Key conjugates, exemplified by the proline conjugate, NU:UB 31 (**208**), were shown to co-target topo I and the individual (α and β) isoforms of human topo II *in vitro* in enzyme inhibition assays using gel electrophoretic methods; furthermore, their pattern of cell-kill correlated with topo levels in a panel of animal and human cancer cell lines. Lead compounds were identified and subsequently shown to have notable antitumour activity in MAC15A experimental colon cancer which is refractory to standard agents. This research programme has made a significant contribution to an understanding of the structural requirements for dual enzyme inhibition and has provided new potential anticancer drugs that have progressed to pre-clinical evaluation. **[PART A]**

In another aspect, the chemistry of spacer-linked anthraquinone-amino acid conjugates was extended to the synthesis and characterisation of oligopeptide derivatives that were designed as matrix metalloproteinase (MMP)-activated prodrugs, in a new approach to tumour-selective drug targeting. A prototype oligopeptide prodrug PL1 [(a D-ala-ala-ala-leu-gly-leu hexapapetide derivative (**293**)] of the *in vivo*-active dual topo inhibitor NU:UB 31, containing MMP-9 sensitive cleavage 'hotspots' in the peptide motif, underwent selective initial cleavage by human recombinant enzyme and by homogenates of a highly MMP-9 expressing HT1080 human fibrosarcoma. Preliminary data suggests that it may be feasible to selectively deliver potent cytotoxic agents to the site of a tumour by exploiting the proteolytic capacity of over-expressed MMPs in the tumour environment. **[PART B]**

CONTENTS

CHAPTER FOUR – PART A – NOVEL SPACER-LINKED ANTHRAQUINONE-AMINO ACID CONJUGATES: RESULTS AND DISCUSSION

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Preface

This thesis presents and discusses data from a research programme that was based in synthetic organic chemistry. The chemical synthesis, compound characterisation and in part, *in vitro* cytotoxicity determinations, were carried out by the author, either in-house or in the collaborating laboratory (Cancer Research Unit, University of Bradford). The thesis is divided into two related parts, **PART A** and **PART B**.

PART A, the major component, is concerned with the development of novel spacer-linked anthraquinone-amino acid/ peptide conjugates as cytotoxic agents and putative dual inhibitors of DNA topoisomerases I and II.

PART B extended the chemistry in **PART A** to include anthraquinone-based oligopeptide prodrugs containing amino acid sequences designed to be cleaved by overexpressed matrix metalloproteinases in the tumour environment.

Chapter one sets out the hypothesis and aims for **PART A** of this research programme and surveys current cancer therapy, the structure and function of topoisomerases and their inhibitors in current clinical use. Whilst inhibitors of either type I or type II enzymes have been reviewed in the literature, the focus of chapter two brings together the wide structural diversity of a growing number of compounds, so called dual topoisomerase inhibitors, that co-target I and II proteins.

Anthraquinones occupy a central position in cancer chemotherapy and topo inhibition is recognised as the principal mechanism by which clinically useful drugs exert their cytotoxic action; severe dose-limiting side effects have stimulated the synthesis of numerous derivatives over the past three decades. Chapter three critically reviews the literature confined to the chemistry of anthraquinone-amino acid conjugates in the context of this research project.

Chapter four discusses the design rationale and the detailed synthesis and spectroscopic characterisation of a library [code-named NU:UB] of novel spacer-linked anthraquinone-amino acid conjugates as candidate topoisomerase inhibitors. For selected key conjugates their *in vitro* and *in vivo* chemosensitivity and topoisomerase inhibitory properties are discussed.

Chapter five sets out the hypothesis and aims for **PART B** of this research programme and analyses the rationale for matrix metalloproteinases (MMPs) as targets in current cancer therapy. Chapter six discusses the design, synthesis and properties of novel prototype anthraquinone-oligopeptide MMP-substrates for tumour activated prodrug therapy. Chapter seven presents summary conclusions for **PARTS A** and **B** of this research.

Chapter eight contains a complete catalogue of novel compound structures (and their descriptors) prepared during this programme of work.

Chapter nine details the experimental procedures used for the synthesis and characterisation of the compound library and cytotoxicity determinations.

Additional experimental protocols used in the provision of biological data in support of this work and selected NCI generated compound data are given in the appendices.

Results from this research programme have been reported, in part, in the following publications:

Novel spacer-linked anthraquinone peptide conjugates: design synthesis and evaluation of *in vitro* cytotoxicity (in experimental colon cancer), and topoisomerase inhibition. Mincher DJ, Bibby MC, Double JA, King H, Lowe G, Philip K, **Turnbull A.** Annal. Oncology, (1996), 7, 109.

Synthesis and development of spacer-linked anthraquinone peptide topoisomerase inhibitors which circumvent drug resistance, Mincher DJ, Bibby MC, Double JA, Gilmour PS, Lowe G, **Turnbull A**, Annal. Oncology, (1998), 9, 28.

Biochemistry of topoisomerase II-alpha and II-beta inhibition by novel anthraquinone-peptide conjugates: Design of isoform-specific agents. Gilmour PS, Austin CA, Lowe G, **Turnbull A**, Mincher DJ, Annal. Oncol., (1998), 9(Suppl. 2), 236.

Design and development of a new class of topoisomerase inhibitor: Preliminary in vivo evaluation in experimental colon cancer. Mincher DJ, **Turnbull A**, Bibby MC, Double JA, Gilmour PS, Lowe G, Br. J. Cancer, (1999), 80(Suppl. 2), P88.

In vivo anti-tumour activity and preclinical development of spacer-linked anthraquinonyl-amino acid topoisomerase inhibitors. Mincher DJ, **Turnbull A**, Bibby MC, Double JA, Gilmour PS, Lowe G, Kay G, Hickson ID, Br. J. Cancer, (1999), 80(Suppl. 2), P87.

International Patent WO9965886 [corresponding to PCT/GB/01901, CA2334797 and EP1087935] Anthracene derivatives as anticancer agents, *Inventors*: Mincher DJ, **Turnbull A**. Publication Date: 23-12-1999.

Novel dual topoisomerase (I and II) inhibitors displaying high selectivity in colon carcinoma. Mincher DJ, **Turnbull A**, Pettersson S, Bibby MC, Double JA, Br. J. Cancer, (2000), 83(Suppl. 1), P162.

Design, synthesis and development of novel inhibitors of human DNA-topoisomerase I. Mincher DJ, Kay G, McDonald JEL, **Turnbull A**, Bibby MC, Double JA, Br. J. Cancer, (2000), 83(Suppl. 1), P161.

In vivo activity of novel spacer-linked anthraquinone-peptide conjugates. Jackson S, Double JA, Mincher DJ, **Turnbull A**, Bibby MC, Clinical Cancer Research, (2000), 6(Suppl.), 250.

NU:UB 31: A novel in vivo-active dual topoisomerase I and II inhibitor with high selectivity in colon carcinoma. Mincher DJ, **Turnbull A**, Pettersson S, Bibby MC, Double JA, Clinical Cancer Research, (2000), 6(Suppl.), 249.

Design of novel inhibitors of human DNA topoisomerase I. Mincher DJ, Kay G, McDonald JEL, **Turnbull A**, Bibby MC, Double JA, Clinical Cancer Research, (2000), 6(Suppl.),136.

Design of new topoisomerase I inhibitors: Synthesis and in vitro activity. Mincher DJ, Kay G, Pettersson S, **Turnbull A**, Bibby MC, B. J. Cancer, (2001), 85(Suppl. 1), 92.

International Patent WO0144190 [corresponding to PCT/GB00/048, CA2395170 (anti-cancer agents III), EP1244624]. Anthracene derivatives as anti-cancer agents. *Inventors*: Mincher DJ, **Turnbull A,** Kay GG. Publication Date: 21-06-2001.

In vitro and in vivo-active anthraquinone peptide conjugates: A putative

pharmacophore for colon specificity. Pettersson S, **Turnbull A**, Kay G, Bibby MC, Double JA, Mincher DJ, Br. J. Cancer, (2001), 85(Suppl. 1), 92.

NU:UB 199, a new colon selective agent that targets topoisomerase I and II. Mincher DJ, **Turnbull A**, Kay G, Young L, Pettersson S, Bibby MC, Double JA, Clinical Cancer Research, (2001), 7(11)(Suppl.), 452.

Mincher DJ, **Turnbull A**, Kay G, Young L, Pettersson S, Bibby MC, Double JA. NU:UB 199, a new dual inhibitor of topoisomerase I and II with selectivity for colon carcinoma, Proceedings of the American Association for Cancer Research, (2002), 43, 1157.

In vivo antitumour activity and pharmacokinetics of NU:UB 31 a anthraquinone-peptide conjugate. Jackson S, Double JA, Loadman PM, Mincher DJ, **Turnbull A**, Bibby MC, Br. J. Cancer, (2002), 86(Suppl. 1), P259.

NU:UB 199: A new colon selective agent that targets topoisomerase I and the beta-isoform of topoisomerase II. Young L, Mincher DJ, **Turnbull A**, Kay G, Pettersson S, Bibby MC, Double JA, Br. J. Cancer, (2002), 86(Suppl. 1), P230.

Design of tumour-activated oligopeptide prodrugs that exploit the proteolytic activity of matrix metalloproteinases. Mincher DJ, Loadman PM, Lyle J, Di Salvo A, **Turnbull A**, Bibby MC, Double JA, European J. Cancer, (2002), 38(Suppl. 7), 402.

International Patent WO02072620 [corresponding to PCT/GB/02/01069, EP1366064]

Tumour targeting prodrugs activated by metallo matrixproteinases. *Inventors* Mincher DJ, **Turnbull A**, Bibby MC, Loadman PM. Publication Date: 19-09-2002.

New leukaemia-selective dual topoisomerase inhibitors. Mincher DJ, **Turnbull A**, Kay G, Young L, Bibby MC, Double JA, Leukemia, (2003), 17(3), O38.

Design of tumour-activated prodrugs that harness the 'dark side' of MMP-9. Young L, Di Salvo A, **Turnbull A**, Lyle J, Bibby MC, Double JA, Kay G, Loadman PM, Mincher DJ, Br. J. Cancer, (2003), 88(Suppl. 1), S27.
Winner of the BACR best scientific poster prize at the British Cancer Research Meeting (July) 2003, Bournemouth, UK.

Design of tumour-activated prodrugs that exploit the dark side of matrix metalloproteinases. Mincher DJ, **Turnbull A**, Kay G, Di Salvo A, Young L, Bratkova D, Bibby MC, Double JA, Lyle J, Loadman PM. Proceedings of the American Association for Cancer Research, (2003), 44(2nd ed.), 4627.

US Patent US2003130272 [corresponding to PCT/GB00/04829, WO0144190]. Anthracene derivatives as anti-cancer agents. *Inventors*: Mincher DJ, **Turnbull A,** Kay GG. Publication Date: 10-07-2003.

US Patent US2003203975 [corresponding to PCT/GB99/01901, WO99/65866] Anti-cancer Agents. *Inventors*: Mincher DJ, **Turnbull A**. Publication Date 30-10-2003.

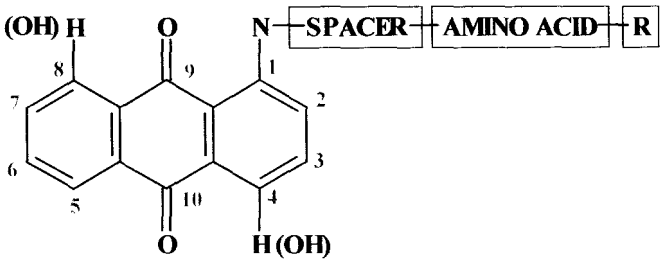
Design, pharmacokinetics and in vivo metabolism of MMP activated prodrugs. Lyle J,

Young L, Di Salvo A, Cooper P, **Turnbull A**, Bibby MC, Mincher DJ, Loadman PM. Clinical Cancer Research, (2003), 9(16)(Suppl.), B180.

NU:UB 73 Synthesis and preliminary in vivo activity of a novel topoisomerase I and IIβ-specific agent. **Turnbull A**, Mincher DJ, Bibby MC, Double JA, Cooper PA, Jackson SM, Gilmour PS, Bioorg.Med.Chem. (in press).

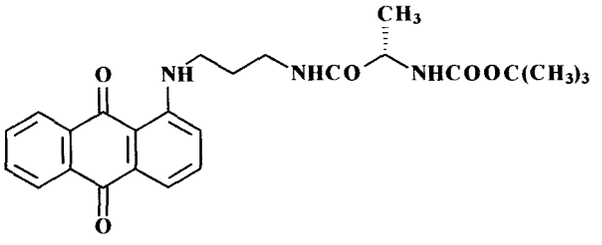
Nomenclature

The structure below represents the compounds prepared in this study in their simplest format.



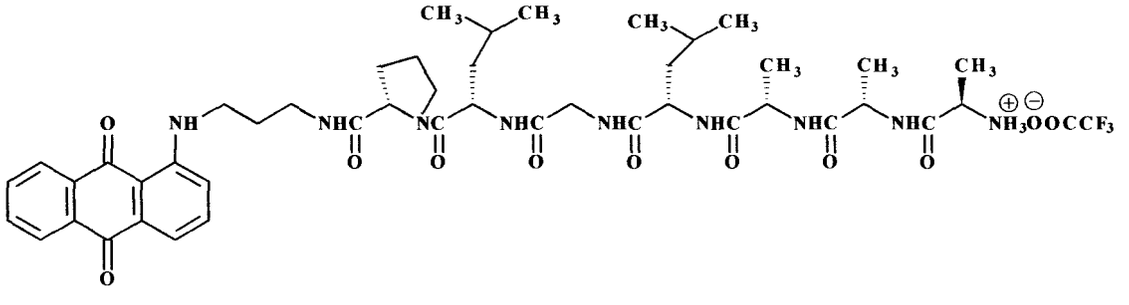
The terms anthraquinone, anthra-9,10-quinone, anthracenedione and anthracene-9,10-dione have been used interchangeably, since they are all in common usage. The compounds synthesised in this study are regarded fundamentally as anthraquinones i.e. anthraquinones substituted with amino (RNH- or R_1R_2N-) side-chains in the 1-position. As such, anthraquinone amino acid/ peptide conjugates derived from simple aminoalkylamino or hydroxyalkylamino spacer groups have been named as substituted anthraquinones, according to the numbering system shown in the figure [This also facilitates comparison with (aminoalkylamino)anthraquinones in clinical use, e.g. mitoxantrone (7)]. The IUPAC system has been used to name conjugates containing more complex (e.g. cyclic or branched) spacer groups when it was more convenient to do so. Aminoalkylamino groups have been abbreviated to, for example, 'propyl' and 'butyl', representing the species -NH-$(CH_2)_3$-NH- and -NH-$(CH_2)_4$-NH-, respectively when set in the context of spacer linked conjugates and are convenient descriptors for the simpler spacer groups in these molecules. Similarly, hydroxyalkylamino spacer groups have been abbreviated to, for example, 'propoxy' which corresponds to -NH-$(CH_2)_3$-O- or 'butoxy' -NH-$(CH_2)_4$-O- etc. It is anticipated that the meaning of these descriptors facilitates communication and will be obvious from the context.

Example A: 1-[3-(N-Tertiarybutoxycarbonyl-L-alanylamino)propylamino]anthraquinone



More generally, the example may be described as 'an N-protected, propyl-linked L-alanine conjugate' wherein *propyl* is a shorthand description for the spacer moiety.

Example B: 1-[3-(D-alanyl-L-Alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)-propylamino]anthraquinone trifluoroacetate

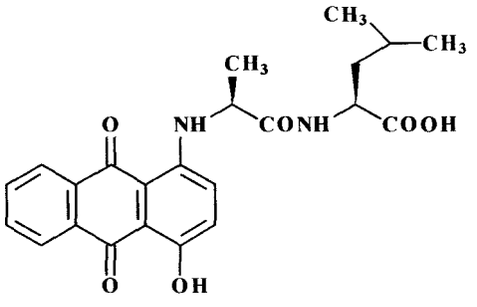


In example B, descriptors for the aminoacyl residues derived from the component amino acids are conveniently used to describe B as: a D-alanyl-L-Alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolyl heptapeptide conjugate. [The peptide is thus described conventionally, with the N-terminus 'to the left']

Note: During discussion of anthraquinone-oligopeptide conjugates of this type, particularly metabolism (degradation) studies of Example B, it was convenient to describe molecules as having residual amino acid fragments attached to the anthraquinone-spacer [AQ-SP] compounds, as in a (truncated) e.g. AQ-SP-pro-leu-gly tripeptide conjugate ("the pro-leu-gly conjugate"), wherein it was understood that

neither the amino acid sequence was altered nor the amino terminus reversed [i.e. that gly was the amino terminus and that the correct interpretation would be unambiguous from the context in which these passages occurred].

Example C:



When the anthraquinone is linked to the N-terminus of an amino acid or peptide (here, a dipeptide), as in Example C, it is convenient, and in keeping with literature precedent, to regard the structure fundamentally as a peptide that bears the anthraquinone substituent, thus affording the systematic name N-(4′-hydroxy-9′,10′-dihydro-C-9′,10′-dioxo-1′-anthryl)alanylleucine for C. This leads to the use of the general descriptive terms, anthracenyl- (or anthraquinonyl) amino acids/peptides for compounds of this type.

When naming hydroxyanthraquinones, the terms quinizarin and 1,4-dihydroxyanthraquinone have been used interchangeably as have leucoquinizarin and leuco-1,4-dihydroxyanthraquinone, and, leuco-1,4,5-trihydroxyanthraquinone and leuco-5-hydroxyquinizarin also as a result of common use of these terms.

Abbreviations

AML	acute myeloid leukaemia
aq	aqueous
AQ-SP	Anthraquinone-spacer (compound or residue)
Boc (or ^{t}Boc)	tertiarybutoxycarbonyl

^{t}Bu	tertiarybutyl
cdk	cyclin dependent kinase
CI	chemical ionisation
CHO	Chinese hamster ovary (cell line)
CML	chronic myeloid leukaemia
DCC	dicyclocarbodiimide
DCU	dicyclohexylurea
DMAP	4-(N,N-dimethylamino)pyridine
DMF	N,N-dimethylformamide
DMSO	dimethysulphoxide
d	doublet
dd	double doublet
DEPT	distortionless enhancement by polarisation transfer
EGFR	epidermal growth factor receptor
EI	electron impact
eq	molar equivalent(s)
ES(I)/(+) or (-)	electrospray (ionisation)/positive or negative mode
ether	diethyl ether
FAB	fast atom bombardment
Fmoc	fluorenylmethoxycarbonyl
m	multiplet
MAC	murine adenocarcinoma of the colon
MDR	multi-drug resistance
mg	milligram(s)
min	minutes

mmol	millimole(s)
MRP	multi-drug resistance protein 1
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nmr	nuclear magnetic resonance
NSCLC	non-small cell lung cancer
NU:ICRF	Napier University: Imperial Cancer Research Fund (compound codes)
NU:UB	Napier University: University of Bradford (compound codes)
Pfp	pentafluorophenyl
Pgp	p-glycoprotein (also known as p-170)
ppm	parts per million
q	quartet
qn	quintet
s	singlet
SCLC	small cell lung cancer
t	triplet
THF	tetrahydrofuran
t.l.c.	thin layer chromatography
TFA	trifluoroacetic acid
topo	topoisomerase
Trt	trityl
VEGFR	vascular endothelial growth factor receptor
Z	benzyloxycarbonyl

CHAPTER ONE

[PART A]

TOPOISOMERASE INHIBITORS AND CANCER:

DESIGN AND PERSPECTIVES

1.1 Hypothesis [Part A]

Anthraquinone-based compounds have successfully been used in the cancer clinic for several years; doxorubicin and mitoxantrone are notable examples that, after their introduction, were shown to target DNA topoisomerase enzymes as a key component of their cytotoxic action. Clinical application of these agents is, however, restricted due to acute toxicity and side effects associated with non-topoisomerase, secondary mechanisms of action, including free radical formation and lipid peroxidation, and the development of either altered topoisomerase drug resistance or multidrug resistance.

In this research programme, it was hypothesised that lead compounds from a novel series of spacer-linked anthraquinone-amino acid conjugates [code-named NU:UB] would be actively cytotoxic and act as 'clean' dual inhibitors of DNA topoisomerase I and II enzymes, and as a consequence of their chemical design features would not suffer from secondary mechanisms of action. It was proposed that nuclear amination of halo- or hydroxy-anthraquinones with bifunctional amine spacer groups followed by amino acid or peptide conjugation to the free spacer terminus, would provide ease of access to a varied compound library for biological evaluation. It was further proposed that dual enzyme inhibitory properties, in contrast to targeting a single enzyme, had the potential to afford compounds capable of circumventing multidrug resistance phenomena.

1.2 Aims [Part A]

The principal aim of this research programme was to synthesise and characterise several series of spacer-linked anthraquinone-amino acid/peptide conjugates as putative topoisomerase inhibitors. Furthermore, the conjugates were to be evaluated for their chemosensitivity in a panel of human and animal tumour cell lines *in vitro* and (in collaboration) *in vivo*. Conjugates were designed either with nuclear un-substituted or

hydroxy-substituted aminoanthraquinone chromophores, conformationally flexible or constrained spacer groups and were either amide-linked or ester-linked to the amino acid or peptide motif. Attempts would be made to correlate chemical structure with pattern of cell kill and observed inhibitory actions against DNA topoisomerase enzymes, to contribute to the rational design of more selective drugs to target topoisomerase proteins.

1.3 Cancer Therapy

The most frequently used cancer therapies (e.g. doxorubicin, cisplatin, taxol) were discovered in the laboratory over two or even three decades ago (Baguley and Kerr 2002).

These therapies were discovered empirically in tumour cell kill assays without bias towards or pre-existing knowledge of the biochemical mechanism of action. Recently, rational approaches to drug design have been applied to cancer therapies. These approaches focus on identifiable molecular targets that are responsible for cell transformation yet to date have been relatively ineffective at curing most malignancies.

Ideally, a molecular target for cancer therapy should have a unique role in the cause of the cancer's pathogenesis and be regulated differently between tumour and normal cycling tissues. Preferably, the target should be located in a biochemical pathway so that a biochemical antagonist of the target will selectively induce growth arrest or apoptosis of the tumour cells. Unfortunately, at present, the identification of all the molecular targets that give rise to the majority of cancers is incomplete. Identification of such targets is further complicated by the genetic instability of cancer cells. Even though multiple genetic changes contribute to the generation of cancer, several irrelevant

genetic differences are commonly observed that complicate identification of the critical molecular target.

Chemotherapeutic treatment of cancer today involving FDA (Food and Drug Administration, US) approved drugs (http://www.fda.gov/cder/cancer/druglistframe.htm) can be grouped into four different categories based upon either their target or the nature of their composition. These groups include: cytotoxics, biologicals, targeted therapeutics, and hormonal therapeutics.

Cytotoxics were discovered primarily due to their abilities to kill cells. Those compounds with antitumour activity against animal tumour models entered the clinic and those with clinical efficacy were approved, all the while without knowledge of their target of action. The cytotoxic targets were later discovered to include: DNA (intercalating, alkylating and cross-linking agents); DNA synthesis pathway enzymes (antimetabolites); tubulin (antimictrotubule and tubulin polymerizing agents); and topoisomerases (topoisomerases I and II). Biologicals can be subdivided into antibody (receptor blocking, ligand binding, and targeted delivery) and natural biological (cytokines and interferons) subgroups. Molecular targeted therapeutics (Buolamwini 1999) are predominantly signal pathway or ancillary enzymes (ras, raf, mek, src, her, cdk, kit, BCR-Abl tyrosine kinase, VEGFR and EGFR tyrosine kinases, metalloproteinase, proteosome, etc.). However, the FDA has approved only one BCR-Abl tyrosine kinase inhibitor, one EGFR tyrosine kinase inhibitor, and one proteosome inhibitor. Hormonal therapeutic agents include gonadotropin releasing hormone analogues, antiandrogens and antiestrogens. The FDA has approved several of these for commercialization.

Confusion may arise from discussions about molecular targets of cytotoxic drugs and molecular targeted drugs that may be cytotoxic. Topoisomerase (enzyme) targeting

drugs are classified as cytotoxics even though they have specific molecular targets. This is because their cytotoxic properties were generally known prior to the discovery of their molecular targets, whereas molecular targeted drugs were discovered from their actions on their respective molecular targets.

Classical cytotoxic drugs continue to dominate the market with respect to the number of prescriptions written because of their proven efficacy in the clinic but their commercial values are limited because most are off-patent. Gemzar (antimetabolite), Navelbine (antimicrotubule) and Camptosar (topoisomerase I) are recent commercial successes in this category. Hormonal therapy is the most rapidly growing category of chemotherapy because of reduced toxicity risks exhibited by members of this class. However, efficacy is directly dependent upon the hormone receptor expression in the tumour and this hormone dependency can be lost with time. Natural biologicals, which include interferon and interleukin IL2, are effective but unexciting in their clinical responses but receptor blocking antibodies [Herceptin (HER-2/neu antibody), Erbitux], ligand binding antibodies (early clinical trials), and targeted delivery antibodies (Rituxan, Rituximab, SGN-15) are commercial successes or show promising results (Kim 2003). The FDA approval of Rituxan in November 1997, for the treatment of relapsed of refractory low-grade CD20 positive follicular lymphoma, represented the first Mab therapy approved in cancer therapy (McLaughlin *et al* 1998). Herceptin has received FDA approval for use in patients with metastatic breast cancer that demonstrates over-expression of HER-2/neu (Baselga 2001). Targeted therapeutic approaches, on the other hand, have met with disappointments until very recently when Gleevec (STI-571, Imatinib) (a tyrosine kinase inhibitor that inhibits *abl*-specific phosphorylation) (Roskoski 2003) and Iressa (Gefitinib, ZD1839) (targeting EGFR tyrosine kinase) (Khalil *et al* 2003) were approved for CML (Druker *et al* 2001) and NSCLC, respectively.

The focus of large and small pharmaceutical companies has been squarely on hormonal, biological, and novel molecular targets in drug discovery and development of anticancer agents in recent years. The resulting fierce competition in the hormonal arena and the general lack of success in the biological and molecular target areas are factors forcing large pharmaceutical companies to seek in-licensing opportunities for drugs that actually work in the clinic. Thus, some re-focusing on cytotoxics and their controlled delivery to tumours is receiving growing attention.

Topoisomerase I (topo I) and topoisomerase II (topo II) are clinically validated targets. The commercially available camptothecin analogues targeting topo I include Camptosar (Irinotecan, CPT-11) and Hycamtin (topotecan). Camptosar is approved for colon carcinoma and Hycamtin is approved for ovarian cancer and SCLC. A number of other camptothecin analogues are in various stages of clinical evaluation (Zunino *et al* 2002). Several topo II targeting drugs were commercially available well before their molecular target was identified. These include etoposide and teniposide, doxorubicin and other anthracyclines, and mitoxantrone. No new class of topoisomerase II targeted agents has been approved or appears in the pipeline; greater interest persists for anti-topoisomerase I agents since clinically approved examples are limited to the camptothecin family and are structurally labile. The clinically active topoisomerase I and II targeting drugs all act by stabilizing a covalent, transient intermediate formed between the genomic DNA of a cancer cell and topoisomerase enzymes.

1.4 DNA Topoisomerases

1.4.1 Function and Mechanism

DNA topoisomerases are essential enzymes found in all living organisms. Topoisomerases control and modify the topological states (such as over- and under-winding, knotting or tangling) of DNA by a complex catalytic process involving strand cleavage, strand passage and finally religation of the DNA strands. Hence, these DNA associated enzymes play a critical role in replication, transcription, recombination and many other cellular processes (Wang 2002). There are two distinct classes of topoisomerase, differentiated by their mechanistic and physical properties (Champoux 2001). Type I cause transient **single-strand** breaks in DNA, allowing controlled rotation about (or passing of a single strand through) the nick before rejoining; a process that does not require an energy cofactor, the energy for this reaction is instead derived from that stored in the supercoiled DNA. The type I enzymes have been further subdivided into type IA and type IB subfamilies based on the type of DNA adduct they form. Type IA topoisomerases (including eukaryotic topoisomerase IIIα and IIIβ) form a transient covalent phosphotyrosine linkage to the 5′ end of DNA whereas type IB topoisomerases (including eukaryotic topoisomerase I and mitochondrial topoisomerase I) attach to the 3′ DNA terminus. Type IA topoisomerases are able to relax only negatively supercoiled DNA, require magnesium and a single-stranded stretch of DNA for function. In contrast, type IB topoisomerases can relax both positively and negatively supercoiled DNA with equal efficiency and do not require a single-stranded region of DNA or metal ion to function (Wang 1996, Champoux 1998).

The type II enzymes modulate topology by passing an intact helix through a transient **double-stranded** break they create in the DNA backbone (Berger 1998). During cleavage, two tyrosines attack opposite stands of the DNA duplex, forming covalent 5′-

phosphotyrosine linkages to DNA, in a four base pair stagger. ATP is required for the catalytic activity of type II topoisomerases.

Topoisomerases I and II have been extensively studied over the past three decades in relation to human cancers and these topoisomerases will be discussed below.

1.4.2 DNA Topoisomerase I

The gene for human topoisomerase I has been mapped to chromosome 20q12-13.2 (Juan *et al* 1988). The topoisomerase I gene product is a ~100kDa monomeric protein that requires phosphorylation for full activity and this protein is most abundant in the nucleus but also found in nucleoplasm (Pommier *et al* 1990, Fleischmann *et al* 1984). Limited proteolysis has shown that the enzyme is composed of four major domains: a highly charged 24kDa NH_2-terminal domain, a positively charged 56kDa core domain, a 7 kDa linker domain, and a 6 kDa COOH-terminal domain which contains the catalytic Tyr-723 (Stewart *et al* 1996). The determination of crystal structures of the core, linker and C-terminal domains of human topoisomerase I in complex with DNA has given greater insights into the catalytic mechanism of action of this enzyme on DNA (Stewart *et al* 1998, Redinbo *et al* 1998). More recently, the x-ray crystal structure of human topoisomerase I covalently bound to duplex DNA and the camptothecin analogue topotecan has been reported (Staker *et al* 2002). This structure can help explain several of the already established structure-activity relationships of the camptothecin family and suggests mutations that are significant in the production of a drug-resitant enzyme. Furthermore, a crystal structure of human topoisomerase I with an oligonucleotide containing Ara-C (1-beta-D-arabinofuranosylcytosine), the potent antineoplastic drug used in the treatment of acute leukaemia, at the +1 position of the non-scissile DNA strand has also been described (Chrencik *et al* 2003). It was speculated that subtle

structural changes, caused by the presence of the Ara-C in the DNA duplex, may contribute to the cytotoxicity of this drug (though not as a topoisomerase poison) by prolonging the lifetime of the covalent human topoisomerase I-DNA complex.

Topoisomerase I is present throughout the cell cycle and its activity varies less than topoisomerase II during the cell cycle (Heck *et al* 1988, Romig and Richter 1990), which makes topoisomerase I an attractive target for drug development.

1.4.3 DNA Topoisomerase II

Relaxation of DNA supercoiling by topoisomerase II is considered to play a major role in DNA replication and transcription. Topoisomerase II also has a critical role in chromosome condensation and separation during mitosis. In fact, it is believed that topoisomerase II partly makes up the chromosome scaffold and the nuclear matrix. The chromosome scaffold is the protein structure that remains after DNA and histones have been removed from chromosomes in mitotic cells, and the nuclear matrix is a similar preparation from cells in interphase (Adolphs *et al* 1977). Two isoforms of human topoisomerase II have been identified and these are referred to as topoisomerase IIα and topoisomerase IIβ, 170 and 180kDa proteins respectively. The topoisomerase IIα gene is located on chromosome 17q21-22 and the topoisomerase IIβ gene on the 3p24 chromosome (Tan *et al* 1992). These enzymes differ in many aspects including cell cycle regulation and nuclear isolation (Austin *et al* 1995). Topoisomerase IIα is localised in the proliferating compartments of all tissues and is detectable in both the cell nucleus and cytoplasm, whereas topoisomerase IIβ is expressed ubiquitously and is localised in the nucleoli and nucleoplasm (Turley *et al* 1997). During mitosis, topoisomerase IIα appears completely bound to the mitotic chromatin, while topoisomerase IIβ diffuses into the cytosol (Meyer *et al* 1997)

Topoisomerase IIβ levels remain relatively constant over the cell cycle and topoisomerase IIα levels are closely linked to the proliferation state of the cell, increasing 2-3 fold during G_2/M phases and in rapidly proliferating cells (Turley *et al* 1997).

1.4.4 Topoisomerases as Targets for Cancer Chemotherapy

Many of the most effective anticancer agents in current clinical use are known to exert their cytotoxic action, at least in part, by targeting DNA topoisomerase enzymes. Since tumour cells in many cases are highly proliferative cells and topoisomerases are involved in replication and proliferation processes, the levels of these enzymes are often increased in growing cancer cells compared to normal cells, providing potential for tumour selectivity.

Topoisomerase levels have been shown to be elevated in many haematological malignancies and solid tumours compared to corresponding normal cells. For example, topo I levels were found to be 14-16 fold higher in cancerous colon tissue than in normal colon (Giovanella *et al* 1989). Both topoisomerase II isoforms can be overexpressed in human tumours. Overexpression of topoisomerase II has been noted in human cervix, lung and colon cancers as well as in a study of ovarian tumours where a 10-fold increase in topoisomerase I and topoisomerase II compared to normal tissue was reported (McLeod *et al* 1994, Van der Zee *et al* 1991). Although elevated topoisomerase levels in tumours might contribute, in part, to this selectivity, it is likely that other factors such as cell cycle checkpoints, deficient DNA repair pathways and apoptotic response represent important factors in selectivity of drugs against cancer cells (Pommier 1999).

The interaction of topoisomerases with DNA can be broken down into several key steps:

1. Enzyme-DNA-binding.
2. Cleavage of DNA by transesterification from the phosphodiester DNA backbone to an enzyme catalytic tyrosine resulting in a covalent bond between the protein and one terminus of the DNA nick (3′ terminus in the case of topo I and 5′ terminus of a double-strand break in the case of topo II).
3. DNA strand passage.
4. Resealing of the DNA break concerted with the release of the topoisomerase enzyme.
5. ATP hydrolysis (topo II only).

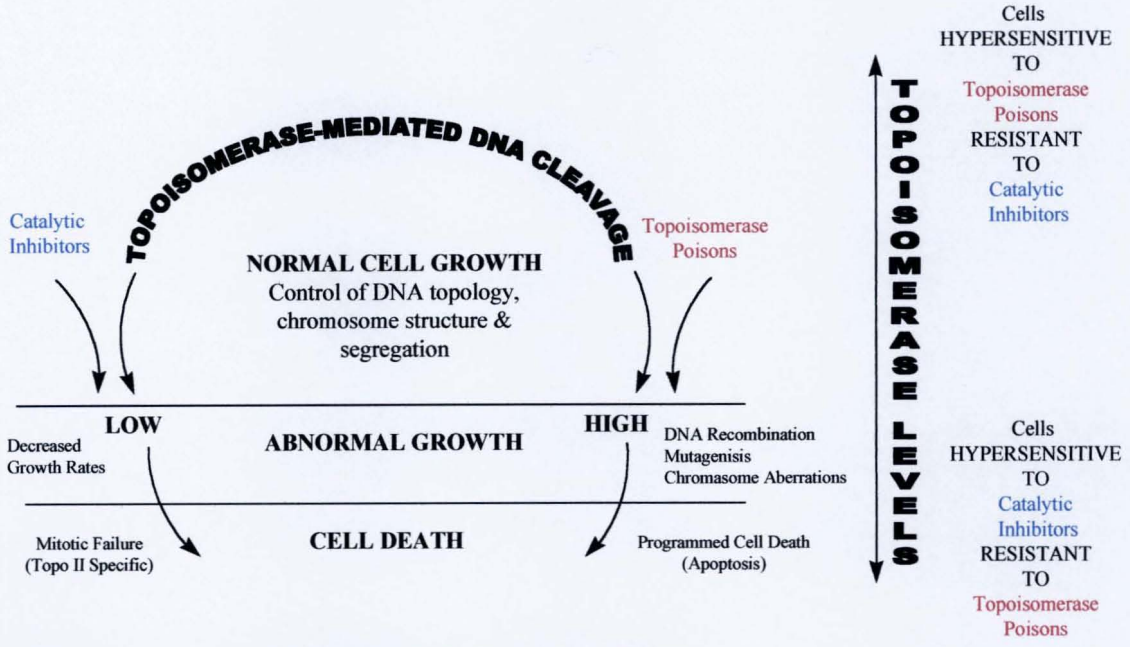
Topoisomerase targeting agents may act at any of the above steps resulting in inhibition or poisoning of the enzyme and subsequent cell death.

The mechanism of action of topoisomerase inhibiting agents can be divided into 2 categories: **catalytic inhibitors** (or suppressors) and **topoisomerase poisons** (although the name topoisomerase inhibitors often refers to both types). Topoisomerase poisons act by stabilisation of a drug-DNA-enzyme ternary complex, preventing religation of the cleaved strand(s). An increase in the concentration of these transient covalent enzyme-DNA complexes to levels which cannot be tolerated in the cell converts topoisomerases into physiological toxins (Froelich-Ammon and Osheroff 1995). Following traverse of replication complexes, transient topoisomerase mediated breaks become permanent double- stranded breaks, triggering events that ultimately culminate in cell death (Fortune and Osheroff 2000). The triggering of these events is sometimes referred to as "programmed cell death syndrome" or apoptosis.

Catalytic inhibitors are agents that act on any other steps in the catalytic cycle, for example, by binding directly to topoisomerases, or binding to DNA and changing its

structure so that it can no longer be recognised by the topoisomerases, or by trapping topoisomerase II in a closed clamp form, preventing enzyme turnover (Roca *et al* 1994).

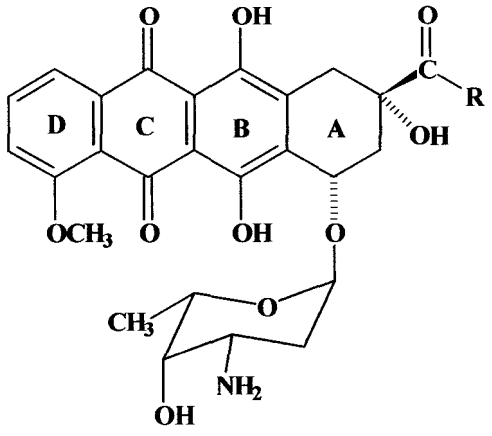
Figure 1: The Role of Topoisomerases in the Life and Death of Cells



[Adapted from Wilson Byl et al, Biochemistry, (1999), 38, 15573-79.]

Figure 1 outlines how topoisomerase poisons can be distinguished from inhibitors by their cytotoxic criteria. Increased levels of topoisomerases render cells hypersensitive to enzyme poisons but resistant to inhibitors. Conversely, decreased enzyme levels render cells resistant to poisons but hypersensitive to inhibitors. Maximal toxicity of topoisomerase poisons occurs during S phase whereas it has been proposed that trapping of the DNA topo II in the closed-clamp form, the mechanism of action of the bis(2,6-dioxopiperazine) class of catalytic inhibitors (Andoh 1998), inhibits cell-cycle progression at G_2-M. For the pure inhibitors of topoisomerase II, mitosis may be the cellular process whereby the damage is instigated; proceeding through this stage of the cell cycle when topoisomerase II is inhibited can result in aneuploidy and chromosomal breakage (Wang 1994a).

1.4.5 Topoisomerase I and II Inhibitors in Current Clinical Use



(1) R = CH_2OH = Doxorubicin

(2) R = CH_3 = Daunorubicin

For the past three decades, the anthracycline antibiotics doxorubicin (adriamycin) (**1**) and daunorubicin (daunomycin) (**2**) have been considered as first-line chemotherapy in the treatment of a variety of solid and haematological tumours (Murphy *et al* 1995, Wiernik and Dutcher 1992), despite serious dose-limiting side effects such as myelosuppression and cardiotoxicity (Zucchi and Danes 2003).

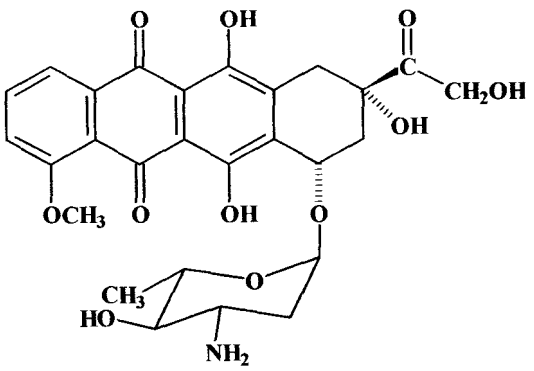
Although topoisomerase II has long been recognised as the primary cellular target of doxorubicin and daunorubicin (Tewey *et al* 1984), a number of additional mechanisms have been identified as contributing to the antiproliferative and cytotoxic actions of these agents. These include DNA intercalation and subsequent inhibition of macromolecular biosynthesis (Fritzache and Wahnert 1987), induction of DNA damage by free radical formation (Muller *et al* 1998), lipid peroxidation (Fukuda *et al* 1992) and direct membrane effects (Tritton and Yee 1982).

Cancer cells are very effective in developing biochemical mechanisms which allow for cellular resistance to a particular antineoplastic agent. In order to overcome the problem of resistance development, clinicians generally use treatment regimens combining several drugs with different cellular targets (combination therapy). A particular drug that produces

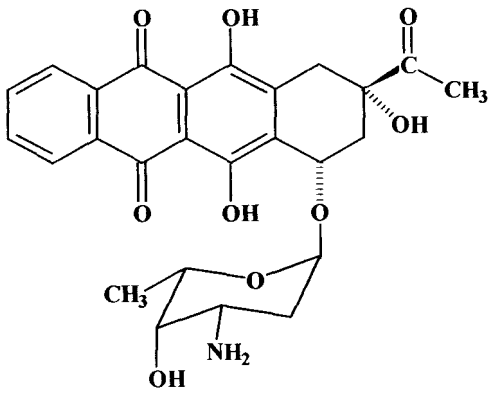
some tumour shrinkage when used alone may produce a cure when used in combination with other antineoplastic drugs. Topoisomerase inhibitors feature in many of the most effective treatment regimens. For example, ABVD [adriamycin (topoisomerase II inhibitor), bleomycin (DNA cleaving agent), vinblastine (antimitotic agent) and dacarbazine (an alkylating agent)] is standard therapy for advanced Hodgkin's disease, with roughly 80% of patients achieving complete remission and 60% of patients cured (Urba and Longo 1992).

More recently additional anthracyclines, including epirubicin (**3**), idarubicin (**4**) valrubicin (**5**) and aclarubicin (**6**) have received clinical approval, based upon modest improvements over doxorubicin and daunorubicin.

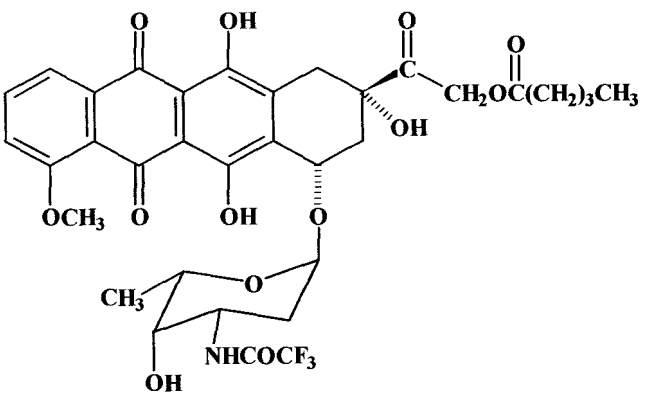
Epirubicin is used mainly in the treatment of breast cancer, either as a single agent or as part of adjuvant therapy (FDA approved September 1999). Although less potent than doxorubicin it is also less cardiotoxic; a cumulative dose of epirubicin of up to 950 mg/m^2 can be administered before the emergence of acute and chronic cardiomyopathy (Ryberg *et al* 1998), compared to 550 mg/m^2 for doxorubicin. A structural feature common to all anthracyclines is a quinone ring which can generate reactive oxygen species (ROS) through one-electron reduction by flavin-centred reductases (Minotti *et al* 2000) or through a non-enzymatic pathway involving coordination of a ferric ion with chromophore rings B and C (Myers 1998). In the presence of water molecules, this complex can initiate redox cycling and the production of superoxide anions and ultimately hydroxyl radicals (Olson and Mushlin 1990). Myocardial cells lack free-radical scavenging defence mechanisms and are therefore particularly susceptible to anthracycline-mediated ROS generation. Additionally, treatment with anthracyclines results in the elimination of glutathione peroxidase activity (Myers 1998).



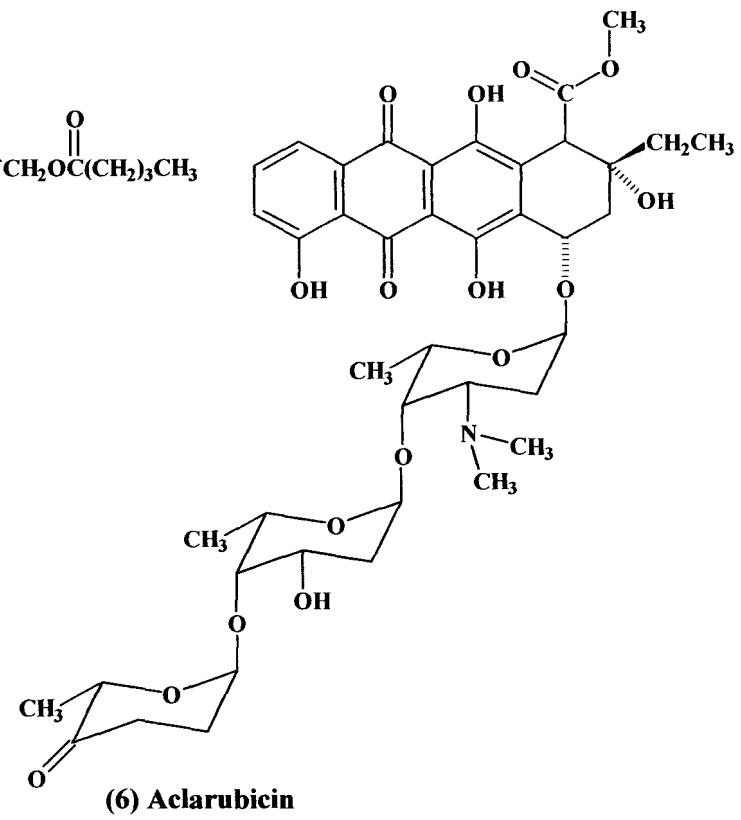
(3) Epirubicin



(4) Idarubicin



(5) Valrubicin

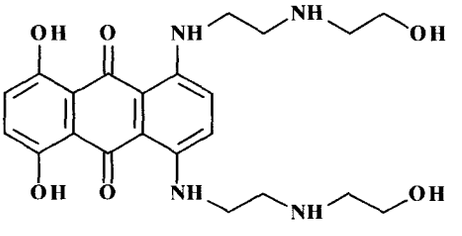


(6) Aclarubicin

Both idarubicin and aclarubicin are used in the treatment of acute leukaemia. Idarubicin (4-demethoxydaunorubicin) has higher lipid solubility, increased cellular uptake and less dependency on P-glycoprotein efflux than its parent compound daunorubicin (Speth *et al* 1989, Roovers *et al* 1999) and is approved for use, in combination with other antileukaemic drugs, for the treatment of acute myeloid leukaemia (AML) in adults. The mechanism of action of both idarubicin and aclarubicin involves, to some extent, targeting

of topoisomerase I in addition to topoisomerase II. Idarubicin has been shown to produce low levels of topoisomerase I-mediated DNA cleavage *in vitro*, however studies in yeast indicate that poisoning of topoisomerase II is the principal mechanism of cell kill (Guano *et al* 1999). Aclarubicin has a dual topoisomerase targeting mechanism; acting as a topoisomerase I poison and a catalytic inhibitor of topoisomerase II. This compound is discussed in greater detail in **Section 2**.

Valrubicin (N-trifluoroacetyldoxorubicin-14-O-valerate) (**5**), a semi-synthetic doxorubicin analogue with improved cellular uptake and reduced cardiotoxicity compared to its parent compound (Onrust and Lamb 1999), has been approved for use (1998) in the treatment of bladder carcinoma, by intravesical administration. Valrubicin readily penetrates the superficial muscle layer of the bladder wall at cytotoxic drug concentrations. Although valrubicin binds less strongly to DNA than doxorubicin its principal mechanism of action is still thought to be through poisoning of topoisomerase II enzymes, however, metabolic activation by non-specific esterases is essential for stabilisation of cleavable complexes (Silber *et al* 1987).

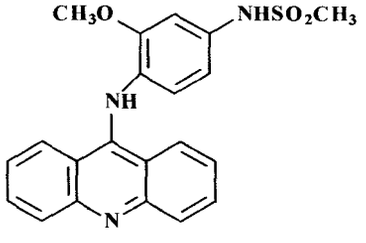


(7)

The proven clinical usefulness of doxorubicin led to an intensive search, in the late 1970s, for new structurally related compounds with antitumour activity but without the dose-limiting cardiotoxicity and which were easier to access, given the lengthy procedures required for total synthesis or modification (semi-synthesis) of doxorubicin analogues. It was believed at that time (wrongly) that the amino sugar moiety was responsible for the

cardiotoxicity of doxorubicin and daunorubicin (Adamson 1974), hence vast numbers of compounds were synthesised which retained a planar anthraquinone ring system and contained amino- or alkylamino-substituted side chains in place of the sugar.

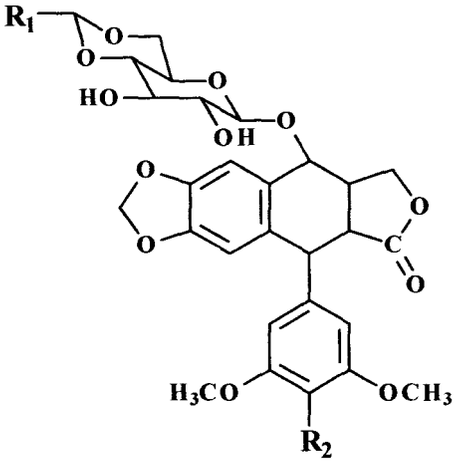
The experimental antitumour activity of one such molecule, 1,4-bis[2-(2-hydroxyethyl)amino]ethylamino]-9,10-anthracenedione (ametantrone), was discovered by random screening at the National Cancer Institute (NCI) and led to the synthesis and extensive investigation of structure-activity relationships on a series of bis(substituted aminoalkylamino)anthraquinones by Zee-Cheng and Cheng (1978). 1,4-Dihydroxy-5,8-bis[2-(2-hydroxyethyl)amino]ethylamino]-9,10-anthracenedione was the most active compound identified and was subsequently prepared as its water soluble hydrochloride salt, mitoxantrone (Novantrone) (**7**) (Murdock *et al* 1979). Mitoxantrone is the only compound of this class (aminoanthracenediones) approved for clinical use. It retains the topoisomerase II poisoning ability and clinical efficacy of doxorubicin but has greatly diminished cardiotoxicity (Faulds *et al* 1991). Mitoxantrone is primarily used for the treatment of breast and prostate cancers, leukaemia and lymphomas and has been incorporated into selected chemotherapy regimens in place of doxorubicin because of its reduced toxicity (Benjamin 1995).

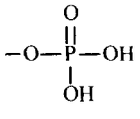


(8)

Amsacrine (mAMSA) (**8**) was prepared by Atwell and co-workers in the early 1970s during a programme of work to design and synthesise anilino-substituted analogues of 9-

anilinoacridine with anti-tumour activity (Atwell *et al* 1972). Amsacrine is a potent topoisomerase II poison but clinical development was hampered due to poor solubility and lack of broad-spectrum clinical activity and its use is limited to the treatment of acute leukaemia (Schaich *et al* 2002).



	R_1	R_2
Etoposide (**9**)	$-CH_3$	-OH
Teniposide (**10**)		-OH
Etopophos (**11**)	$-CH_3$	

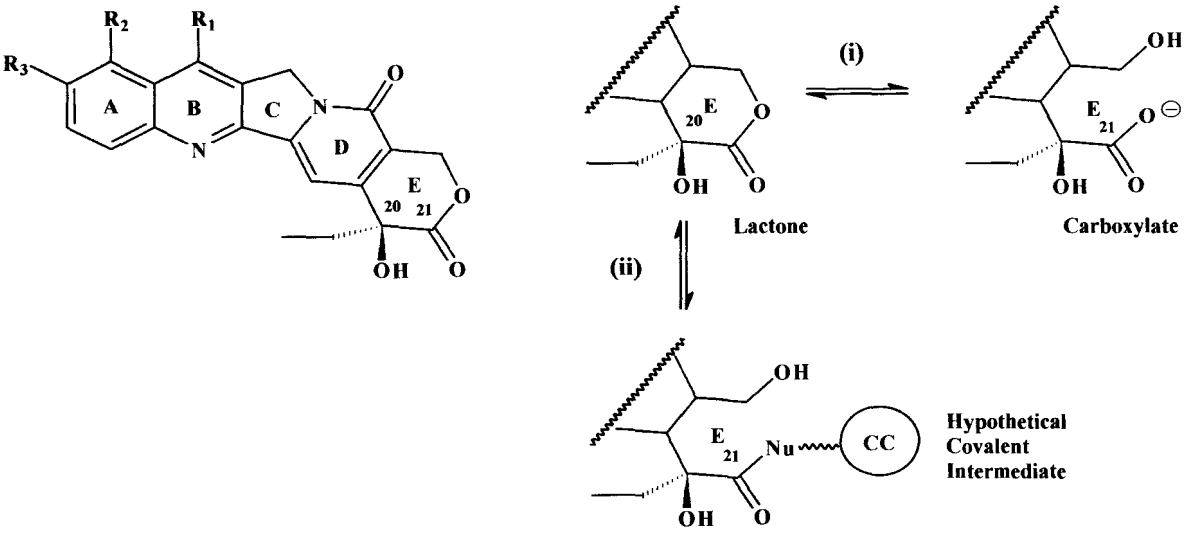
Podophyllotoxins have been used medicinally by various cultures for over 1000 years (Sliven 1991). In the 1940s, podophyllotoxin was found to act as an antimitotic agent by inhibiting mitotic spindle production, resulting in cell death (King and Sullivan 1946). However, podophyllins were too toxic for clinical use and a search began for podophyllotoxin derivatives that retained antineoplastic activity but were less toxic. Two synthetic podophyllotoxin analogues, etoposide (VP-16) (**9**) and teniposide (VM-26) (**10**), were found to be potent antineoplastic agents but almost completely lacked antimitotic activity. These compounds entered clinical trial in the early 1970s, and it was reported in 1976 that etoposide caused dose-dependent single-strand and double-strand DNA breaks (Loike *et al* 1976), however it was not until 1984 (one year after FDA approval) that topoisomerase II was identified as the molecular target of etoposide (Chen *et al* 1984). In fact, etoposide was the first clinical anticancer agent demonstrated to act through inhibition of topoisomerase II.

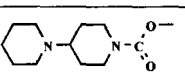
Etoposide has widespread clinical use against a variety of neoplasms, including lymphomas, leukaemias, neuroblastoma and soft-tissue sarcomas, and it is the first choice drug for the treatment of testicular and small cell lung cancers (Belani *et al* 1994).

Despite teniposide being 10-fold more cytotoxic *in vitro* than etoposide, its use is limited, mainly due to a higher incidence of hypersensitivity reactions, to the treatment of refractory childhood acute lymphoblastic leukaemia. Myelosuppression is the dose-limiting toxicity for both compounds and there have been many reports of the late (1-5 years) development of acute non-lymphocytic leukaemia (with 11q23 chromosomal abnormalities) in patients treated with either etoposide or teniposide (Stine 1997).

Due to the poor aqueous solubility of etoposide, a phosphate prodrug etopophos (**11**) was developed and given FDA approval (1996) for use in combination therapy in the treatment of testicular and small cell lung cancer. Etopophos is soluble in water at concentrations up to 20 mg/ml, is rapidly converted in the blood to etoposide within 15 minutes of administration and has equivalent antitumour activity to etoposide (Schacter 1996).

Figure 2: Structure of camptothecins and **(i)** inactivation by opening of the lactone E ring (at physiological pH) and **(ii)** formation of the hypothetical covalent intermediate between camptothecin and the topoisomerase I-DNA cleavage complex. [Adapted from Pommier et al. Biochim. Biophys. Acta, (1998a), 1400, 83-106].



A and B ring modifications			
	R_1	R_2	R_3
Camptothecin (**12**)	H	H	H
Topotecan (**13**)	H	$-CH_2-N(CH_3)_2$	OH
Irinotecan (**14**)	$-CH_2-CH_3$	H	
SN38 (**15**)	$-CH_2-CH_3$	H	OH

The potent antitumour activity of camptothecin (**12**) was discovered in the mid 1960s, however, early clinical trials were discontinued because of unmanageable clinical toxicities. The identification of topoisomerase I as the cellular target of camptothecin (Liu 1989) led to the development of water-soluble derivatives with fewer side effects, such as topotecan (Hycamtin) (**13**) and irinotecan (Camptosar, CPT-11) (**14**). All clinically used camptothecins are potent topoisomerase I poisons with the exception of irinotecan; a prodrug that requires conversion to the active metabolite SN-38 (**15**), by a

carboxylesterase converting enzyme. Camptothecin and its derivatives poison topoisomerase I by stabilising the transient covalent DNA-topoisomerase I [Figure **2 (ii)**] cleavage complex and preventing religation of the cleaved DNA strand. SN-38 is amongst the most potent cleavable complex inducing compounds in this class. Single-strand breaks induced by topoisomerase I are considered non-toxic to cells because the DNA lesions can be efficiently and rapidly repaired. However, their conversion into double strand breaks, believed to be formed by collision of the stabilised cleavable complex with proceeding replication forks during the S phase of the cell cycle, results in the inhibition of DNA synthesis, G2 arrest and eventual cell death (Hsiang *et al* 1989). The camptothecins in current clinical use have two major limitations:

1. The instability of the six-membered lactone ring; at physiological pH camptothecins are in equilibrium with their inactive (carboxylate) form [see Figure **2 (i)**].
2. Their topoisomerase I cleavage complexes reverse within minutes after removal of the drug (Covey *et al* 1989), leading to long infusion times for patients during cancer therapy.

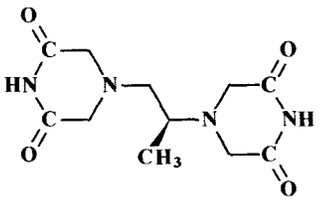
Topotecan (Hycamtin) first received FDA approval in 1996 for the treatment of patients with advanced metastatic ovarian carcinoma and has since been approved for use in the treatment of small cell lung cancer. This drug has the shortest plasma half-life of any camptothecin (reported to date) and requires repeated daily administration or continuous infusion over several days or even weeks (Takimoto *et al* 1998). Irinotecan, (CPT-11, Camptosar) is approved for the treatment of metastatic colon carcinoma which has relapsed or progressed following failure of 5-FU-based therapy (Cunningham 1999).

Although the majority of topoisomerase II-targeting agents are poisons, examples of catalytic inhibitors of the enzyme with clinical applications are known.

In contrast to poisons, catalytic inhibitors do not directly induce strand breaks and may even prevent their formation; they can act at any stage of the catalytic cycle other than cleavable complex formation, either by interfering with enzyme-DNA binding or by trapping the DNA-bound enzyme in the closed clamp conformation. Two, controversial, therapeutic approaches have emerged based on the combination of a poison with a catalytic inhibitor.

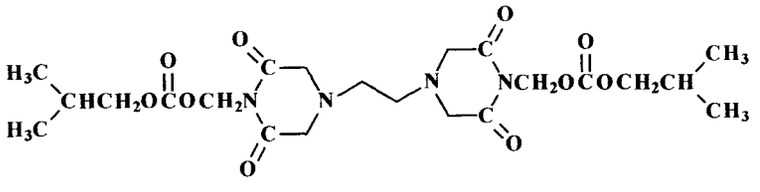
One approach is pharmacological modulation of poisoning effects by a catalytic inhibitor. Since a pure inhibitor can abolish the toxic effect of a poison, inhibitors of this type may be used to direct the toxic effect. For example, the bisdioxopiperazine, dexrazoxane (ICRF-187) (**16**), that does not cross the blood-brain barrier, can be used to increase the tolerated dose of etoposide, which does (Holm *et al* 1996). The principle is being evaluated in phase II trials in small-cell lung cancer patients with central nervous system metastases.

A potentially broader application of combining a protective catalytic inhibitor with a poison is suggested by the known acidic extracellular environment associated with solid tumours. Whereas (neutral) etoposide can permeate plasma membranes, weak bases cannot; weakly basic non-toxic catalytic inhibitors could protect normal tissues without compromising the antitumour efficacy of etoposide. This concept has been demonstrated in preclinical models using the weakly basic topoisomerase II catalytic inhibitor chloroquine, although the latter is too toxic for *in vivo* use (Jensen *et al* 1994) and clearly more suitable compounds need to be found.



(16)

In another approach, the sequential use of an inhibitor with a poison, catalytic inhibitors are attractive agents in second-line therapy of malignant tumours, since they display no cross-resistance in cell lines selected for resistance to topoisomerase II poisons. A clinically approved example has been advanced in Japan; sobuzoxane (MST-16) (**17**) is another member of the class of bisdioxopiperazine catalytic inhibitors (Andoh 1998).



(17)

Catalytic inhibitors, although generally predicted to be of lesser value in therapy than poisons, due to decreased levels of DNA damage, may find application (other than in the alternative senses outlined above) given that some catalytic inhibitors, including merberone and dexrazoxane are able to induce apoptosis in the absence of DNA breaks (Khelifa and Beck 1999). The clinical significance of these properties is yet to be established but may become significant in circumventing drug resistance syndromes.

1.4.6 The Need for New Topoisomerase Inhibitors?

The wide structural diversity of topoisomerase inhibitors and their mechanisms of action have been the subject of recent comprehensive reviews (Pommier 1998b, Malonne and Atassi 1997, Gatto *et al* 1999, Bailly 2000). The development of topoisomerase inhibitors as clinically useful anticancer drugs, their performance in the clinic and limitations of their

clinical application have been discussed in detail (Takimoto *et al* 1998, Holden 2001, Larsen *et al* 2003).

The major clinical limitation of existing topoisomerase inhibitor-based chemotherapy is the problem of multi-drug resistance (MDR) whereby the development of resistance to one drug results in the simultaneous development of resistance, not only other topoisomerase inhibitors but to a variety of other, often structurally and mechanistically unrelated compounds (Kartner and Ling 1989; Kaye 1988; Ueda *et al* 1999). One of the most important and well characterised mechanisms of MDR involves increased expression of P-glycoprotein (Pgp), a transmembrane glycoprotein, which acts as a drug efflux pump for many of the most important classes of anticancer drugs such as the anthracyclines, vinca alkaloids, epipodophyllotoxins and taxanes.

Whereas MDR is inherently expressed in some cancers, in others it develops in response to treatment and is the main reason for the failure of chemotherapy.

Another mechanism associated with resistance against topoisomerase-interactive agents is a decreased level or activity of the target enzyme (known as altered topoisomerase resistance or at-MDR). However, since the topoisomerase I enzyme function is essential to very basic cell survival requirements, commonly decreased activity of topo I is compensated for by an increase in topo II expression, with subsequent enhanced cytotoxicity of cells to topo II inhibitors (Whitacre *et al* 1997). Similarly, resistance to topo II inhibitors has been linked to a reduction in the catalytic activity of topoisomerase II (Beck *et al* 1993) or a reduction in levels of topo IIα mRNA. For example, in a study of 66 etoposide- and mAMSA- resistant cell lines (Matsumoto *et al* 1997) reduced expression of topo II α mRNA was observed in 95% of resistant cell lines in comparison to parental cell lines, with a compensatory increase in topo I and topo II-β mRNA levels.

CHAPTER TWO

[PART A]

DUAL TOPOISOMERASE INHIBITION

2 Dual Topoisomerase Inhibition

While most topoisomerase interacting drugs target either topo I or topo II, several classes of compounds have more recently been shown to act against both enzymes often exhibiting a complex pattern of activities including inhibition and poisoning of the two enzymes.

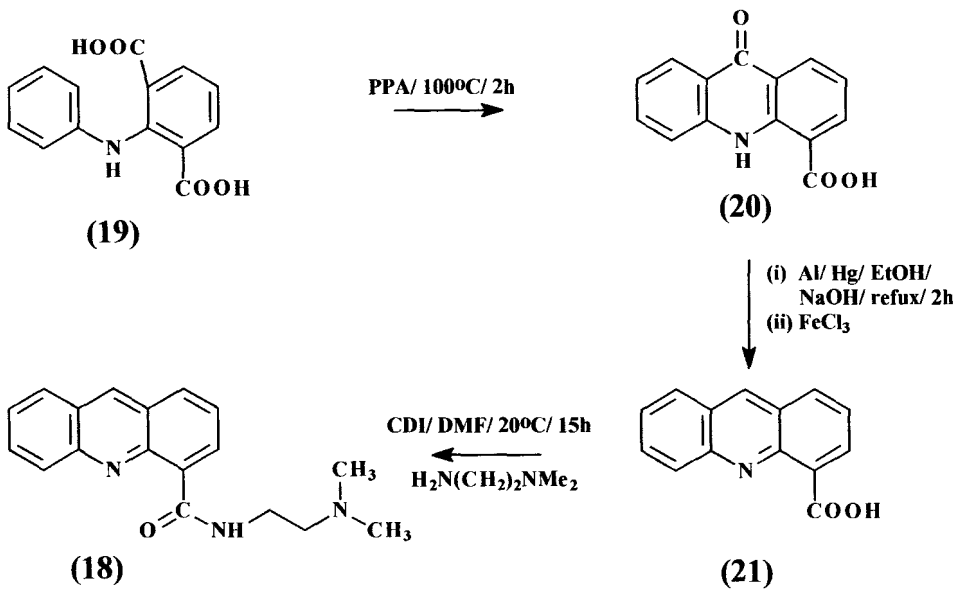
Simultaneous targeting of both topo I and topo II (α- and/or β- isoforms) by a drug may be an important contributing factor for circumventing resistance mechanisms due to alteration of a single target enzyme.

The principal classes of topoisomerase inhibitors are described below.

DACA (N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide) (**18**) and related tri- and tetracyclic carboxamides have been extensively studied over the past two decades and form the largest class of dual topoisomerase I and II inhibitors.

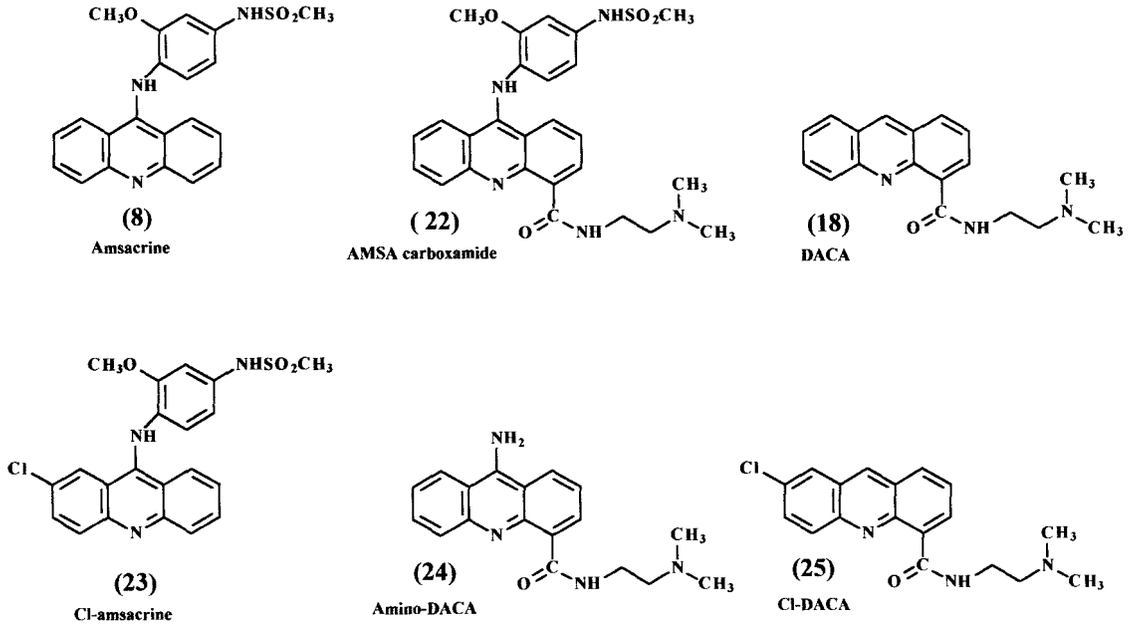
DACA was developed by Baguley and co-workers during a programme to design and synthesise acridine derivatives with selective activity against solid tumours (Atwell *et al* 1987). The synthesis of DACA is outlined in **Scheme 1**. Cyclisation of the N-phenyl-aniline-dicarboxylic acid (**19**) using polyphosphoric acid gave the acridone (**20**). Reduction of compound (**20**) using aluminium/mercury amalgam, followed by reoxidation of the resulting intermediate acridan with $FeCl_3$ afforded the acridine-4-carboxylic acid (**21**). Reaction of (**21**) with 1,1′-carbonyldiimidazole followed by addition of N,N-dimethylethylenediamine gave the acridine carboxamide, DACA (**18**).

Scheme 1



Early studies indicated that topoisomerase II (Schneider *et al* 1988) was the main target for acridine-4-carboxamides (DACA etc), while pointing out their non-classical mechanism of action (Woynarowski *et al* 1994). In contrast to the majority of topo II poisons, DACA displayed a wide spectrum of activity against solid tumours in animals but was only moderately active against experimental leukaemia. The ability of DACA to overcome both p-glycoprotein-mediated and atypical multidrug resistance together with a complex relationship between cytotoxicity and drug concentration and exposure times, including self-inhibition at high drug concentrations, suggested a unique mode of action (Finlay *et al* 1993).

Figure 3: Structure of selected acridines



It has since been shown that DACA is in fact a dual poison of both topoisomerases I and II. Structure activity studies on a series of acridine derivatives, (**Figure 3**) related to both amsacrine and DACA, have identified molecular features which are important in controlling the ability of acridines to stimulate DNA cleavage with either topoisomerases I or II. The main findings of the study were that amsacrine (**8**) and its 7-chlorinated derivative (**23**) poison only topo II. The addition of the N-2-(dimethylamino)ethyl group alone, in compounds (**22**) and (**24**), slightly increased activity towards topo I, whilst addition of the charged side-chain in combination with the removal of the anilino group (DACA) (**18**) resulted in a pronounced change in the pattern of topo II induced cleavage, promoted topo I cleavage and gave compounds with biological activity against "atypical" multidrug resistant cell lines. The addition of the 7-chloro substituent to DACA (**25**) suppressed topo II cleavage but increased stimulation of topo I cleavage and activity in multidrug resistant cell lines (Finlay *et al* 1996). Further studies extending the SARs for

acridine-4-carboxylic acids, substituted in the 5-, 6-, 7-, or 8- position, found that steric bulk was more important than the electronic properties of the substituent, with larger groups leading to loss of cytotoxic activity (Spicer *et al* 1997). More recently, Bridewell *et al* (1999) have carried out a detailed investigation into the relative roles of topoisomerases I and II in the cytotoxic mechanism of DACA and its 7-chloro derivative. In cell-free systems DACA produced no evidence of topoisomerase I-mediated DNA cleavage but inhibited enzymatic activity at concentrations >10μM; poisoning of topoisomerase II occurred at drug concentrations >5μM. Cl-DACA induced topoisomerase I-mediated DNA cleavage at 5μM but inhibited DNA relaxation at 10μM, consistent with suppression (self-inhibition) of poisoning. This compound produced only very weak topoisomerase II-mediated DNA cleavage bands.

Hence, although both DACA and its 7-chloro derivative have dual topoisomerase I/II specificity, DACA preferentially poisons topoisomerase II and Cl-DACA preferentially poisons topoisomerase I.

More recently, the TARDIS (trapped in agarose DNA immunostaining) assay has been used (Padget *et al* 2000) to determine whether DACA stabilised topoisomerase-DNA complexes formed *in situ* in individual human leukaemia CCRF-CEM cells.

The results confirmed the findings of Bridewell *et al* indicating the preferential role of topoisomerases II in the cytotoxic mechanism of DACA. Under the conditions used in the TARDIS assay DACA appeared to be selective for topoisomerase IIα. DACA was more effective in forming cleavable complexes with topoisomerase IIα than with either the II-β isoform or topoisomerase I. However, the authors concluded that even formation of low levels of cleavable complexes with these enzymes might still play a role in cell death.

A panel of three human leukaemia (Jurkat) cell lines, displaying a range of resistance mechanisms (Finlay *et al* 1990), has been used in a number of studies (Spicer *et al* 1997; Gamage *et al* 1999; Gamage *et al* 2002; Spicer *et al* 2002) to predict possible topoisomerase-mediated mechanisms of action for compounds structurally related to DACA.

JL_C is the wild-type (sensitive) cell line, JL_A is resistant to the DNA intercalator amsacrine and similar agents due to reduced levels of topoisomerase II. The doxorubicin-resistant cell line JL_D also has altered levels of topoisomerase II. A compound with ratios (JL_A/ JL_C and JL_D/ JL_C) of IC_{50} values less than about 2-fold would possibly be expected to act by a novel, non- pure topoisomerase II-mediated mechanism of action. In this screen, the dual topoisomerase I and II inhibitor DACA has JL_A/ JL_C and JL_D/ JL_C ratios of 2.3 and 2.5 respectively, while its 7-chloro derivative, which has been shown to preferentially poison topoisomerase I, has ratios of 1.2 and 1.3. The topoisomerase II poisons amsacrine and doxorubicin have JL_A/ JL_C and JL_D/ JL_C ratios of 85 and 74 (amsacrine) and 4.4 and 13 (doxorubicin) respectively. Hence, this panel can provide an initial screen for selecting analogues of DACA with greater absolute potency but similar or lower JL_A/ JL_C and JL_D/ JL_C ratios. Examples of its use to identify compounds with a mixed topoisomerase I/ II mechanism of action are discussed below, particularly where actual topoisomerase inhibitory data confirms the suspected mode of action.

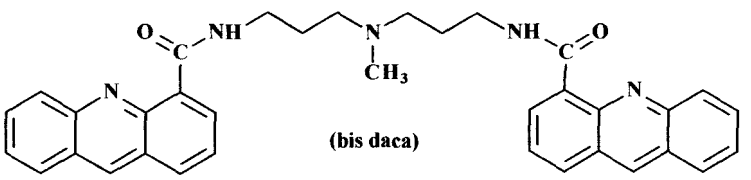
Several studies have shown that the relative activity of DACA (and acridines in general) against topoisomerase I and II enzymes can be modulated by appropriate substitution of the acridine chromophore (Spicer *et al* 1999a; Denny *et al* 1982).

Bridewell *et al* (2001) used a series of DACA analogues (mainly halogen derivatives, monosubstituted in the 5-, 6- or 7- positions) in an attempt to correlate *in vitro* and *in vivo*

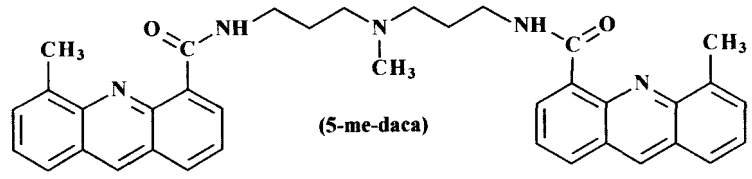
biological activity. Topoisomerase I and II-mediated cleavage and relaxation assays and the panel of three human leukaemia (Jurkat) cell lines were used to identify compounds in which topoisomerase II might play a greater role in their mechanism of action. The compounds displayed a very complex relationship between topoisomerase poisoning and inhibition, in vitro cytotoxicity and in vivo antitumour activity. The authors hypothesised that DACA analogues can act both *in vitro* and *in vivo* to simultaneously poison topoisomerase II and inhibit topoisomerase I catalytic activity, and that this combination of dual activity contributed to the high antitumour activity of this class of compound.

Compounds containing two neutral, relatively lipophilic DNA monointercalating chromophores, such as naphthalimides (Nitiss *et al* 1998) and anthracyclinones (Chaires *et al* 1997), linked by a flexible chain are currently being developed as anticancer drugs. A comparative study of a variety of these bis(chromophores) with their corresponding monomers found variable but significant gains in potency for the dimeric species (Spicer *et al* 1999b).

Bis(DACA) (**26**) (Spicer *et al* 1999b) and its 5-methyl analogue (**27**) (Gamage *et al* 1999) were prepared during a programme of work to develop a series of dimeric tricyclic carboxamides, including substituted bis(acridine-4-carboxamides). The bis(DACA) analogue was found to be 5-fold more cytotoxic than its monomer.

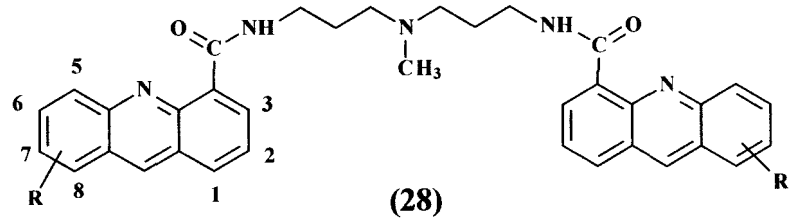


(26)



(27)

Compound (**27**) was the most cytotoxic member from this series of acridine-substituted bis(acridine-4-carboxamides) with general structure (**28**).

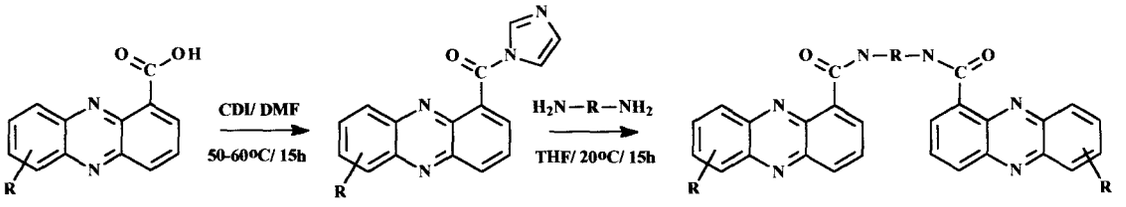


(28)

Over 40 analogues were prepared containing a wide range of substituents in one or two positions of the acridine ring system. Analogues with small substituents in the 5-position were most potent with IC_{50} values in the nanomolar range against many cell lines in the NCI cell line panel. Larger substituents in any position caused a significant decrease in potency. For example, a 5-phenyl analogue was almost 250-fold less potent than compound (**27**) in the NCI screen (mean GI_{50} over the whole cell line panel). All compounds were tested in the human leukaemia (Jurkat) cell line screen, in which the results indicated that they were likely to have a greater effect on topoisomerase I inhibition than topoisomerase II. It was confirmed that compound (**27**) inhibited topoisomerase I activity in a cell-free system, however any effect on topoisomerase II enzymes was not reported.

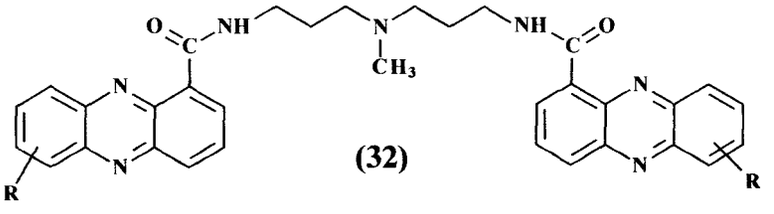
The development of dimeric analogues of lipophilic neutral DNA intercalators has been extended by Spicer *et al* (2000) to include bis(phenazines). The synthesis of compounds with general structure (**31**) is outlined in **Scheme 2.** Activation of the acid (**29**) with 1,1'-carbonyldiimidazole gave the resultant N-imidazolide (**30**) which was isolated, purified and reacted with a stoichiometric amount of an appropriate α,ω-bis-amine to give the bis(phenazine-1-carboxamide) (**31**).

Scheme 2. General reaction scheme for the synthesis of bis(phenazines).
[Applicable to Spicer *et al* 2000 and Gamage *et al* 2001]

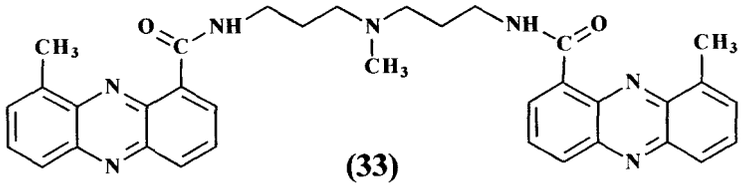


(29) (30) (31)

A series of monocationic ring-substituted bis(phenazine-1-carboxamides), joined by the same $-(CH_2)_3NMe(CH_2)_3-$ linker chain used in the aforementioned bis(acridine-4-carboxamide) series, has been prepared. These compounds, with general structure (**32**), were evaluated for cytotoxic activity in a panel of tumour cell lines, including the panel of three human leukaemia (Jurkat) cell lines used to identify compounds with a possible mixed topoisomerase I/ II mechanism of action. All compounds had JL_A/ JL_C and JL_D/ JL_C ratios <2, consistent with topoisomerase II inhibition not being their primary mechanism of action.



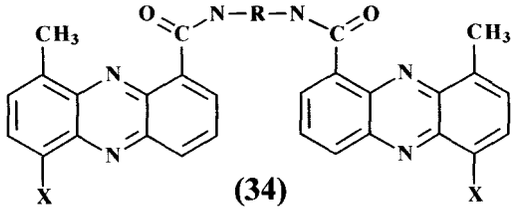
(32)



(33)

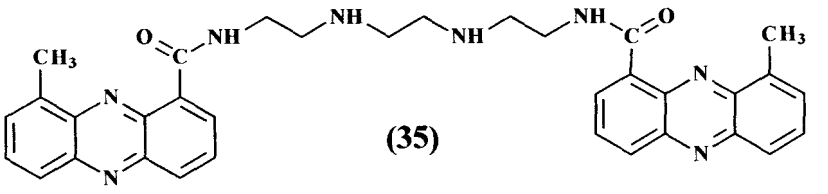
The most cytotoxic compound (**33**) was shown to (slightly) stimulate topoisomerase I-mediated cleavage of plasmid pBR322 DNA at low drug concentrations (0.1 and 0.25 μM). Higher drug concentrations (1 and 5 μM) were found to inhibit the relaxation of plasmid DNA by both topoisomerase I and II.

All bis(phenazines) included in this study were preferentially active towards colon tumour cell lines in the NCI 60 cell line screen, being on average almost 10-fold more active in the HT29 colon cell line than in the panel as a whole; significant growth delays were produced *in vivo* in the subcutaneous murine colon 38 tumour model by several bis(phenazine) analogues.



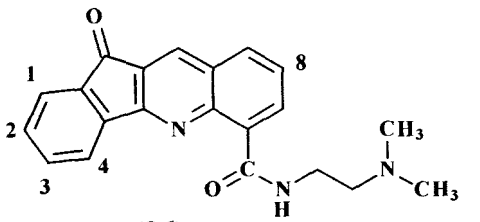
(34)

Studies were extended to the include of a series of dicationic bis(9-methylphenazine-1-carboxamides) joined by a variety of dicationic linkers of varying length and conformational rigidity. Compounds with general structure (**34**), have been prepared and evaluated for their *in vitro* growth inhibitory effects in human and murine cell lines, including the panel of three human leukaemia (Jurkat) cell lines. (Gamage *et al* 2001). All compounds had JL_A/ JL_C and JL_D/ JL_C ratios <1, consistent with topoisomerase II inhibition not being their primary mechanism of action.



(35)

This series of bis-phenazines is exemplified by compound (**35**) which was potently cytotoxic, with IC_{50} values in the low/sub nanomolar range against a variety of human cell lines, and poisoned both topoisomerase I and II in a purified enzyme system (Stewart *et al* 2000).

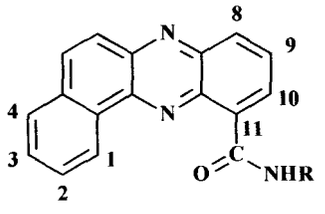


(36)

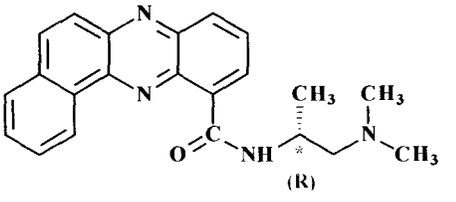
Deady *et al* (1999) reported the synthesis and structure-activity relationships of chromophore-substituted analogues of the prototype indenoquinoline (**36**); a potent cytotoxin which displayed patterns of cell line activity consistent with dual topoisomerase I/II inhibition (Deady *et al* 1997). A series of compounds containing mainly methoxy and chlorine groups in the 1-, 2-, 3-, 4- and 8- positions of the ring system were prepared. Most compounds retained both cytotoxic potency and JL_A/ JL_C and JL_D/ JL_C ratios in the human leukaemia (Jurkat) screen predictive of a dual topoisomerase I/II mechanism of action, except for the 4-substituted analogues which were less effective in the resistant cell lines than the wild type, suggesting a mode of action mainly mediated by interaction with topoisomerase II. The authors did not include any actual topoisomerase inhibitory data to confirm the proposed mechanism of action by this series of indenoquinolines.

Vicker *et al* (2002) have reported the development of second generation, orally active, dual topoisomerase I and II inhibitors, structurally related to DACA. Extensive structure

activity relationships on >75 novel angular benzophenazines, conforming to general structure (**37**) were carried out.



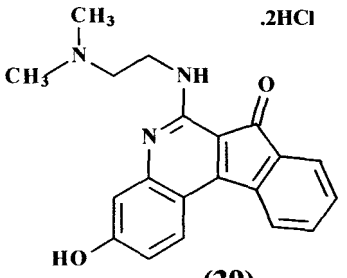
(37)



(38)

The effects on cytotoxic potency of substituents in the 1-, 2-, 3-, 4-, 8-, 9- and 10-positions of the benzo[*a*]phenazine fused ring system, and variation in the nature of the amide side chain at the C-11 position were investigated.

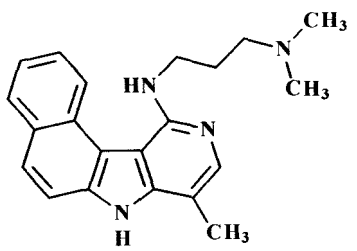
The introduction of chirality into the carboxamide side chain resulted in a series of enantiospecific cytotoxic agents, exemplified by compound (**38**), coded XR11576. The (R)-enantiomer was ~4-fold more potent than the (S)-enantiomer with IC_{50} values of 23 nM and 29 nM in the H69 parental human small cell lung carcinoma and H69/LX4 (P-glycoprotein over-expressing) resistant cell lines respectively. The authors believed this to be the first reported example wherein side chain chirality in DNA intercalating agents had an important influence on biological activity. XR11576 stabilised both topoisomerase I- and II-mediated cleavable complex formation in a dose-dependent manner between 0.03 and 1μM (Dangerfield *et al* 2001); cleavage patterns obtained differed from those induced by camptothecin and etoposide. This compound has also been shown to be unaffected by various mechanisms of multi-drug resistance, including down-regulation of topoisomerase II and displays marked *in vivo* efficacy against a number of tumour xenografts. (Mistry *et al* 2002).



(39)

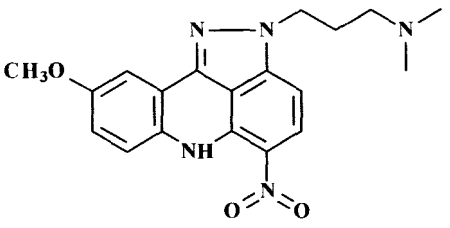
TAS-103, in common with DACA (**39**), bears a cationic (dimethylamino)ethylamino side chain (but not amide-linked to the nucleus) and shares structurally similar features with intoplicine (**40**) which also contains a (longer) aminoalkylamino side-chain. This substituent appears to be important in the ability of these compounds to interact with topoisomerases and in drug transport through cell membranes (Pastwa *et al* 1998; Haldane *et al* 1999). A 1997 paper by Utsugi et al reported that TAS-103 was a dual topoisomerase I and II poison and also interfered with the catalytic activities of both enzymes in the low micromolar range *in vitro*. Later studies by Wilson Byl and co-workers re-classified TAS-103 as a topoisomerase II poison; dual topoisomerase poisoning was confirmed in cell free systems, however, results from yeast genetic models revealed that poisoning of topoisomerase I made virtually no contribution towards the cytotoxicity of TAS-103 (Wilson Byl *et al* 1999). Additionally, Fortune *et al* (1999) reported that the apparent inhibition of topoisomerase I catalytic activity by this compound was more probably as a result of strong intercalative binding of the drug to DNA and not by the inhibition of enzymatic activity. The authors confirmed that TAS-103 does inhibit the catalytic activity of human topoisomerase IIα by blocking the DNA religation reaction of the enzyme. Results from the TARDIS assay (Padget *et al* 2000) also indicated preferential targeting of topoisomerase IIα by TAS-103 in whole human leukaemia CCRF-CEM cells; similar topoisomerase IIα selectivity was also reported for DACA (**18**).

Despite conflicting reports on the relative contribution of topoisomerase I and II poisoning towards the cytotoxicity of TAS-103, activity was retained in camptothecin and etoposide resistant cell lines and was not affected by P-glycoprotein-mediated, MRP or LRP mechanisms of multidrug resistance (Aoyagi *et al* 1999, Mindermann *et al* 2000). TAS-103 had a broad spectrum of antitumour activity *in vivo* against a variety of human xenografts, derived from lung, colon, stomach and pancreatic cancer, and has proceeded to Phase I clinical trial (Utsugi *et al* 1997).



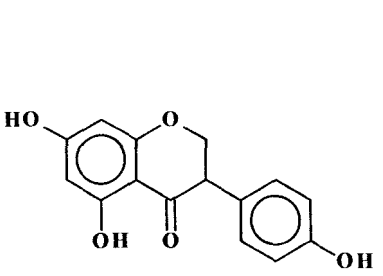
(40)

Intoplicine (RP-60475) (**40**) is an *in vivo* active dual topoisomerase I and II poison that underwent early clinical trials. In cell free systems and living cells intoplicine induced high levels of concentration-dependent topoisomerase I- and II- mediated strand breaks and was active in m-AMSA- and camptothecin-resistant but not multidrug-resistant cell lines (Poddevin *et al* 1993). Riou and co-workers reported structure activity relationships for a series of 22 intoplicine analogues that displayed a range of topoisomerase I- and/or II-mediated cleavage activity. Compounds with dual topoisomerase I and II poisoning ability were more active *in vivo* in the P388 leukaemia model than those which selectively inhibited either topoisomerase I or II alone. It was proposed that dual topoisomerase poisoning is crucial for antitumour activity within this series of compounds (Riou *et al* 1993).

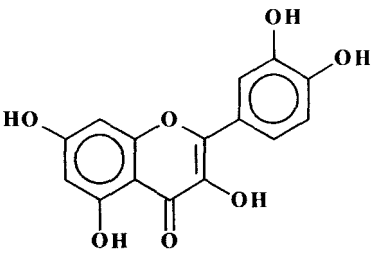


(41)

The pyrazoloacridine (NSC 366140) (**41**) has undergone broad phase II clinical trial in a number of tumour types (Adjei 1999) and has shown selective activity against solid tumour cells, cytotoxicity in non-cycling and hypoxic cells and can circumvent P-glycoprotein and multidrug resistance-associated protein (MRP) mechanisms of drug resistance. NSC 366140 was shown to be a potent catalytic inhibitor of both topoisomerases I and II at low micro-molar concentrations *in vitro* (Adjei *et al* 1998) but had no effect on cleavable complex stabilisation.



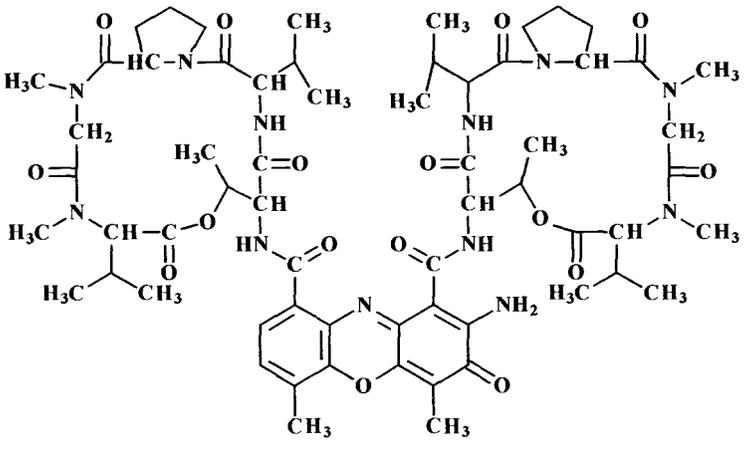
(42)



(43)

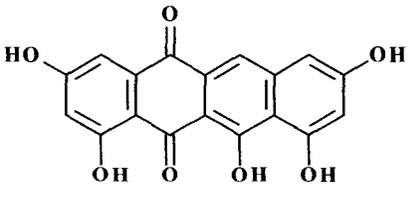
Dual targeting of topoisomerases I and II has been shown for some flavones and isoflavones at high (usually milli- rather than micro-molar) drug concentrations. For example, the isoflavone genistein (4', 5,7-trihydroxyisoflavone) (**42**) induced mammalian topoisomerase II dependent DNA cleavage *in vitro*; the cleavage activity was comparable to that of the standard topoisomerase II poisons m-AMSA and etoposide (Yamashita *et al* 1990). Boege *et al* (1996) reported that both quercitin (**43**) and, to a lesser extent genistein, inhibited topoisomerase I-catalysed religation of DNA. Quercitin produced a moderate amount of topoisomerase II-mediated DNA cleavage at 50μg/ml drug concentration,

however, this value was well above that required for cytotoxicity by quercitin which had, for example, an IC_{50} of 7μg/ml against the leukaemic CCRF-CEM cell line (Austin *et al* 1992).



(44)

Wassermann (1990) *et al* reported perhaps the first example of dual topoisomerase I and II poisoning by an antitumour agent. Actinomycin D (**44**), a natural antitumour antibiotic with limited clinical use, was found to stimulate both topoisomerase I- and II- induced DNA cleavage.



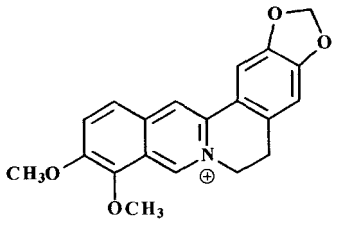
(45)

A 1991 paper by Yamashita *et al* reported dual topoisomerase I and II poisoning by the antitumour antibiotic saintopin (**45**), which produced levels of topoisomerase I-mediated DNA cleavage comparable to that of camptothecin and topoisomerase II-mediated DNA cleavage activity equipotent with m-AMSA and etoposide, in cell free systems. Later studies by Fujii *et al*, using immunoband depletion experiments with whole-cell lysates,

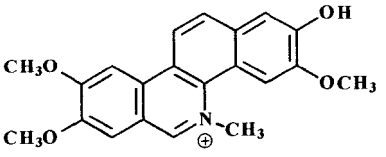
indicated that topoisomerase I was likely to be the principal cellular target of saintopin (Fujii *et al* 1997). Saintopin was used as a probe for the study of drug-enzyme interactions by dual topoisomerase inhibitors. Leteurtre *et al* (1994) proposed a 'drug-stacking' model that can accommodate a common topo I-topo II pharmacophore with the drug binding through hydrogen bonding and/or stacking with a base flanking the DNA termini and stacking with the catalytic tyrosine within the active-site pocket of the enzyme.

Protoberberine alkaloids, for example berberine (**46**), and structurally related organic cations display a range of topoisomerase I- and/or II poisoning abilities. The benzophenanthridine natural products nitidine (**48**) and fagaronine (**47**) are dual topo I and II poisons (Larsen *et al* 1993, Wang *et al* 1993), whereas the protoberberine analogue coralyne (**49**) poisons only topo I (Makhey *et al* 1996).

Structure-activity relationships on coralyne and a series of related compounds have shown that small structural changes can have a large effect on biological properties.

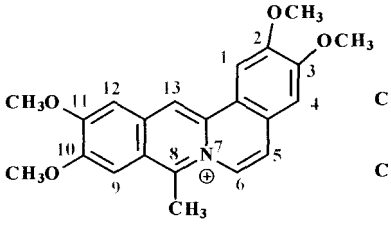


(46) Berberine

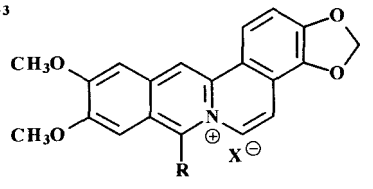


(47) Fagaronine

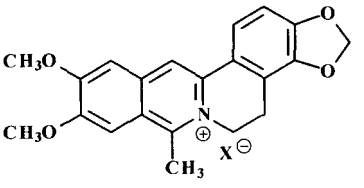
(48) Nitidine



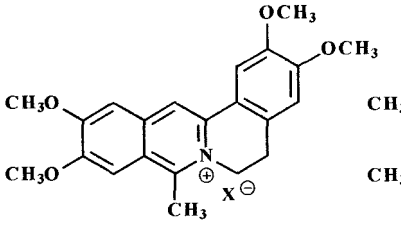
(49) Coralyne



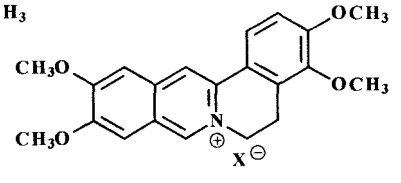
(50) 2c R=CH_3
(51) 10c R=H



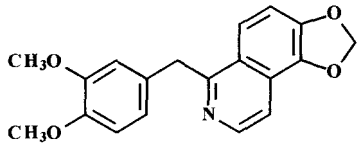
(52) 7c R=CH_3
(53) 8c R=H



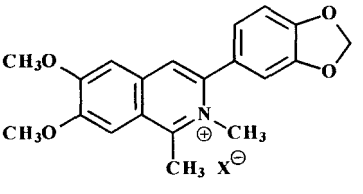
(54) 7a



(55) 8b



(56) 9c

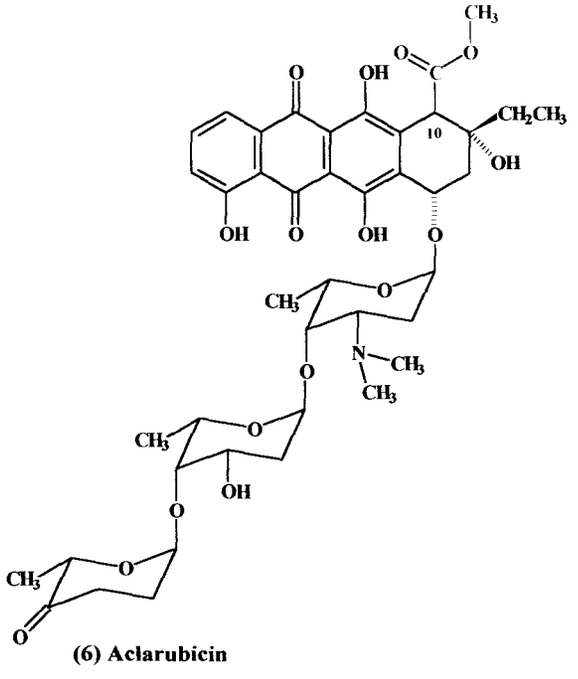


(57) 14b

Certain protoberberines, including (**50**), (**51**), (**52**) and (**53**) exhibited selective *in vitro* cytotoxicity against some solid tumour-derived cell lines, including SF-268 glioblastoma, compared to the RPMI 8402 leukaemia cell line (Sanders 1998). Despite these compounds being potent dual topoisomerase I and II poisons (Makhey *et al* 1996), this selective cytotoxicity was not linked to their interaction with topoisomerases, rather, it was

associated with the presence of an imminium ion and other structural features of protoberberines, principally 3,4-methylenedioxy substitution of the A-ring.

Compound (**54**), which differed from compound (**52**) only by its pattern of A-ring substitution, poisoned only topoisomerase I but retained selective *in vitro* cytotoxicity. Removal of the 8-methyl substituent and switching the A-ring dimethoxy substitution pattern from the 2,3- positions to 3,4- in compound (**55**) abolished all topoisomerase poisoning ability. The ring-opened and N-methylated quaternized coralyne analogues, (**56**) and (**57**) respectively, were inactive against both topoisomerases I and II.

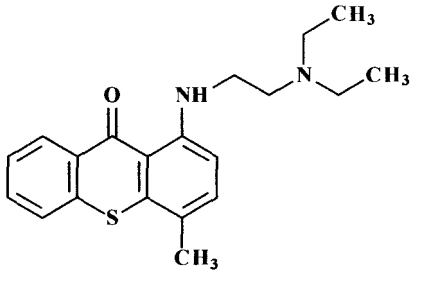


(6) Aclarubicin

The trisaccharide anthracycline aclarubicin (aclacinomycin A) (**6**), used clinically in the treatment of acute myelocytic leukaemia, has been shown to act as a (concentration dependent) topoisomerase I poison (Nitiss *et al* 1997) and a catalytic inhibitor of topoisomerase II (Sehested and Jensen 1996). This unusual pattern of dual topoisomerase activity contrasts greatly with that of the anthracyclines doxorubicin and daunorubicin; 'classical' topoisomerase II poisons that have no effect on topoisomerase I. The switch

between topoisomerase II poisoning and catalytic inhibition has been linked to the presence of a carboxymethyl group at C-10 (Jensen *et al* 1993).

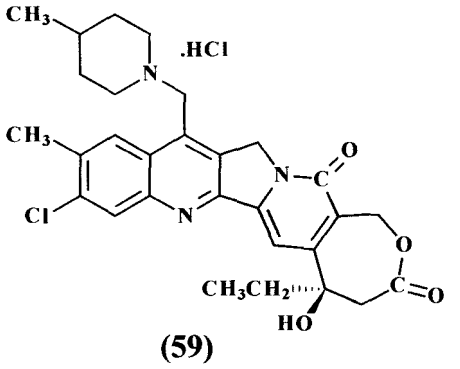
The observation that aclarubicin can kill both exponentially growing and plateau phase cells by a non-cell cycle-selective mechanism may be a consequence of simultaneous topoisomerase I and II inhibition (Bridewell *et al* 1997).



(58)

Lucanthone (**58**), an antitumour drug used as an adjuvant in radiation therapy, was reported to inhibit the catalytic activity of topoisomerases I and II at micromolar concentrations and stabilize topoisomerase II-DNA cleavable complexes, although the level of DNA double strand breaks induced by lucanthone was not clear (Bases and Mendez 1997).

Later work by Dassonneville *et al* (1999) confirmed that lucanthone produced significant topoisomerase II-mediated cleavage of plasmid DNA at 20 and 50μM drug concentrations.



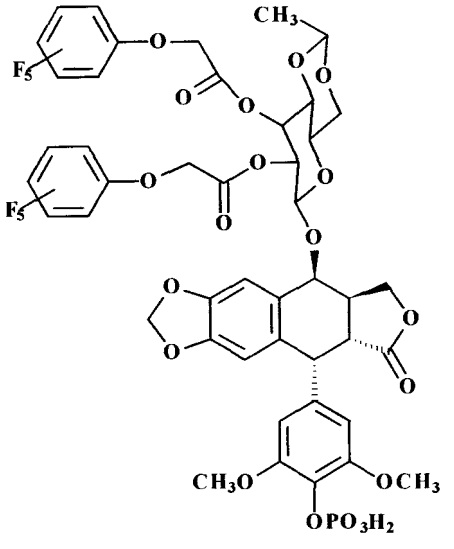
(59)

The water-soluble homocamptothecin, BN 80927 (**59**), which possesses an expanded (seven-membered) E-ring lactone (in contrast to the usual six-membered ring in the camptothecins), has been shown to be a potent topoisomerase I poison and a catalytic inhibitor of both topoisomerases I and II (Lavergne *et al* 1999). In cell-free systems, BN 80927 inhibited the topo I-mediated relaxation of supercoiled pUK19 plasmid DNA in a dose-dependent manner, giving a maximum of 70% relaxed DNA at 1μM. In immunoband depletion experiments in intact colon HT29 cells BN 80927 induced 3-fold higher levels of topo I-cleavable complex formation than SN-38, the active metabolite of the clinically used drug CPT-11 (irinotecan) (Demarquay *et al* 2000). BN 80927 also inhibited topo II-mediated relaxation of supercoiled pUK19 plasmid DNA producing >80% relaxed DNA at 1 and 10μM drug concentrations, an inhibitory activity equivalent to that of etoposide. This dual mechanism of action is unique for a compound so structurally related to camptothecin.

BN 80927 retained activity in PgP and MRP over-expressing cell lines and was potently cytotoxic on populations of resting G_0/G_1 HT29 cells; neither camptothecin, SN38 nor etoposide showed any activity against these cells (Huchet *et al* 2000).

Significantly higher plasma concentrations of the active form were achieved (90% remaining after 3h) than for camptothecin ($t_{1/2}$ 30 min) due to greater stability of the seven membered lactone ring. Insertion of the methylene spacer between the alcohol moiety and

the carbonyl group in the conventional six-membered α-hydroxylactone ring, found in camptothecin (**12**) and its clinically active analogues topotecan (**13**) and irinotecan (**14**), considerably reduced conversion into its inactive carboxylate form. Furthermore, BN 80927, administered orally, was more efficacious than the clinically used camptothecin derivative topotecan, administered intraperitoneally, in xenograft studies against PC3 and DU145 transplantable prostate tumours in mice and has proceeded to clinical trial.

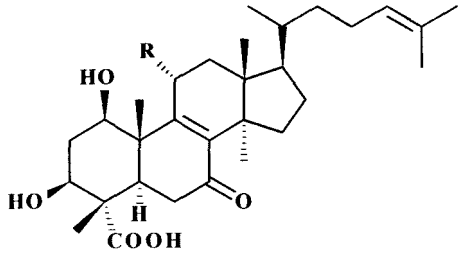


N-methyl-D-glucamine salt

(60)

F11782 (**60**), a fluorinated lipophilic epipodophylloid (Guminski *et al* 1999) currently in preclinical development by Pierre Fabre, has been shown to be a potent dual catalytic inhibitor of topoisomerases I and II (α and β). F11782 inhibited topoisomerase I mediated relaxation of plasmid DNA (IC_{50} 4.2μM) and inhibited the kDNA decatenation (of kDNA) activity of topoisomerase IIα and IIβ (IC_{50} values of 1.8μM and 1.3μM respectively) but did not stabilise either topoisomerase I- or II-mediated cleavable complex formation (Perrin *et al* 2000). This mechanism of action is in marked contrast to that of its parent compound etoposide; a classical topoisomerase II poison which does not act as a catalytic inhibitor of either topoisomerase I or II (Hande 1998).

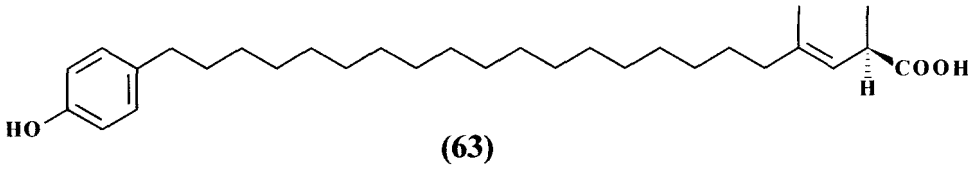
It has been proposed that F11782 inhibits the binding of topoisomerases to DNA by a direct interaction between the drug and enzyme. F11782 neither binds to DNA nor stabilises cleavable complex formation but has been shown to induce DNA damage in wild-type Chinese Hamster Ovary CHO-K1 cells by the formation of DNA double-strand breaks, a feature normally associated with topo poisons rather than catalytic inhibitors and appears to be unique to F11782 (Barret *et al* 2002a). Despite having only moderate cytotoxicity *in vitro* with, for example, IC_{50} values against the human GCT27 testicular teratoma and A2780 non-small cell lung cancer cell lines of 0.18μM and 67μM respectively (Barret *et al* 2002b), F11782 displayed significant *in vivo* antitumour activity against a variety of murine and human tumour models (Kruczynski *et al* 2000). This compound also showed synergistic cytotoxicity *in vitro* when incubated simultaneously with many standard clinical agents including the topoisomerase II poisons doxorubicin and etoposide.



(61) R = OH
(62) R = H

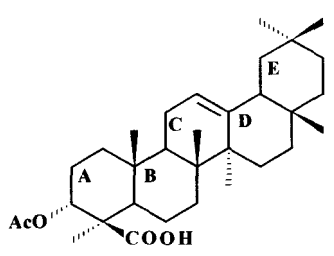
Mizushina and co-workers (2000) have investigated the effects of novel natural triterpenoids (**61**) and (**62**) on the activities of many DNA associated enzymes including human topoisomerases I and II. Concentrations of 100μM for each compound inhibited the topoisomerase I and topoisomerase II mediated relaxation of plasmid DNA by 85% and 95% respectively. The activities of eukaryotic DNA polymerases were also inhibited by > 70% despite neither compound binding to DNA, suggesting that they act directly on

these enzymes even though the mode of action, amino acid sequences and three-dimensional structures of topoisomerases I and II and DNA polymerase are markedly different. The authors did not report whether the compounds had any effect on cleavable complex formation and suggested that they inhibited the catalytic activity of topoisomerase I and II enzymes prior to DNA binding. Despite compound (**61**) being cytotoxic *in vitro* against the human stomach cancer cell line NUGC with an LD_{50} value of 38μM no further anti-cancer studies were reported for these triterpenoids, perhaps due to insufficient compound isolation.

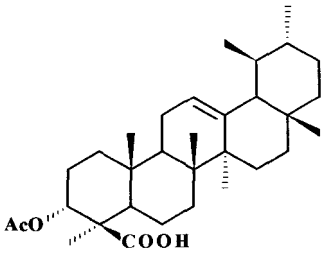


(63)

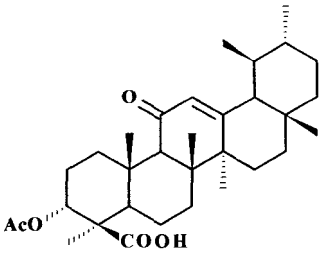
Later studies from the same laboratory described analogous inhibition of topoisomerases I and II and eukaryotic DNA polymerases by (R)-(−)-elenic acid (**63**), an alkylphenol produced by an Indonesian sponge and synthesised in bulk by the authors (Mizushina *et al* 2002). Elenic acid completely inhibited the catalytic activity of both topoisomerase I and II at 2μM and calf DNA polymerase at 15μM but had no effect on other DNA metabolising enzymes, for example, prokaryotic DNA polymerase or HIV-1 reverse transcriptase. Compound (**63**) was moderately cytotoxic against a human gastric cancer cell line NUGC-3, with a LD_{50} value of 22.5μM. The authors erroneously appeared to assume that lack of DNA binding by this compound (determined by uv thermal melt analysis) would preclude cleavable complex stabilisation; the topoisomerase I poison camptothecin binds neither to DNA nor the enzyme alone, rather only to the 'cleavable complex'.



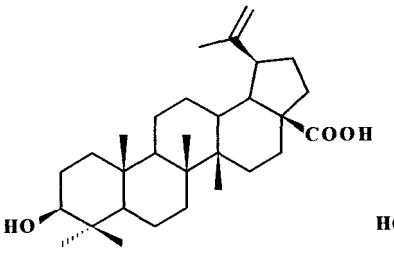
(64) 3-O-Acetyl-α-boswellic acid (AαBA)



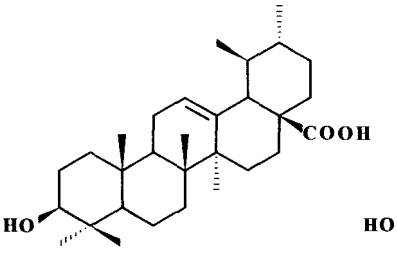
(65) 3-O-Acetyl-β-boswellic acid (AβBA)



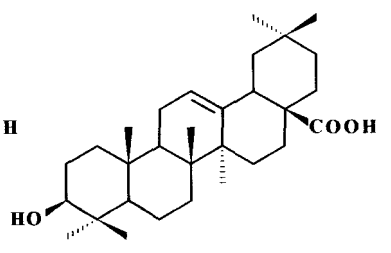
(66) 3-O-Acetyl-11keto-β-boswellic acid (AKβBA)



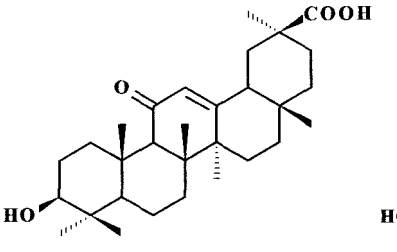
(67) Betulinic acid



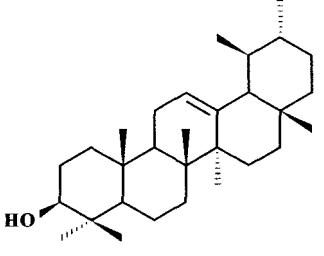
(68) Ursolic acid



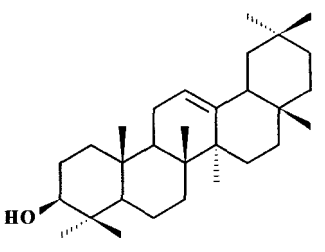
(69) Oleanolic acid



(70) 18-β-Glycyrrhetinic acid



(71) α-Amyrin

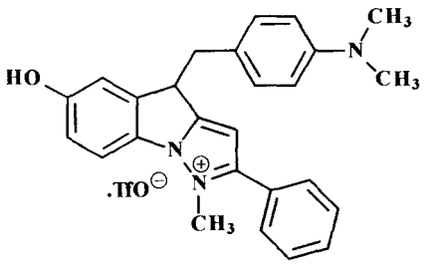


(72) β-Amyrin

Acetyl-boswellic acids and related pentacyclic triterpenes; natural products with antiviral (Pavlova *et al* 2003) and potent anti-inflammatory activity (Safayhi *et al* 1997), have recently been shown to be cytotoxic *in vitro* against a variety of human cancer cell lines. Studies have been carried out by Syrovets and co-workers to determine a possible mechanism of action for this class of compound. The ability of nine structurally related pentacyclic triterpenes to inhibit the topoisomerase I- and IIα- mediated relaxation of DNA was determined by gel electrophoresis. All three acetyl-α-boswellic acids [(**64**), (**65**) and (**66**)] inhibited human topoisomerase I and IIα in a concentration-dependent manner. Compound (**64**) was most potent with IC_{50} values for the inhibition of the catalytic activity

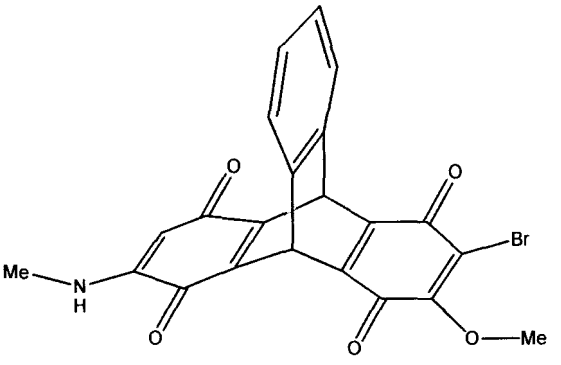
of topoisomerase I and IIα of ~3μM and ~1μM respectively. Betulinic acid (**67**), which was the most effective of the other pentacyclic triterpenes [compounds (**67**) to (**72**)], inhibited topoisomerase I and IIα with IC_{50} values of ~43μM and ~5μM respectively. None of the compounds stabilized cleavable complexes.

Within this series of triterpenes carboxylation of the pentacyclic ring structure, particularly on rings A and D, was necessary for topoisomerase inhibition; compounds (**70**), (**71**) and (**72**) lacking a carboxylic group on these rings were inactive against both topoisomerases I and IIα. The authors found that inhibition of topoisomerases by these compounds was independent of either DNA intercalation or minor groove binding and proposed that the mechanism of inhibition was by direct binding to topoisomerases (Syrovets *et al* 2000). A similar mechanism of dual topoisomerase inhibition has been proposed for the aforementioned triterpenoids (**61**) and (**62**), studied by Mizushina *et al*. Experiments to determine the ability of these compounds to inhibit the topoisomerase IIβ- mediated relaxation of DNA were not carried out. In a 1998 paper by Perrin *et al* topoisomerase interacting antitumour agents were tested for their ability to selectively inhibit the catalytic activity of either the α or β isoforms of topoisomerase II. Although the catalytic inhibitors tested in this study were mostly more sensitive towards the α form, the topoisomerase II poisons doxorubicin and mitoxantrone were three times more sensitive towards the β isoform (Perrin *et al* 1998). Topoisomerase IIβ- as a target for pentacyclic triterpenes should not be overlooked.



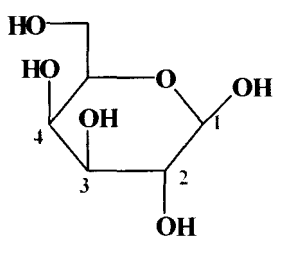
(73)

The pyrazolo[I,5-*a*]indole derivative (**73**) has been shown to be a potent dual catalytic inhibitor of topoisomerases I and II (IC_{50} values of 10μM and 20μM respectively) in cell free assays and in living cells but did not stabilize DNA-topo I/II cleavable complexes. In immunoband depletion experiments in DLD-1 human colon carcinoma cells, pre-treatment of the cells with 150μM of compound (**73**) prior to incubation with either camptothecin or etoposide, resulted in restoration of free topoisomerases I and II to near original levels. This indicates that compound (**73**) targets these enzymes in living cells preventing their incorporation into the cleavable complexes by the topoisomerase poisons camptothecin and etoposide. Compound (**73**) was active *in vitro* in the NCI human cancer cell line panel (47 cell lines) with a mean GI_{50} value of 1.2μM although the authors did not indicate whether the COMPARE programme confirmed a possible topoisomerase-mediated mechanism of action (Umemura *et al* 2002).

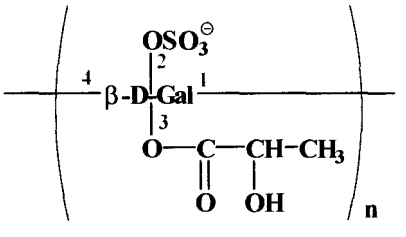


(74)

A synthetic triptycene analogue (**74**), with potent cytotoxicity in the nanomolar range *in vitro*, has recently been shown to be a dual catalytic inhibitor of topoisomerases I and II (Wang *et al* 2003). This compound was more effective at inhibiting topoisomerase II activity than m-AMSA and was equipotent with camptothecin in topoisomerase I relaxation assays.



β-D-galactopyranose

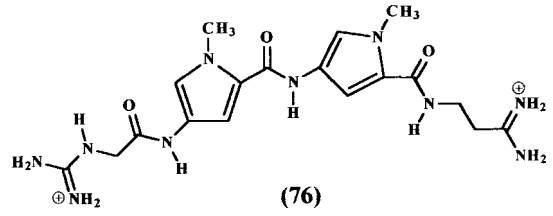


(75) GA3P
(the numbers indicate the position on the galactopyranose ring)

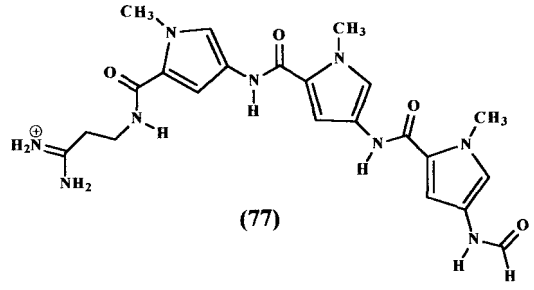
A cytotoxic marine microalgal polysaccharide (**75**), consisting of a D-galactan sulphate unit associated with L-(+)-lactic acid, has been shown to be an extremely potent dual catalytic inhibitor of topoisomerases I and II, irrespective of the presence or absence of the lactate group (Umemura *et al* 2003). Compound (**75**) inhibited the topoisomerase I-mediated relaxation of pT2GN plasmid DNA with an IC_{50} of ~0.017μg/mL; dextran sulphate, a related polysaccharide had an IC_{50} of ~0.006μg/mL. IC_{50} values of

~0.048μg/mL and ~0.024μg/mL were obtained for the inhibition of topoisomerase II–mediated decatenation of k-DNA by (**75**) and dextran sulphate respectively. Neither dextran sulphate nor compound (**75**) stabilised DNA-topoismerase I or II cleavable complexes. Despite being a potent catalytic inhibitor of both topoisomases I and II dextran sulphate was not cytotoxic, whereas compound (**75**) had GI_{50} values ranging from 0.67-11μg/mL in a panel of 38 human cancer cell lines; it seems unlikely that the mechanism of cell kill for compound (**75**) involves topoisomerase inhibition.

Additionally, a 1998 paper by Fung *et al.* described the isolation of a novel antitumour compound, codenamed S2, from the mucus of a coral, *galaxea fascicularis*, thought to contain a Galβ(1-4)GclNAc dissacharide linkage at its active centre, though full structural characterization was incomplete. The authors claimed that extract S2 inhibited the relaxation of supercoiled DNA by topoisomerases I and II and stabilised topoisomerase I-DNA cleavable complexes however, as the quality of the gel photographs was extremely poor and comparator topoisomerase inhibitors were not included in any of the experiments the validity of the quantifiable data must be called into question (Fung *et al* 1998).

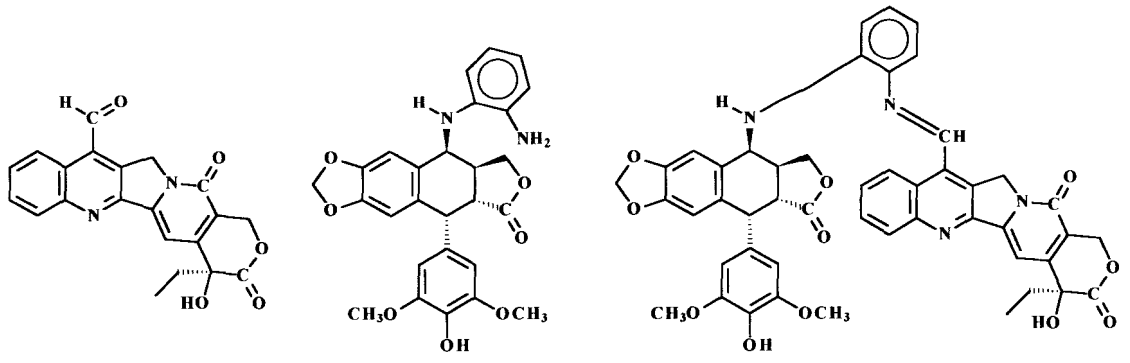


(76)



(77)

The experimental antibiotics netropsin (**76**) and distamycin (**77**), along with many other groove binding molecules, have been shown to modulate the activities of topoisomerases I and II. Both are crescent shaped, N-methyl pyrrole-2-carboxamide containing oligoamides, which bind with great specificity to narrow AT regions of the minor groove following the turn of the DNA helix. This pronounced sequence selectivity of binding to AT rich regions of DNA is due to a combination of steric, conformational and electrostatic factors. McHugh *et al* reported that distamycin stimulated topoisomerase I relaxation of supercoiled DNA at low drug concentrations whilst drug concentrations of greater than 2μM resulted in inhibition of topoisomerase I activity (McHugh *et al* 1989). Distamycin was found to stimulate topoisomerase II-mediated DNA relaxation, antagonised the inhibitory effect of etoposide and modulated topoisomerase II-mediated cleavage of SV40 DNA induced by etoposide (Fesen and Pommier 1989). Both netropsin and distamycin were reported to stabilize topoisomerase I-DNA ternary complexes, inducing limited but highly specific cleavage of DNA in regions with extremely high AT content (Chen *et al* 1993).



(78) (79) (80)

Finally, a 'twin drug' approach adopted by several groups (Sondhi *et al* 1997) involved the physical linking of separate inhibitors of topoisomerase I and II, or the attachment of pure topoisomerase inhibitors to other DNA-interactive carriers, in an attempt to design molecules with dual topoisomerase targeting ability. Compound (**80**) is given as a typical example of a hybrid molecule consisting of a topoisomerase I poisoning camptothecin derivative (**78**) joined via by an imine linker to a topoisomerase II poisoning 4′-O-demethyl epipodophyllotoxin derivative (**79**). The hybrid molecule (**80**) stabilised cleavable complexes with both topoisomerase I and II, however, the level of cleavage was considerably lower than for the unconjugated compounds (**78**) and (**79**). Compound (**80**) was cytotoxic *in vitro* and retained activity against cell lines resistant to either standard topoisomerase II poisons, camptothecin or the antimitotic agent vincristine. The circumvention of topoisomerase-mediated mechanisms of drug resistance may be a result of simultaneous targeting of topoisomerases I and II by this compound. To date, no experimental agents of this type have entered clinical trial.

CHAPTER THREE

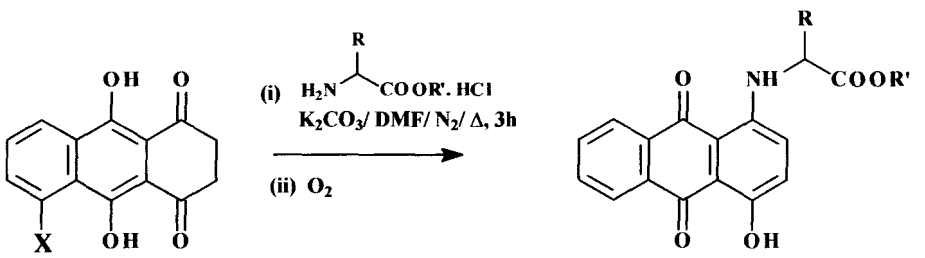
[PART A]

ANTHRAQUINONE-AMINO ACID CONJUGATES

3 Anthraquinone-Amino Acid Conjugates

Previous studies from this laboratory have shown that 1:1 conjugates of anthraquinones and amino acids (C-terminally free) constitute a new class of cytotoxic agent (Mincher 1993). Compounds with general structure (**83**) were prepared by reacting leucoquinizarin (**81**) or leuco-5-hydroxyquinizarin (**82**) with the free amino acid ester (liberated from the hydrochloride salt by the addition of an excess of potassium carbonate) in DMF, under nitrogen [**Scheme 3**]. Derivatisation reactions were carried out to form hydrazides by reaction of the derived anthraquinone amino ester conjugates with hydrazine hydrate in methanol.

Scheme 3



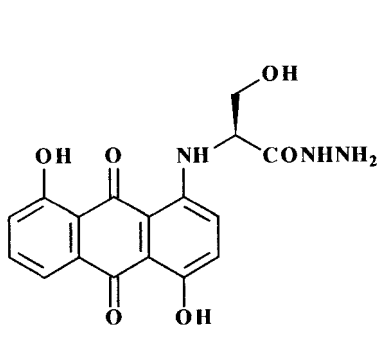
(81) X = H = leucoquinizarin
(82) X = OH = leuco-5-hydroxyquinizarin

(83)

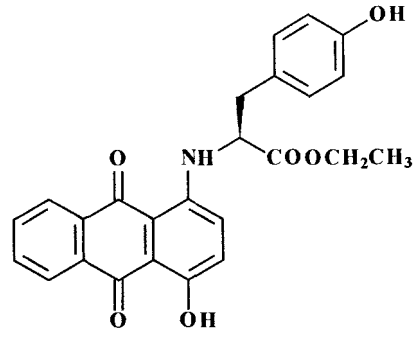
The more cytotoxic members of the series were shown to exert their antitumour effect, in part, by selective inhibition of human DNA-topoisomerase enzymes. For example, the serine hydrazide conjugate NU:ICRF 506 (**84**) inhibited the *in vitro* catalytic activity of topoisomerase I (IC_{50} < 5 μg/ml) and topoisomerase II (IC_{50} < 5 μg/ml) in plasmid DNA relaxation experiments, determined by gel electrophoresis, but did not stabilise the cleavable complex (in contrast to the mechanism of action of the topo I poison camptothecin or the topo II poisons amsacrine and adriamycin). Furthermore, this

conjugate inhibited the topoisomerase II (predominately the α-form, purified from HeLa cells) -mediated decatenation of kinetoplast k-DNA, with an IC_{50} of less than 1 μg/ml.

In vitro cytotoxicity studies against a variety of human cell lines have shown that compound NU:ICRF 506 (**84**) had mean IC_{50} values in the low μM range with, for example, IC_{50} values against human MCF-7 and ZR-75-1 human breast cancer cell lines of 8μM and 5.5 μM respectively and 1.4 μM against the A2780 human ovarian cancer cell line.



NU:ICRF 506
(84)



NU:ICRF 505
(85)

Significant broad spectrum in vitro activity was also displayed by compound NU:ICRF 505 (**85**), a tyrosine ethyl ester conjugate with, for example, IC_{50} values against the A549 human non-small cell lung cancer cell line and the daudi human lymphoma cell line of 8μM and 8.3 μM respectively. This compound was found to be an inhibitor of topoisomerase I through stabilisation of the cleavable complex formed between DNA and the enzyme in a manner similar to camptothecin with induction of 38% nicked DNA lesions for compound (**85**) and 58% for camptothecin compared to drug-free enzyme only controls. (Meikle *et al* 1995a). NU:ICRF 505 (**85**) has also been shown to circumvent Pgp-mediated and altered topoisomerase drug resistance mechanisms *in vitro*. NU:ICRF 505 was noncross-resistant against a camptothecin resistant (3.4-fold) Chinese hamster ovarian cell line CHO ADR-3 and only 1.8-fold resistant in the doxorubicin-resistant human

ovarian cell line A2780^{AD}, generally considered to possess the classic MDR phenotype. Hypersensitivity to the topoisomerase I overexpressing CHO ADR-r (doxorubicin-resistant) cell line was shown by NU:ICRF 505 (Cummings *et al* 1996).

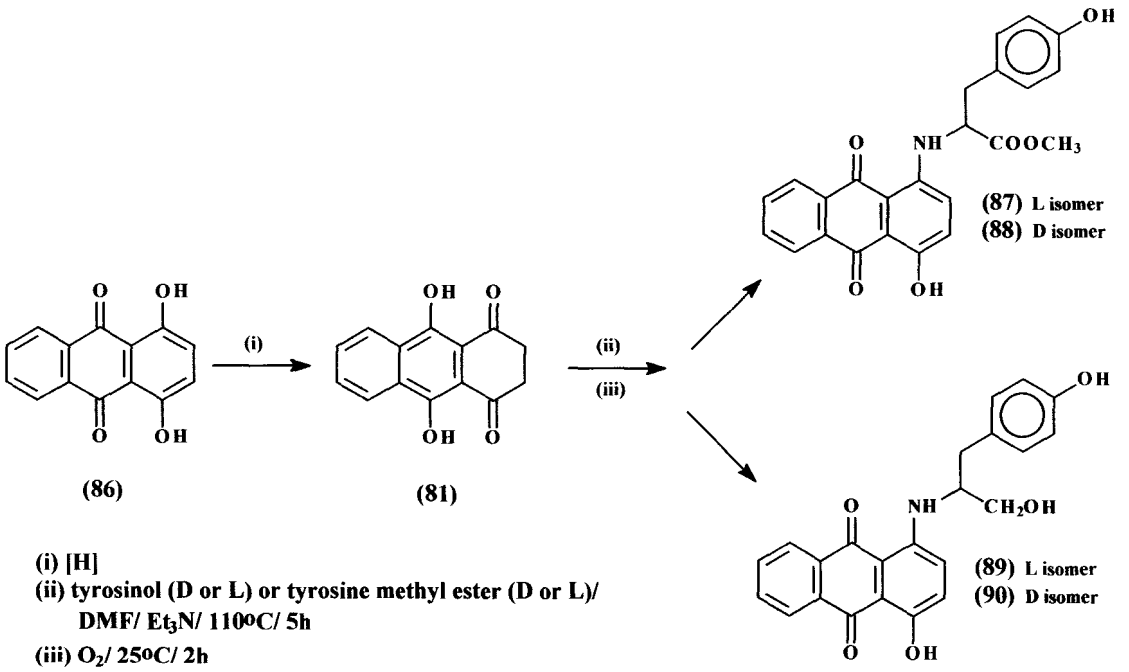
As a consequence of these selective topoisomerase interactions the foregoing compounds progressed to in vivo experiments where activity was demonstrated against human breast, colon and lung xenografts, however poor aqueous solubility and insufficient bioavailability prevented further development.

Limited molecular modelling studies (Meikle *et al* 1995a) were carried out on eight anthracenyl-amino acid conjugates, which though structurally similar, differed greatly in their topoisomerase inhibitory profiles. The eight equivalent atoms of the anthraquinone ring system were overlaid, leaving each amino acid substituent in its preferred energy minimised conformation. Compounds used in this study included the tyrosine ethyl ester conjugate, the topo I poison NU/ICRF 505, NU/ICRF 513 (a dihydroxyphenylalanine methyl ester conjugate, and topo II catalytic inhibitor) and NU/ICRF 514 (a tyrosine methyl ester conjugate, and weak topo II catalytic inhibitor). It was found that each amino acid substituent projected out from the overlaid anthraquinone ring system in a different conformation with the dihydroxyphenyl ring in NU/ICRF 513 parallel with the anthraquinone and the phenyl ring system in both NU/ICRF 505 and NU/ICRF 514 projecting from the anthraquinone in completely opposing directions. It was concluded that the observed differences in topoisomerase inhibition for this class of compound are a consequence of major conformational alterations brought about by small changes in the amino acid substituent, suggesting that the structural requirements for topo I cleavage are much more rigid than for topo II inhibition.

Zagotto *et al* have synthesised a small series of D- and L- aminoacyl-anthraquinone derivatives, analogues of the aforementioned NU:ICRF 505 (**85**), to investigate the effects of chirality on DNA sequence recognition (Zagotto *et al* 2000).

1,4-Dihydroxyanthraquinone (quinizarin) (**86**) was converted to its leuco form (**81**) using potassium carbonate and sodium hydrosulphite under nitrogen and reacted with either tyrosine methyl ester (D or L) or tyrosinol (D or L), prepared by sodium borohydride reduction of the corresponding methyl esters, to give compounds (**87**), (**88**), (**89**) and (**90**). The synthesis is outlined below in [**Scheme 4**].

Scheme 4



In vitro cytotoxicity testing found that the compounds were almost completely inactive against human prostate (PC3) and lung (H460) carcinoma with IC_{50} values >> 100μM. These results contrast with studies carried out in this laboratory on the L-tyrosine methyl ester (**87**); it was not acknowledged by the authors that a compound previously synthesised in this laboratory and codenamed NU:ICRF 514 (Mincher 1993) had, for

example, IC_{50} values against the COLO-320DM human colon cancer cell line and the MCF-7 human breast cancer cell line of 12μM and 13.5μM respectively. Zagotto does not report the protocol for solubilisation of the test compounds for cytotoxicity assays; an explanation of the discrepancy may be that the agents were not truly in solution, although phenotypic differences between cell lines may account for the observed lack of cytotoxic potency.

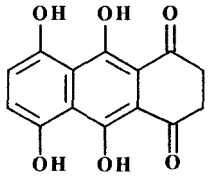
Indeed, the authors claimed that DNA binding studies of the compounds could not be performed due to serious solubility problems with precipitates forming even at micromolar concentrations.

In a further attempt to determine possible DNA-recognition by the compounds molecular modelling studies were carried out. The eight equivalent atoms of the anthraquinone ring system were overlaid whilst leaving the chiral amino acid substituent in its preferred energy minimised conformation. It was found that the stereochemistry at the α-amino acid chiral centre had little effect on the three-dimensional structure of each isomer. (Again, these results contrast with the molecular studies of Meikle *et al* in which it was reported that small changes in the amino acid substituent had a major effect on its conformation). The authors summarily concluded that a longer peptide would be required for chiral recognition of DNA.

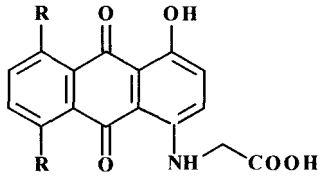
Morier-Teissier and co-workers have investigated the potential use of anthraquinone-peptide conjugates as redox active drugs by exploiting the ability of the quinone moiety to undergo enzyme mediated reduction leading to the formation of hydroxy radicals and thereby to effect DNA cleavage.

Initial studies reported the attempted 1,4- bis-amination of leucoquinizarin with glycine, based upon the method of Greenhalgh and Hughes (Greenhalgh and Hughes 1968).

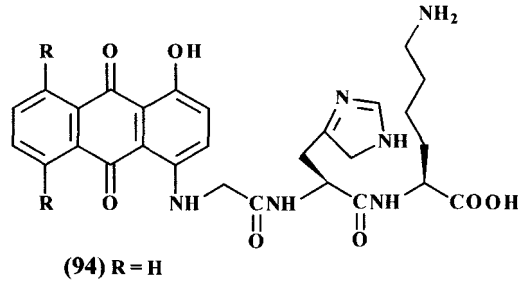
When either leucoquinizarin (**81**) (2,3-dihydro-9,10-dihydroxy-1,4-anthracenedione) or 5,8-dihydroxyleucoquinizarin (2,3-dihydro-5,8,9,10-tetrahydroxy-1,4-anthraquinone) (**91**) were refluxed with glycine and triethylamine in de-aerated ethanol only the monosubstituted products (**92**) and (**93**) were obtained. The observed nuclear monosubstitution (with the free amino acid) was thus consistent with the aminations conducted with amino acid esters, even when the amine was used in excess (Mincher 1993). Dicyclohexylcarbodiimide and hydroxybenzotriazole mediated coupling of compounds (**92**) and (**93**) with a preformed dipeptide His-Lys (as the Z-protected benzyl ester) gave the protected anthraquinone-tripeptide intermediates. Co-removal of the Z-protecting group and the benzyl ester using hydrogen bromide-saturated acetic acid gave compounds (**94**) and (**95**) (Morier-Teissier *et al* 1990).



(91)



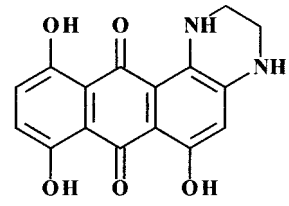
(92) R = H
(93) R = OH



(94) R = H
(95) R = OH

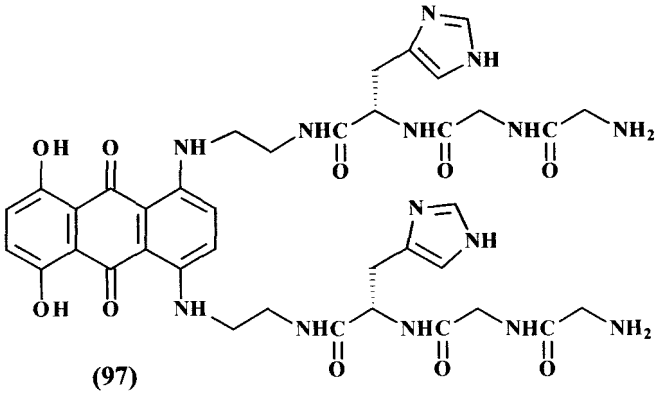
The gly-his-lys conjugates incorporated the known human plasma copper-binding growth factor glycyl-L-histidyl-L-lysine, a metal carrier with high affinity for copper and iron (Pickart and Lovejoy 1987). The monosubstituted compounds had weak DNA-intercalative properties but the peptide part of the molecule was shown to complex with copper ions and under somewhat strict conditions, with hydrogen peroxide and ascorbate produced free radicals (ultimately hydroxy radicals) that induced DNA breakage. It is interesting to note that this approach attempted to exploit the redox properties of anthraquinones to effect cell kill by free radicals against the trend to eliminate free radical production in the anthraquinone and anthracycline families because of the association with

undesirable side effects. None of the monosubstituted compounds had any indication of potential use in cancer chemotherapy. Further studies in the same laboratory (Morier-Teissier *et al* 1993) were aimed at mitoxantrone analogues incorporating two side chains (to provide improved affinity for DNA) and the metal-chelating peptide. The side chains had diaminoethane spacers (the NCH_2CH_2N- pharmacophore in mitoxantrone). It was found that 1,4-bis-amination of 5,8-dihydroxyleucoquinizarin was achieved by insertion of the (aminoethyl)amino spacer groups; the authors did not record that these compounds were the subject of a patented invention by the American Cyanamid Company (see below). The method of Greenhalgh and Hughes was again adopted, this time to ensure bis-amination, and used mono-^{t}Boc-protected ethylenediamine during the nuclear amination step to prevent formation of the unwanted cyclization product 6-[2-aminoethyl)amino]-8,11-dihydroxy-1,2,3,4-tetrahydronaphtho[2,3-*f*]quinoxaline-7,12-dione (**96**).



(96)

Removal of the ^{t}Boc protecting group with trifluoroacetic acid followed by reaction with a preformed protected tripeptide Z-Gly-Gly-L-His-OH (replacing the earlier gly-his-lys), using the same coupling and deprotection procedure described in the previous reaction, gave compound (**97**) as the dihydrobromide salt. The tripeptide gly-gly-his had earlier been used as a metal chelating cleavage agent (Mack *et al* 1988). It was speculated that such a molecule might mimic known oxidases that contain a copper-chelating group associated with a covalently bound hydroquinone cofactor (Ito *et al* 1991).



(97)

The ability of the anthraquinone gly-gly-his conjugate (**97**) to interact with calf thymus DNA was determined using UV thermal melt analysis. Compound (**97**) was found to bind strongly to DNA with a ΔT_m of 17.1°C compared to 15.9°C for mitoxantrone; fluorescence and viscometry experiments indicated an intercalative mode of binding to DNA. This compound also formed several types of pH dependant copper-complexes, produced a substantial amount of free radical formation and was potently cytotoxic in vitro against the murine L1210 leukaemia and human MCF-7 breast cancer cell lines with IC_{50} values of 0.05μM and 0.80μM respectively.

In contrast to the monosubstituted gly-his-lys conjugate (**95**), which was cytotoxic in vitro but inactive in vivo, the gly-gly-his containing conjugate (**97**) showed significant in vivo activity in the P388 leukaemia model, with a T/C value of 230 at 25mg/Kg compared to a T/C value of 190 for mitoxantrone at its optimal dose of 1.6mg/Kg.

It was speculated that the lack of *in vivo* activity observed with the gly-his-lys conjugate was either due to rapid degradation of the peptide or poor cell penetration. In fact, it is most likely that the C-terminally free carboxylic acid group would lead to poor cellular uptake of these compounds due to ionisation at physiological pH.

Similar 4-hydroxy-1-aminoanthraquinone dipeptide conjugates, terminating in negatively charged carboxylate groups were devoid of *in vitro* cytotoxicity (Meikle *et al* 1995b). This was attributed to the charge preventing cellular uptake, a process that would most likely

proceed by passive diffusion as for many of the established anti-tumour drugs (Baguley 1991) in contrast to their hydrophobic ester derivatives which were taken up by cells and were actively cytotoxic.

Gatto and co-workers have prepared a series of peptidyl anthraquinones containing 1 or 2 side chains at positions 1 and/ or 4 of the ring system (Gatto *et al* 1996). All compounds possessed an amido linkage between the anthraquinone chromophore and the first amino acid (always glycine). The mono-substituted compound (**99**) was prepared by the DCC mediated coupling of Boc-glycine to 1,4-diaminoanthraquinone (**98**) in the ratio of 1:2. [**SCHEME 5**].

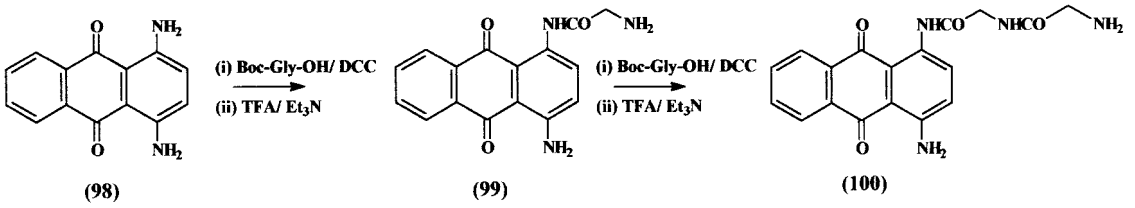
Synthesis of the 1,4-bis-substituted glycine conjugate (**101**) required formation of ω-(bromoacetamido)-1,4-diaminoanthraquinone which was reacted with potassium phthalimide followed by hydrazinolysis with methyl hydrazine [**SCHEME 6**]. Stepwise addition of the appropriate N-protected, C-activated amino acid (gly, D-lys, L-lys, D-trp and L-trp) further extended the peptide side chain up to three amino acids.

Compounds (**99-107**) were studied for their ability to stimulate topoisomerase II mediated cleavage of ^{32}P-labeled SV40 DNA. The glycine and tryptophan conjugates (**99**), (**101**), (**100**), (**102**), (**103**), (**104**) and (**105**) displayed cleavage patterns similar to mitoxantrone although the cleavage band intensity was considerably lower. These compounds were also shown to be moderately cytotoxic in vitro. The glycyl conjugates (**99**), (**100**), (**101**), (**102**) and (**103**) were comparable in cytotoxic potency to ametantrone with IC_{50} values in the low μM range against three tumour cell lines (HL60, HeLa and L1210), whether one or two anthraquinone side-chains or glycine residues were present. However, the conjugates containing D- and L-tryptophan were an order of magnitude less active against the human leukaemia HeLa cell line (IC_{50} values in the human HL60

and murine L1210 cell lines were not determined). In contrast, the lysine-containing conjugates, (**106**) and (**107**), were completely inactive (IC_{50} > 100μM), despite inhibiting topoisomerase II functions and binding strongly to DNA (to some extent) in a sequence specific manner (preferentially binding to alternating GC base sequences) (Gatto *et al* 1997). These results are consistent with the observation that strong sequence specific binding to DNA alone is not sufficient for anticancer activity. Interestingly, the higher binding affinity (2-3 fold) displayed by the D- enantiomer compared to its L-isomer (and mitoxantrone) was thought not to be purely electrostatic in nature, as would be expected, but was attributed to more favourable hydrophobic contacts between the D-lysyl side chains and DNA base pairs.

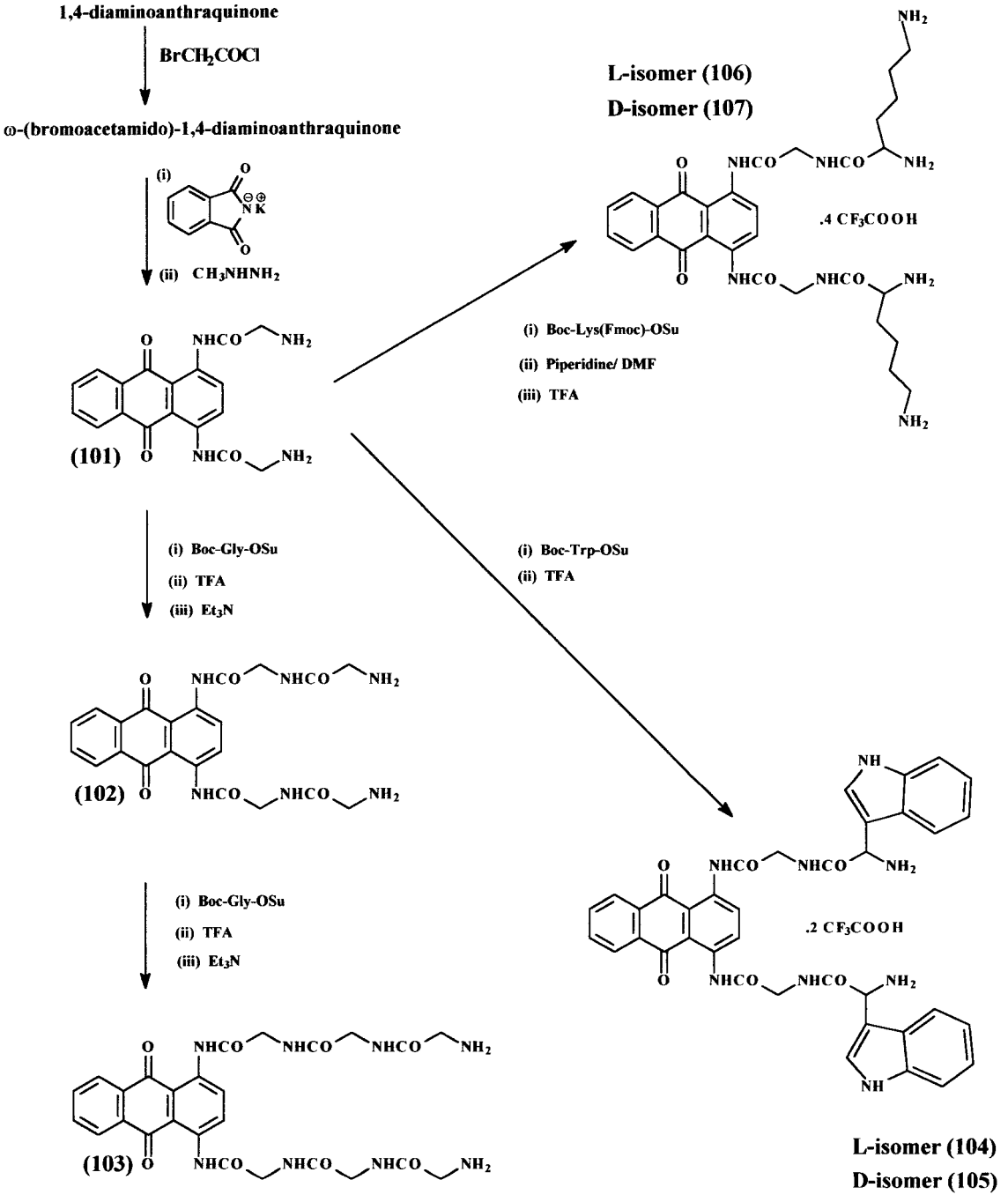
The authors correlated cytotoxicity, and the ability to stimulate topoisomerase II-mediated DNA cleavage, to the nature of the amino acids in the anthraquinone side chains and attributed the lack of activity for the tryptophan and lysine conjugates to the steric bulk of the amino acid side-chains and, in the case of lysine, the presence of additional positive charges.

Scheme 5



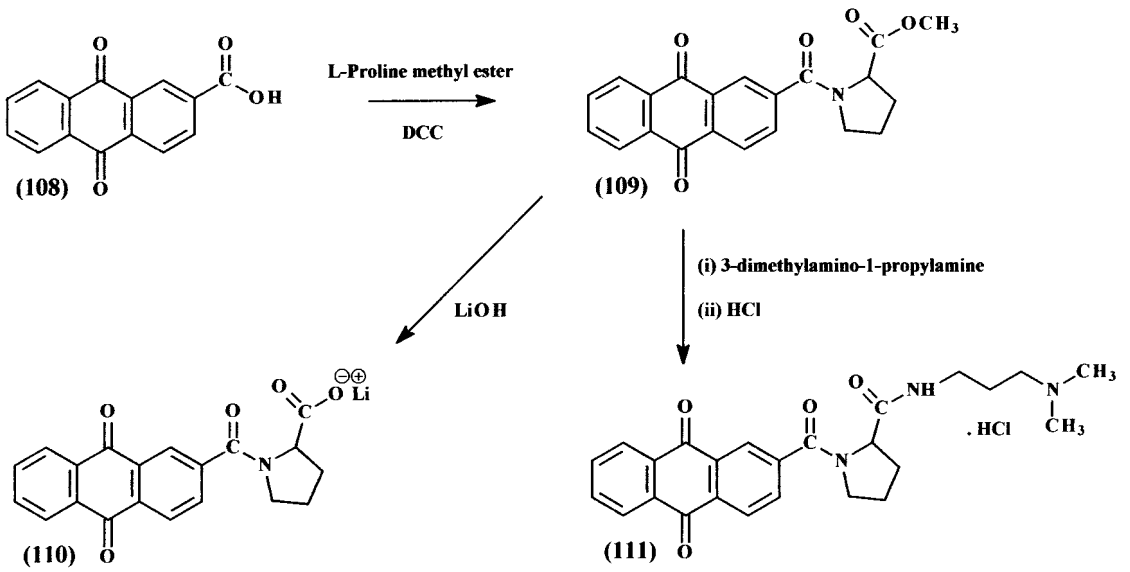
(98) (99) (100)

Scheme 6



Bielawska and co-workers have synthesised [**Scheme 7**] three structurally diverse L-proline analogues of anthraquinone-2-carboxylic acid (**108**) containing electrostatically neutral, anionic and basic amino acid C-termini [(**109**), (**110**) and (**111**) respectively] (Bielawska *et al* 2001a).

Scheme 7

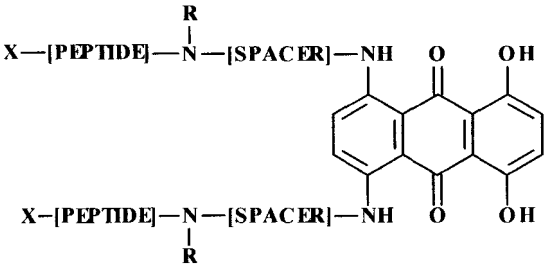


Compound (**109**) was prepared by carbodiimide mediated coupling of L-proline methyl ester with anthraquinone-2-carboxylic acid. Saponification of (**109**) using LiOH gave compound (**110**). The methyl ester conjugate (**109**) was aminated with 3-dimethylamino-1-propylamine and acidified with HCl affording compound (**111**).

The compounds were designed as potential substrates for prolidase [E.C.3.4.13.9], an enzyme overexpressed in some neoplastic tissues, including breast cancer. Prolidase has the ability to hydrolyse the imido bond of various low molecular weight compounds coupled to proline (Bielawska *et al* 2001b). Treatment of the compounds with prolidase generated anthraquinone-2-carboxylic acid and L-proline.

Cytotoxicity was determined in the MCF-7 breast cancer cell line by inhibition of [^{3}H]thymidine incorporation into DNA. All compounds were poorly cytotoxic, compound (**109**), the L-proline methyl ester, was least active with an IC_{50} of 185μM and did not inhibit topoisomerase I or II mediated pPR322 plasmid DNA at concentrations from 5-120μM. The N,N-dimethylaminopropyl containing conjugate (**111**) was found to be most active with an IC_{50} of 87μM against the MCF-7 cell line. This compound was also found to inhibit the catalytic activity of both topoisomerases I and II at 30μM and 60μM respectively. The authors did not include comparator topoisomerase inhibitors in the enzyme-mediated pBR322 plasmid DNA experiments; inspection of the gel photographs suggests that the quality of the supercoiled plasmid was poor, calling into question the validity of the quantifiable data.

Novel antitumour amino acid and peptide derivatives of 1,4-bis[(aminoalkyl and hydroxyaminoalkyl)amino]-5,8-dihydroxyanthaquinones were the subject of a 1988 European patent application by the American Cyanamid Company, EP 0295316 (Fields *et al* 1988). The divalent spacer moiety contained either straight chain or branched alkyl groups; where the spacer was $-CH_2-CH_2-$ and R was $-CH_2-CH_2-OH$ then the compounds were peptide conjugates of mitoxantrone. Peptides typically contained 2 to 5 D or L amino acids. The patent described the synthesis of compounds belonging to general structure (**112**), and reported their *in vivo* activity in the murine P388 lymphocytic leukaemia model.

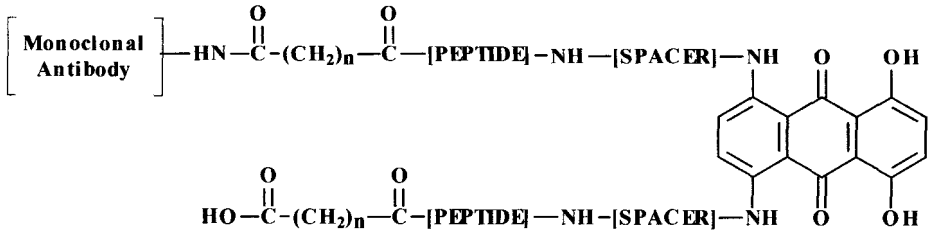


(112)

Where R = CH_2CH_2OH or H
and X = H or ^tBoc, Z or Fmoc

A later European patent application EP0489220, published in 1992, extended the scope of the 1988 patent to include N,N'-bis(succinylpeptide) derivatives of 1,4-bis-(aminoalkyl)-5,8-dihydroxyanthraquinones and their antibody conjugates (Fields *et al* 1992). The invention described the synthesis of novel compounds belonging to general structure (**113**). Peptides, typically 3 to 5 amino acid residues long, were coupled to 1,4-bis[(aminoalkyl)amino]-5,8-dihydroxyanthaquinones, containing either straight chain or branched alkyl groups. Addition of a C_1 to C_4 dicarboxylic anhydride to the peptide N-termini and esterification with N-hydroxysuccinimide/ dicyclohexylcarbodiimide allowed reaction with antibody amino groups.

The *in vivo* activity of the compounds in the lymphocytic leukaemia P388 model was reported. The inhibition of tumour growth by selected p96.5 anti-melanoma derived monoclonal antibody conjugates was also reported in human melanoma SK-Mel-28 tumours grown in athymic mice.



(113)

Ijaz and co-workers have reported the synthesis of a series of eight peptide-1-[N-(2-succinamidylethyl)amino]anthraquinones as potential AP-1 transcription factor inhibitors (Ijaz *et al* 2001). AP-1 proteins have been reported to play an important role in cell proliferation and malignant transformation. Truncated AP-1-like peptides, of five-seven residues, containing a lys-cys-arg motif, highly conserved in the DNA binding domain of AP-1 proteins (Abate *et al* 1990), were attached to the anthraquinone moiety via a flexible linker. It was proposed that the anthraquinone chromophore would facilitate weak initial DNA binding whilst allowing specific interaction of the peptide at the AP-1 binding domain.

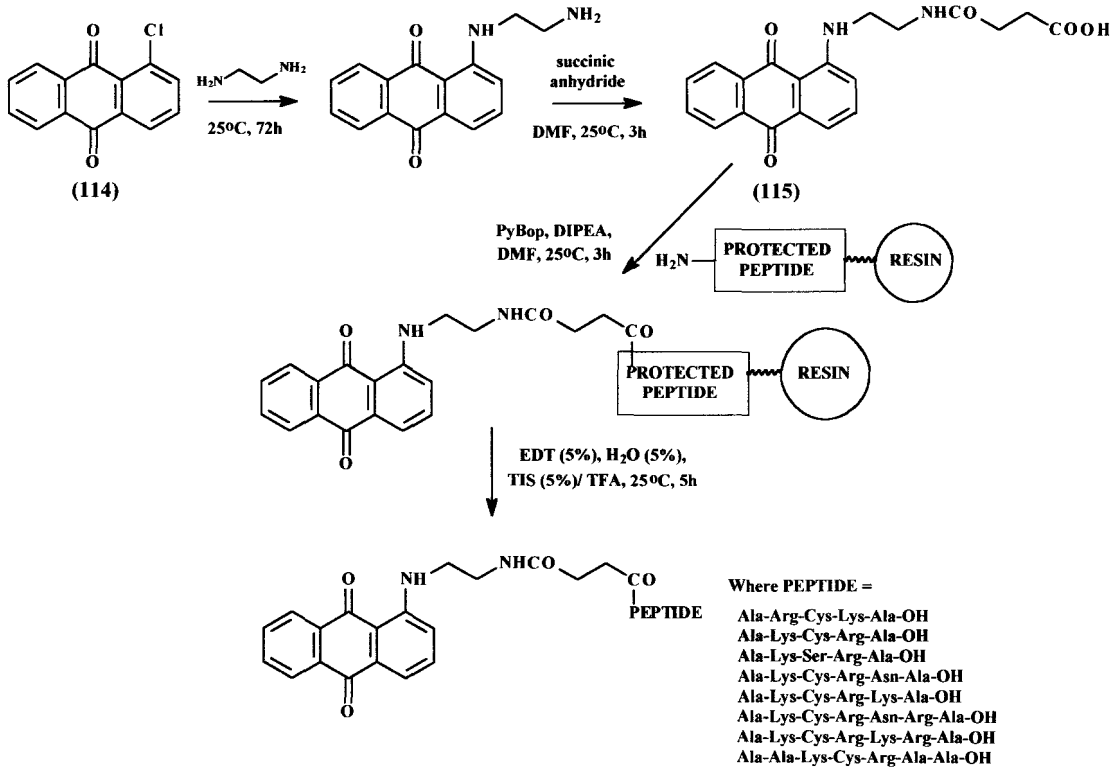
Amination of 1-chloroanthraquinone (**114**) followed by reaction with succinic anhydride gave 1-[N-(2-succinamidylethyl)amino]anthraquinone (**115**) which was attached to the (custom-synthesised) resin-bound peptide using standard peptide coupling conditions. Simultaneous amino acid side chain deprotection and resin cleavage was achieved using trifluoroacetic acid [**Scheme 8**].

The ability of the eight peptide conjugates to interact with calf thymus DNA was determined using UV thermal melt analysis. The ΔT_m values obtained ranged from 4.8 to 10.3 °C with, unsurprisingly, higher DNA melting temperatures for peptide conjugates containing increasing numbers of basic amino acid residues. An electrophoretic mobility shift assay (EMSA) was used to investigate the ability of the compounds, and their respective free peptides, to displace the binding of the AP-1 protein from its DNA consensus sequence. A concentration dependent AP-1 displacement was shown for all eight peptide conjugates. The presence of the anthraquinone moiety increased the efficacy of AP-1 displacement relative to the free peptides.

Although the anthraquinone-peptide conjugates were assembled using solid phase techniques, the compound structures were claimed in the objects of invention of an earlier

patent (Mincher 1998) wherein solution methods were used; specific examples were spacer-linked anthraquinone conjugates of amino acids containing flexible α,ω-diaminoalkane spacers that constitute the foundations of the compound libraries of the present study [the NU:UB conjugate libraries].

Scheme 8



CHAPTER FOUR

[PART A]

NOVEL SPACER-LINKED ANTHRAQUINONE-AMINO ACID CONJUGATES: RESULTS AND DISCUSSION

PART A

NOVEL SPACER-LINKED ANTHRAQUINONE-AMINO ACID CONJUGATES

4.1 Background

Earlier studies from this laboratory had shown that some members of a series of N-linked 1:1 conjugates of amino acids and anthraquinones interacted with either DNA topoisomerase I or DNA topoisomerase II and at least in part, the cytotoxic properties of these compounds correlated with enzyme inhibition *in vitro*. Key compounds are exemplified by the moderate topoisomerase I poison, NU:ICRF 505 (**85**) and the catalytic inhibitor of DNA topoisomerase II, NU:ICRF 506 (**84**) [**Section 3.1**]. Despite promising *in vivo* activity in a number of human xenografts none of this series progressed beyond early pre-clinical studies due to low bioavailability and poor aqueous solubility; the latter was clearly a consequence of the presence of a terminal carboxylic acid ester or hydrazide group in the amino acid moiety. Solubility was increased in the corresponding sodium salts of the carboxy terminus, however, these derivatives were devoid of cytotoxic activity, presumably as a result of poor cellular uptake of the anionic species; similarly, anthraquinone dipeptide conjugates were also inactive and wherein the carboxylic acid group was likely ionised at physiological pH, despite topoisomerase I poisoning activity in cell free *in vitro* enzyme inhibition assays (Meikle *et al* 1995b)

These conjugates were mutually exclusive for either the type I or type II enzyme and the patterns of enzyme inhibition observed for individual compounds were, in part, rationalised on the basis of their ability to interact with or failure to bind to DNA, either by intercalation or groove binding; in general, intercalating compounds favour interaction with topoisomerase II whereas groove binding agents favour interaction with topoisomerase I. The predictive value of DNA binding properties for topoisomerase

interaction is severely limited given that ability to interact with the changed geometry of the DNA-topoisomerase cleavable complex supersedes DNA or enzyme binding as a determinant of biological activity. Indeed, the camptothecins bind neither to DNA nor topoisomerases yet are stabilisers of the cleavable complex (Pommier *et al* 1998a).

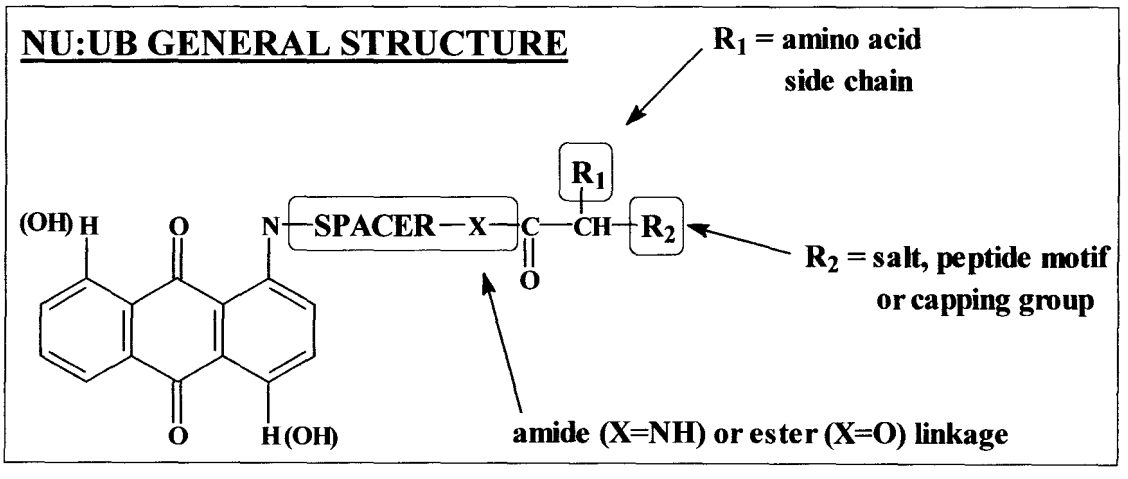
4.2 Spacer-Linked Anthraquinone-Amino Acid/ Peptide Conjugates:

Synthetic Strategy

4.2.1 Design rationale

In an attempt to overcome the drawbacks which were associated with the foregoing compounds, spacer-linked anthraquinone amino acid/ peptide conjugates have been synthesised, in this research programme, in which the amino acid or peptide motif is reversed to afford C-linked, N-terminally free conjugates [code-named NU:UB] of the general structure shown in **Figure 4**.

Figure 4



This design rationale was adopted in order to facilitate:

(i) improved water solubility by salt formation (usually trifluoroacetate or acetate) at the free N terminal amino group of the peptide motif and, where appropriate, on the side chain of basic amino acids such as lysine.

(ii) the introduction of a flexible spacer between the anthraquinone and amino acid to potentially allow an unrestricted bimodal (part intercalative, part groove binding) interaction with nucleic acids.

(iii) improved contact with topoisomerase enzymes as a result of the separation of potential DNA and protein binding domains, towards the design of dual topoisomerase I and II inhibitors.

(iv) the ease of access to oligo-peptide derivatives by stepwise addition of readily available N-protected amino acids to the free N-terminus of the growing peptide using standard peptide coupling reactions.

A main objective of the research programme was to correlate cytotoxicity and topoisomerase inhibition with amino acid structure, spacer length, composition and sequence of the peptide motif.

4.2.2 Preparation of Conjugates: Overview

The preparation of the compounds can be broken down into the following key steps:

(i) Formation of the anthraquinone spacer compound by nuclear amination of halo- or hydroxy- anthraquinones with bifunctional amines (α,ω-diamines or amino alcohols).

EITHER

(ii) Activation of an N-protected amino acid by conversion to the active ester (O-pentafluorophenolate, or N-hydroxysuccinimide) and coupling of the activated amino acid to a free amino terminus of the anthraquinone spacer compound to afford **amide linked** N-protected conjugates.

OR

(iii) DCC mediated coupling of an N-protected amino acid to a free hydroxy terminus of the anthraquinone spacer compound to afford **ester linked** N-protected conjugates.

(iv) Deprotection of the N-protected, amide- or ester- linked conjugates, using the appropriate reagents, to give the candidate drugs as water-soluble salts.

(v) Where appropriate, sequential coupling of additional amino acids (or peptides) by an analogous sequence of reactions.

4.2.3 Anthraquinone Spacer Compounds: Scope of Synthesis

The structures of the anthraquinone-spacer compounds were chosen to differ greatly both in the substitution pattern of the anthraquinone chromophore and the structure of the spacer group. Compounds prepared were unsubstituted aminoanthraquinones or were further substituted with hydroxy groups in the 4- or 4,8- positions. Spacer groups could be further sub-divided as to whether they terminated in an amino or hydroxy group and hence coupled to amino acids via an amide or ester linkage.

The majority of compounds synthesised contained flexible spacer groups derived from straight chain α,ω-diaminoalkanes or ω-aminoalkanols (commonly 3 or 4 methylene groups in length). The main function of the linker group was to distance the anthraquinone (potentially intercalative DNA-binding domain) and peptide motif

(potentially groove binding domain) to create the potential for a bimodal mechanism of binding to DNA, considered to be important in the design of compounds with a dual action against topoisomerase type I and II enzymes.

A limited number of conjugates were prepared where the spacer group comprised:

(i) a shorter alkyl chain.

(ii) bulky, branched (including chiral) or cyclic groups in close proximity to the anthraquinone nucleus.

(iii) direct attachment of a cyclic amine to the N-1 position of the anthraquinone.

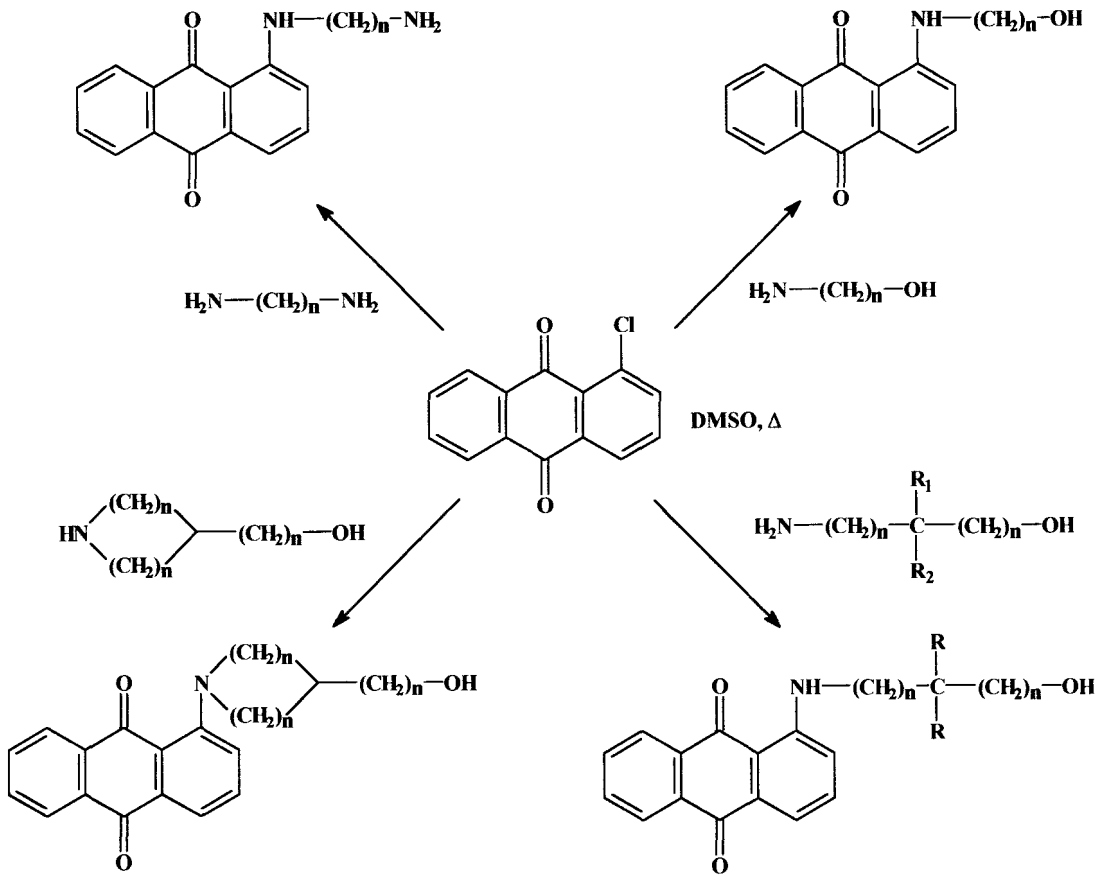
Reduction in spacer length and increase in steric bulk and resultant conformational restriction are features that might preclude intercalation of the anthraquinone chromophore and result in a switch to a more groove binding mode of DNA interaction. It was realised that the choice of spacer could also have an important effect on cell permeability, solubility and bioavailability of the conjugates.

4.2.4 Aminoanthraquinone-Spacer Compounds Derived from Haloanthraquinones: Synthetic Procedures

Scheme 9 outlines the scope of the chemical syntheses and general structures of each of the types of spacer compound prepared by the nucleophilic displacement of chlorine from readily available 1-chloroanthraquinone with either an α,ω-diaminoalkane or ω-aminoalkanol (in excess) in DMSO, typically at 100°C or under reflux as required for 0.5h. Subsequent cooling and addition of water gave red precipitates of the crude (hydroxyalkyl)aminoanthraquinones or (aminoalkyl)aminoanthraquinones [the spacer compounds]; chloroform soluble compounds could be extracted and purified by column chromatography.

Scheme 9:

Spacer compounds prepared by nucleophilic substitution of 1-chloroanthraquinone



Where each n is independently variable

Nucleophilic substitution of haloanthraquinones is a simple and effective procedure to access many aminoanthraquinones in good or high yields. Although several impurities are introduced, as judged by highly coloured trace spots on thin layer chromatograms, the crude product is usually sufficiently pure for subsequent reactions. Katzhendler *et al* (1989) conducted aminations of 1-chloroanthraquinone to give (aminoalkyl)aminoanthraquinones as simple analogues of ametantrone and mitoxantrone (**7**) that were shown to be relatively inactive *in vivo* in the murine P388 tumour model, the most active being 1-[(2-aminoethyl)amino]anthraquinone which was 85-fold less active than the clinically useful anthraquinone doxorubicin (**1**). Several conclusions were made

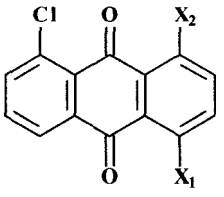
concerning the antitumour activity of 1-(aminoalkyl)aminoanthraquinones unsubstituted in other nuclear positions which can be summarised as:

1. an amino group in the side chain was essential for activity and the optimal number of carbon atoms separating the nitrogens was two; increasing the carbon chain to C_3 or C_4 decreased activity 1.6- and 3.2- fold respectively. This trend was also observed for an analogous series of bis-aminated anthraquinones (Zee-Cheng and Cheng 1983).
2. a terminal primary amine was more active than a tertiary amine.
3. insertion of an additional ethylamino group into the side chain [more closely related to mitoxantrone (**7**)] did not increase activity and, in some cases slightly decreased it.

In the present study, the purpose of aminating anthraquinones was to provide a platform for attachment of amino acid and peptide motifs rather than to identify antineoplastic spacer compounds.

Given the observation that the presence of hydroxy groups substantially increased antitumour activity of mitoxantrone (**7**) compared to its unhydroxylated analogue ametantrone, Zee-Cheng *et al* (1987) had prepared a series of (aminoalkyl)aminoanthraquinones either with or without hydroxy groups and in which chlorine substitution was retained; the compound general structure for non-hydroxylated examples is given in **Figure 5.**

Figure 5

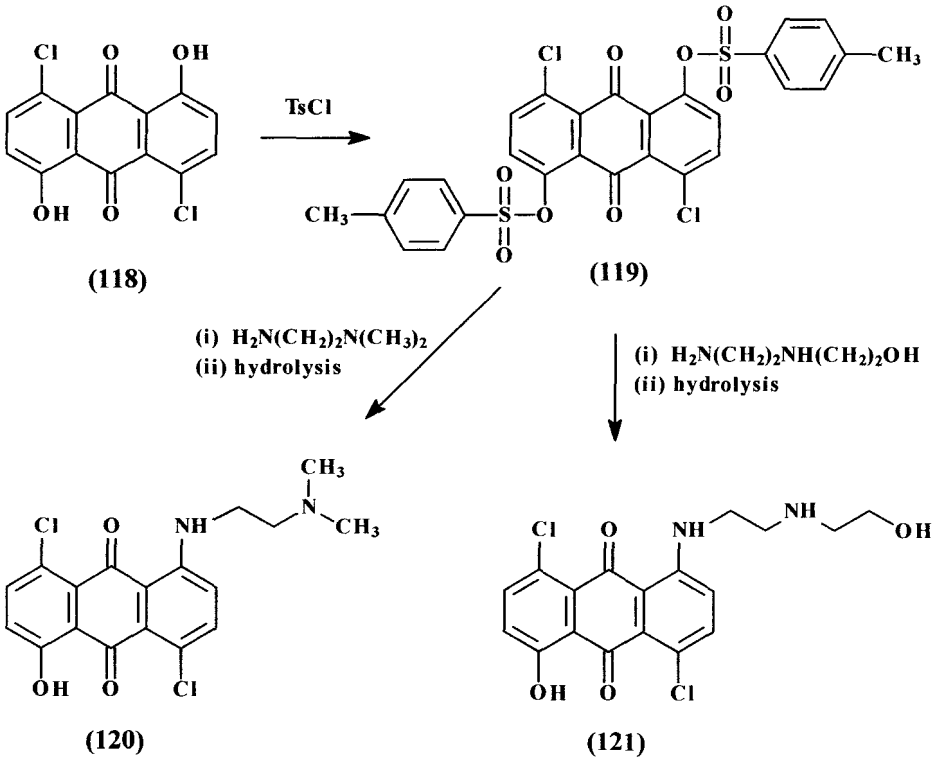


(116) $X_1 = NH(CH_2)_2NH(CH_2)_2OH$; $X_2 = H$

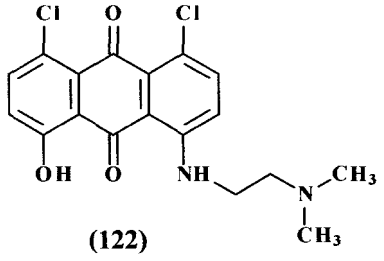
(117) $X_1 = H$; $X_2 = NH(CH_2)_2NH(CH_2)_2OH$

The monoaminated compounds (**116**) and (**117**) were synthesised by reacting 2-[(2-aminoethyl)amino]ethanol with 1,5-dichloroanthraquinone and 1,8-dichloroanthraquinone respectively in competition with bis-amination. Furthermore, the hydroxylated examples were obtained from 1,5-dichloro-4,8-dihydroxyanthraquinone (**118**), as outlined in **Scheme 10**. Monoamination of (**118**) was achieved by reacting the derived ditosylate (**119**) with one equivalent of the appropriate amine to give compounds (**120**) and (**121**), after hydrolysis of the remaining tosyl group.

Scheme 10

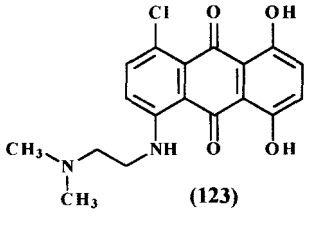


Compound (**122**) was prepared from the ditosylate of 1,8-dichloro-4,5-dihydroxyanthraquinone in a directly analogous fashion to the preparation of (**120**) by reaction with one equivalent of N,N-dimethylethylenediamine followed by hydrolysis.

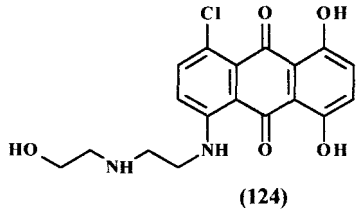


(122)

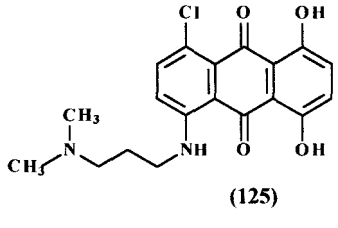
Interesting monoamination reactions occurred with 1,4-dichloro-5,8-dihydroxyanthraquinones. When treated with aliphatic amines in pyridine, the latter anthraquinones gave compounds of structures (**123**), (**124**) and (**125**), i.e. displacement of a chlorine atom occurred.



(123)

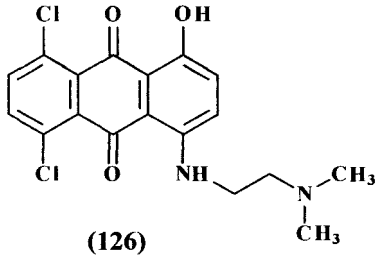


(124)

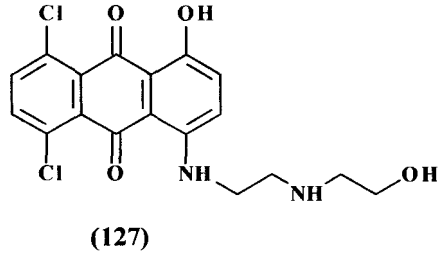


(125)

However, the same reactions carried out in butanol gave compounds with structures (**126**) and (**127**), in which the hydroxy group had been substituted.



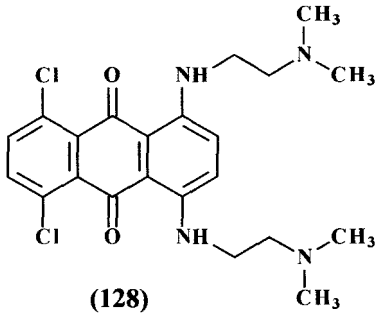
(126)



(127)

When aromatic amines were used, both chlorine atoms were substituted to give 1,4-dihydroxy-5,8-bis(arylamino)anthraquinones. Furthermore, when 1,4-dichloro-5,8-dihydroxyanthraquinone was converted to its leuco derivative with tin and hydrochloric

acid followed by reaction with N,N-dimethylethylenediamine, bis-amination took place. After re-oxidation to the anthraquinone system compound (**128**) was obtained.



(128)

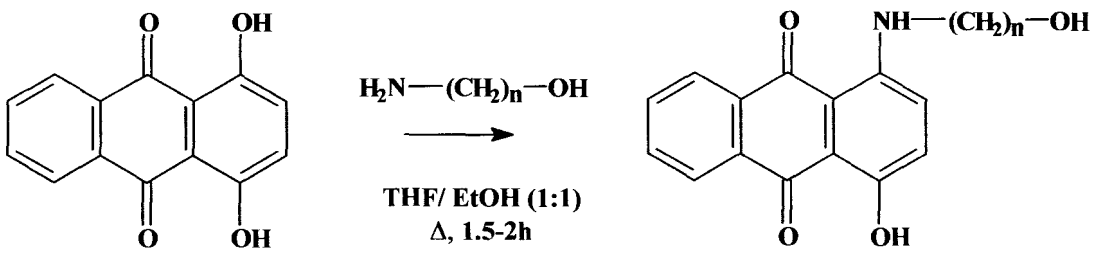
The compounds were tested for biological activity against P388 leukaemia (*in vivo*) and murine L1210 and human colon carcinoma (*in vitro*). Compound (**127**) was most active whilst compounds (**126**), (**123**) and (**124**) also had notable activity against P388 (*in vivo*) and L1210 (*in vitro*). The authors concluded that the optimum number of carbon atoms between the two amino nitrogen atoms was two [consistent with the observations of (Katzhendler *et al* 1989)], and suggested that a hydroxy group para to the amino(alkylamino) group was a necessary but insufficient criterion for activity. The most active compounds have these hydroxy groups, however, compounds (**120**), (**121**) and (**122**) also have these hydroxy groups yet are inactive. It was pointed out that aryl chloro-substituted (aminoalkyl)aminoanthraquinones were generally less active than their corresponding hydroxy-substituted compounds in the *in vivo* P388 model. It is worth noting that the P388 tumour model has been largely discredited by the oncology community as an indicator of likely performance in the human clinical setting.

4.2.5 Aminoanthraquinone-Spacer Compounds Derived from Hydroxyanthraquinones: Synthetic Procedures

Examples of 4-hydroxylated and 4,8-dihydroxylated aminoanthraquinone spacer compounds were synthesised in this research programme as platforms for amino acid conjugation, using adaptations of literature procedures.

Because, in general, the introduction of hydroxy or alkoxy groups into the anthracenedione ring system enhances cytotoxic potency it was considered important to parallel the non-hydroxylated series with hydroxylated analogues. Hydroxylation of the nucleus, as in the case of mitoxantrone may be expected to increase cytotoxicity associated with stronger binding to DNA and slower dissociation kinetics due to the presence of the hydroxy groups. The method of nucleophilic displacement of chlorine from chlorohydroxyanthraquinones to prepare aminohydroxylated compounds is generally unsuitable. This is due either to the non-availability of starting materials with the correct substitution pattern or because of intrinsic resistance to nucleophilic displacement of chlorine from these more electron rich compounds; chlorine in 1-chloro-4-hydroxyanthraquinone, for example, is unreactive to amines. The strategy adopted for the synthesis of 4-hydroxylated aminoanthraquinone-spacer compounds (**130**) was based on the controlled mono-amination of quinizarin (1,4-dihydroxyanthraquinone) (**129**) with primary amines [**Scheme 11**] for an appropriate reaction time.

Scheme 11

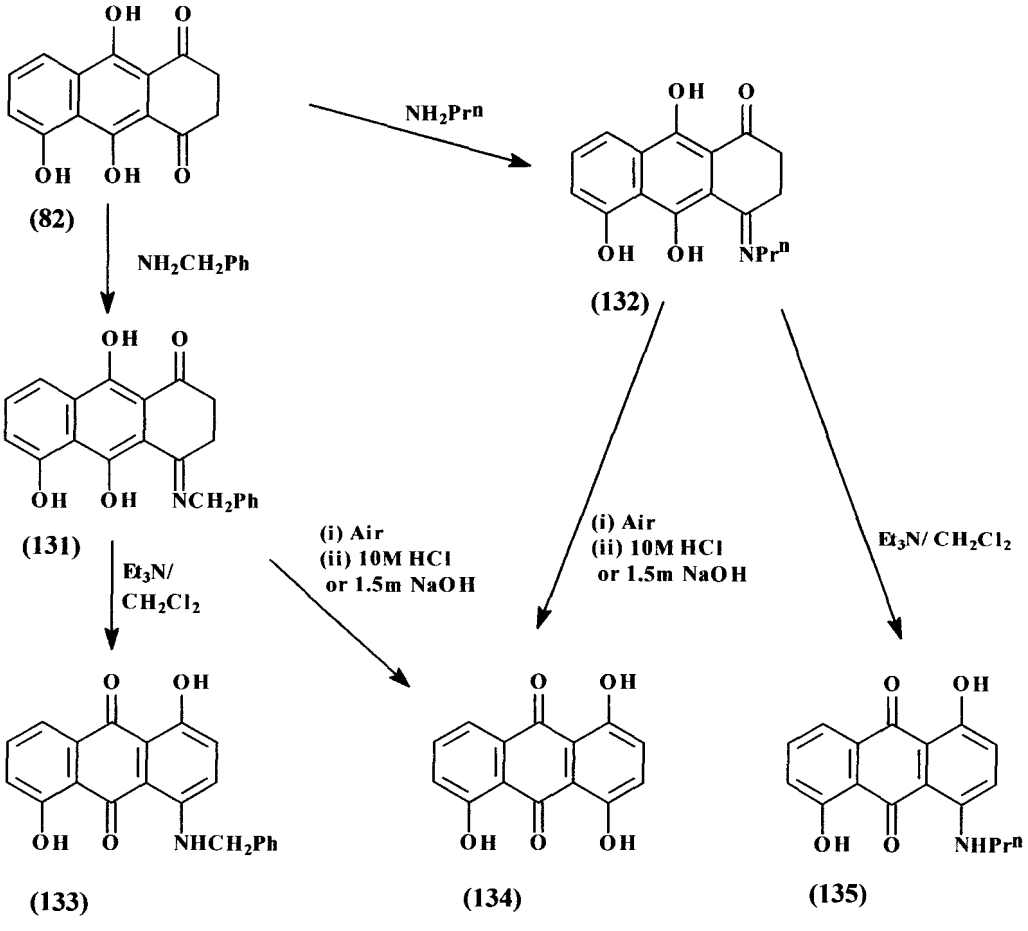


(129) (130)

The reactions in **scheme 12** outline the steps taken in the preparation of 4,8-dihydroxyaminoanthraquinone spacer compounds, for use in amino acid conjugation, from leuco-1,4,5-trihydroxyanthraquinone (leuco-5-hydroxyquinizarin). Methods were based upon the reported regiospecific amination of this leuco-trihydroxyanthraquinone.

Morris *et al* (1986) synthesised (though not as anticancer compounds) (**133**) and (**135**) from leuco-5-hydroxyquinizarin (**82**). It was found that if the intermediates (**131**) and (**132**) were oxidised and then hydrolysed using HCl or NaOH, they each gave 5-hydroxyquinizarin (**134**). However, if these intermediates were stirred with triethylamine in dichloromethane in the presence of air, the aminoanthraquinones (**133**) and (**135**) were formed.

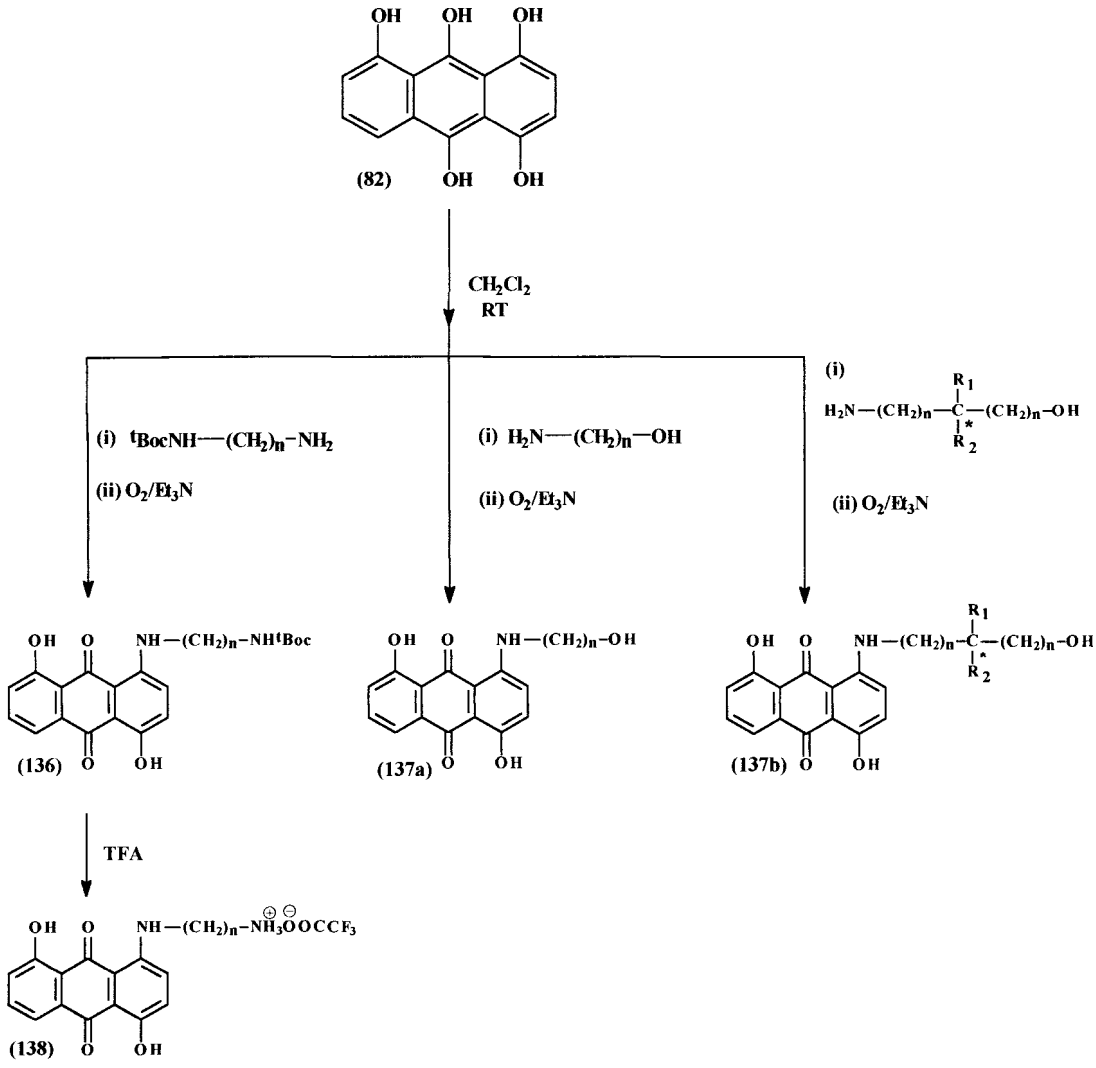
Scheme 12: Regiospecific amination of leuco-1,4,5-trihydroxyanthraquinone



Applying this methodology in this project, a mono N-^{t}Boc protected diaminoalkane or aminoalkanol was added to a solution of leuco-1,4,5-trihydroxyanthraquinone (**82**) in dichloromethane and stirred for 6h. Oxidation was achieved by addition of triethylamine and aeration [**Scheme 13**]. N-deprotection with trifluoroacetic acid converted ^{t}Boc protected intermediates (**136**) into aminoalkylamino-spacer compounds (**138**). These methods allowed access to nuclear-hydroxylated versions of the corresponding unsubstituted spacers [see **Scheme 9**]. Alternatively, amination using either straight chain α,ω-aminoalkanols or branched chain (including chiral) α,ω-aminoalkanols afforded the nuclear-hydroxylated, hydroxyalkylamino-spacer compounds (**137a**) and (**137b**) respectively.

Scheme 13:

Spacer compounds prepared by amination of (leuco) hydroxyanthraquinones



4.2.6 Conjugation of Amino Acids/ Peptides to Anthraquinone Spacer Compounds

The foregoing sections outlined the scope and synthetic methods used to prepare nuclear-substituted and unsubstituted anthraquinone spacer compounds possessing either free amino or alcohol groups that served as points of attachment of N-protected amino acids; the following sections describe the selection, nature and methods used to obtain the library of water-soluble amino acid conjugates.

4.2.7 Choice of Amino Acid or Peptide Motif

Amino acids were chosen to cover a wide range of neutral, polar, basic, hydrophobic, hydrophilic, aliphatic and aromatic side chains which, as in the choice of spacer group, could affect both drug uptake and delivery as well as affinity and specificity for DNA and topoisomerase proteins. It was speculated that the inclusion of unnatural amino acids and D-isomers (most proteolytic enzymes have greatly reduced activity against D-amino acid residues) would result in improved enzymatic stability *in-vivo*.

For efficient coupling of amino acids to the spacer compounds it was clearly important to use N-protected derivatives that would require prior or *in situ* activation.

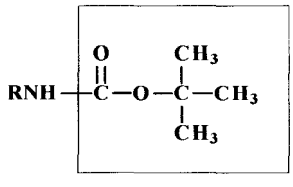
4.2.8 Amino Acid Protection and Deprotection

The success of synthetic organic reaction chemistry is very dependent on protection or blocking of key functional groups to permit selective reaction at other sites in a molecule and although some protecting groups have been used for some considerable time, for example isopropylidene protection of 1,2-diols in sugars, modern protective group chemistry has developed over the last 30 years (McOmie 1973). Several versatile protecting group methods with facile deprotection have been developed for solution

phase and solid phase reactions and have been applied to combinatorial peptide and non-peptide libraries (Jung 1996, Fenniri 2000).

A vast number of N-α and (where necessary) side chain protected amino acids are now commercially available. However, as these have been prepared principally for use in solid phase synthesis, some of the harsh methods used for removal of many of the protecting groups may not always be suitable for use with anthraquinone-peptide conjugates (hydrogen bromide and acetic acid, for example, induces nuclear reactions). Hydrogenolytic reductive methods were unsuitable due to facile reduction of the quinone system (although it is conceivable that re-oxidation at a later stage may prove feasible). In some cases, although removal of the amino acid side chain protecting group was not performed, it was decided that the resulting partially protected conjugate may be of interest due to the hydrophobic/ lipophilic nature of the group. The most conveniently handled protecting groups for the α-amino group were the widely applicable N-tertiarybutoxycarbonyl (^{t}Boc) and Fluorenylmethoxycarbonyl (Fmoc) groups.

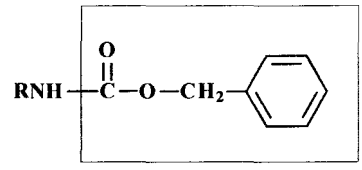
(i) N-tertiarybutoxycarbonyl (N-^{t}Boc) protection



Wherever possible the amino acids used in this research programme were protected on the α-amino group by the N-tertiarybutoxycarbonyl (N-^{t}Boc) group (Bodanszky and Bodanszky 1994). After amino acid coupling, the ^{t}Boc group was removed by dissolving the protected product in trifluoroacetic acid for 0.25h at room temperature; evaporation and

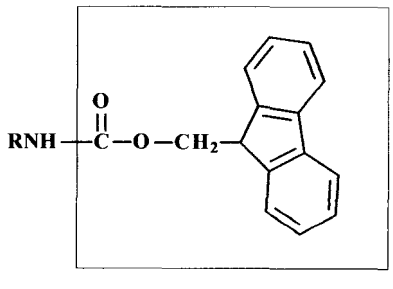
treatment of the residue with ethanolic diethyl ether with cooling conveniently afforded solid or crystalline, water-soluble trifluoroacetate salts of the target conjugates.

(ii) Benzyloxycarbonyl (Z) protection



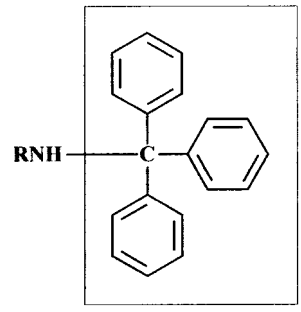
To prevent unwanted reaction on the ε- amino group of the lysine side chain (and the δ-amino group of ornithine) double protection on both the α- and ε- amino groups was required. Where the α-amino protection was with the ^{t}Boc group, differential protection of the ε-group could be achieved by using the N-Benzyloxycarbonyl (Z) group (Bodanszky 1993), allowing selective removal of the ^{t}Boc group and possible subsequent addition of further amino acids, or alternative modification, to the newly liberated α-amino terminus. In the literature, the removal of the Z group is normally carried out using HBr/ glacial AcOH, however this method was found to give a mixture of products and considered too reactive to the anthraquinone system. Fortunately, when used to protect the ε-amino group of lysine (or the δ-amino group of ornithine), the Z group is slightly more acid labile than normal and it was found that prolonged exposure to trifluoroacetic acid (48h) was sufficient for cleavage of the Z protecting group to give clean reaction products.

(iii) Fluorenylmethoxycarbonyl (Fmoc) protection



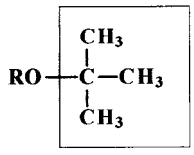
The fluorenylmethoxycarbonyl (Fmoc) protecting group has also been used to provide differential α-amino protection for amino acids such as hydroxyproline, asparagine and histidine where side chain protection was achieved with (acid labile) O-tertiarybutyl, N-β-trityl and N-im-trityl protection respectively. The Fmoc protecting group was removed using 20% piperidine (by volume) in DMF (for approximately 5 minutes) and was completely stable to trifluoroacetic acid (Carpino and Han 1972). It was necessary to remove the Fmoc-piperidine adduct from the anthraquinone conjugate products by column chromatography.

(iv) Trityl (Trt) protection



The trityl (triphenylmethyl) group has been used very widely for the protection of primary hydroxy groups in carbohydrate and nucleoside chemistry. This group was also conveniently used here for N-protection of imidazole nitrogen in histidine and for the side chain amide nitrogen function in asparagine. This protecting group could be easily removed from anthraquinone conjugates of these amino acids by trifluoroacetic acid at room temperature for the required number of hours (longer reaction times than for ^{t}Boc removal).

(v) Tertiarybutyl (^{t}Bu) protection



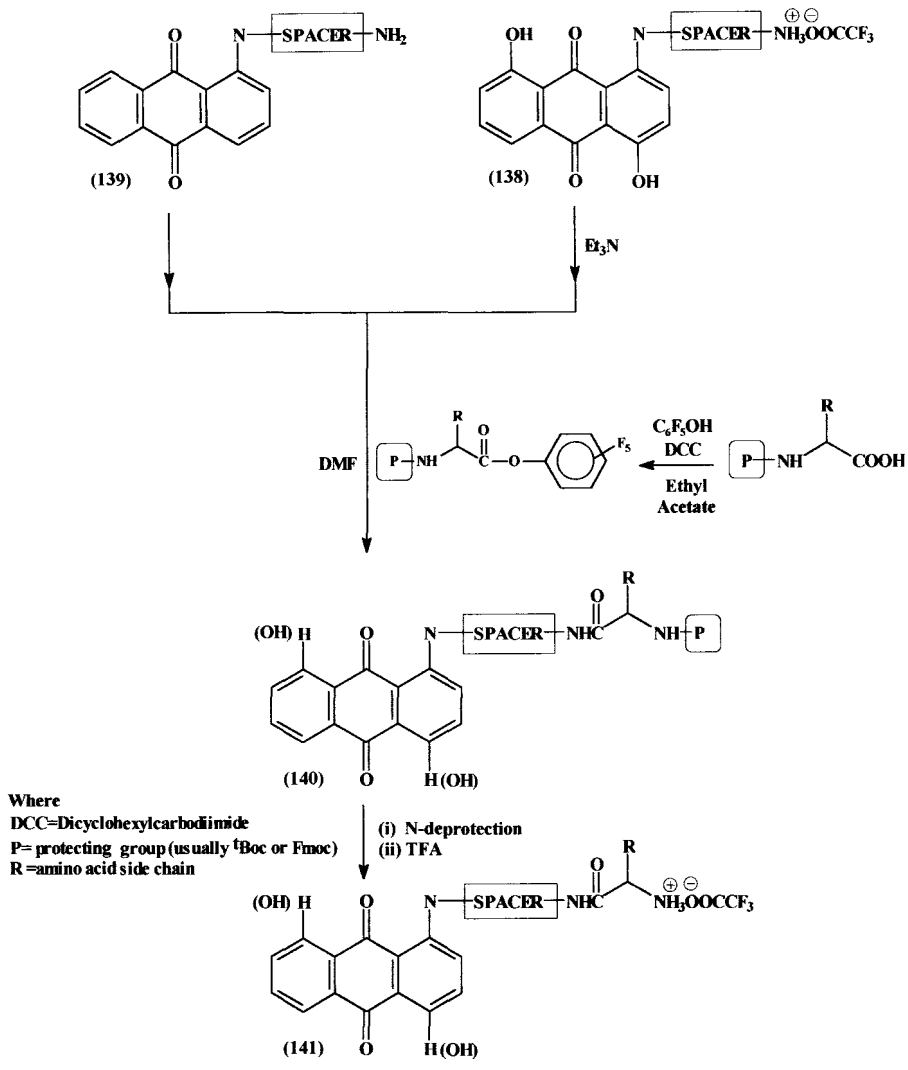
Tertiary butyl esters are often used as protecting groups for carboxylic acids including amino acids and the hydroxy groups of serine, threonine and 4-hydroxyproline are reliably protected in peptide synthesis as the O-tertiarybutyl (O-^{t}Bu) ethers. Here, examples of anthraquinone conjugates incorporating these residues were successfully deprotected with trifluoroacetic acid at 0°C to room temperature over 12-24h.

4.2.9 Coupling of Amino Acids/ Peptides to Anthraquinone Spacer Compounds

The synthesis of **amide-linked** anthraquinone peptide conjugates is outlined in **Scheme 14**. Pentafluorophenolate active esters of amino acids were prepared from pentafluorophenol and a suitable N- (and if necessary side chain) protected amino acid in dry ethyl acetate using dicyclohexylcarbodiimide (DCC) as the coupling agent. The precipitated dicyclohexylurea (DCU) was filtered off and after evaporation, the residual pentafluorophenolate ester was reacted with the appropriate spacer compound [for example (**138**) and (**139**)] in DMF. Solvent extraction and column chromatography gave an analytically pure sample of the protected intermediate (**140**). N-Deprotection and conversion to the trifluoroacetates (or acetate) afforded the water soluble **amide-linked** anthraquinone-amino acid conjugate salts (**141**) [the NU:UB library (amides)].

Scheme 14:

Simplified Synthesis of **Amide-Linked** Anthraquinone-Amino Acid Conjugates

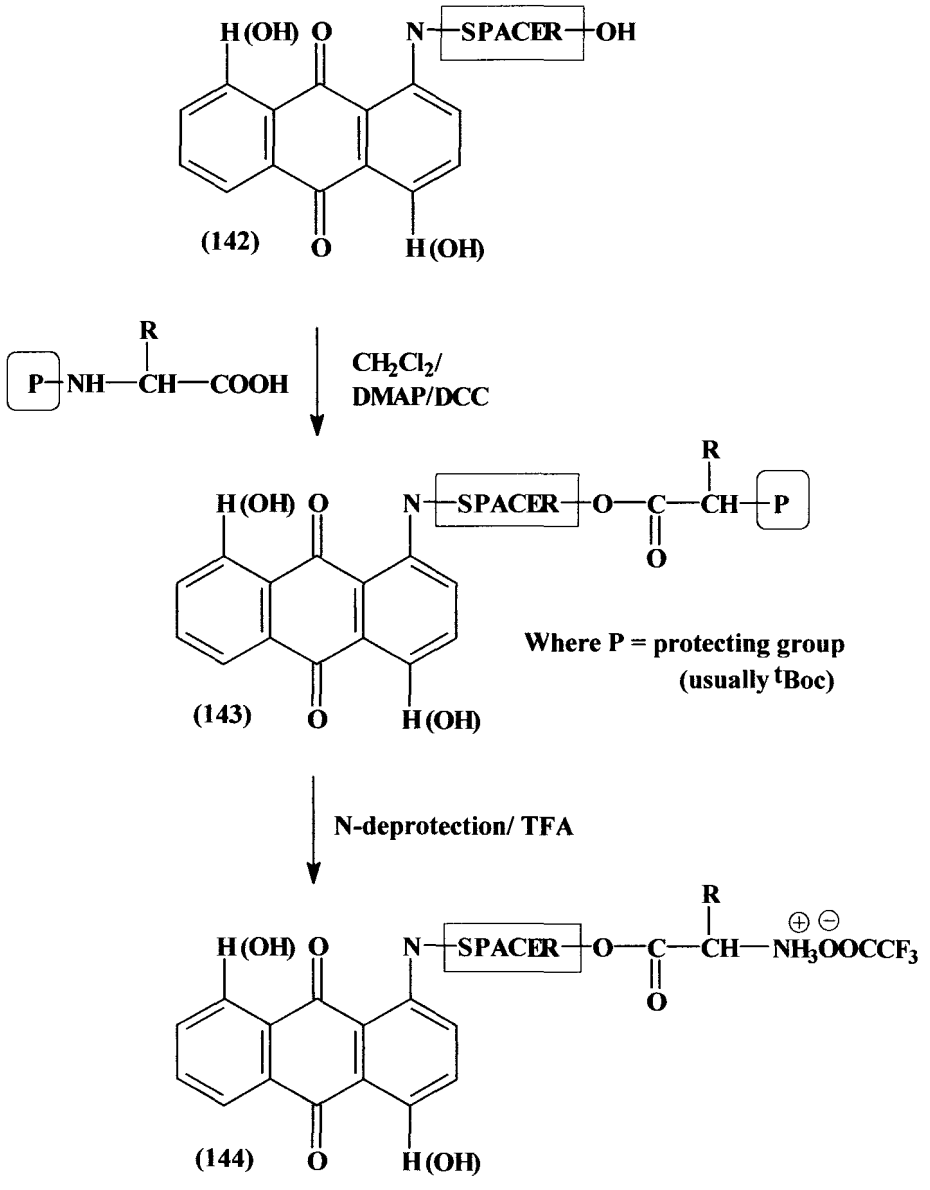


The synthesis of **ester-linked** anthraquinone-amino acid conjugates is outlined in **Scheme 15**. Spacer compounds terminating in a free hydroxy group (**142**) were coupled with the appropriate N- (and if necessary side chain) protected amino acid in dry dichloromethane using dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP). The

protected intermediates (**143**) were purified by solvent extraction and column chromatography to give analytically pure samples. N-Deprotection, typically to remove ^{t}Boc with TFA, afforded the water soluble **ester-linked** anthraquinone-amino acid conjugate salts (**144**) [the NU:UB library (esters)].

Scheme 15:

Simplified Synthesis of **Ester-Linked** Anthraquinone-Amino Acid Conjugates



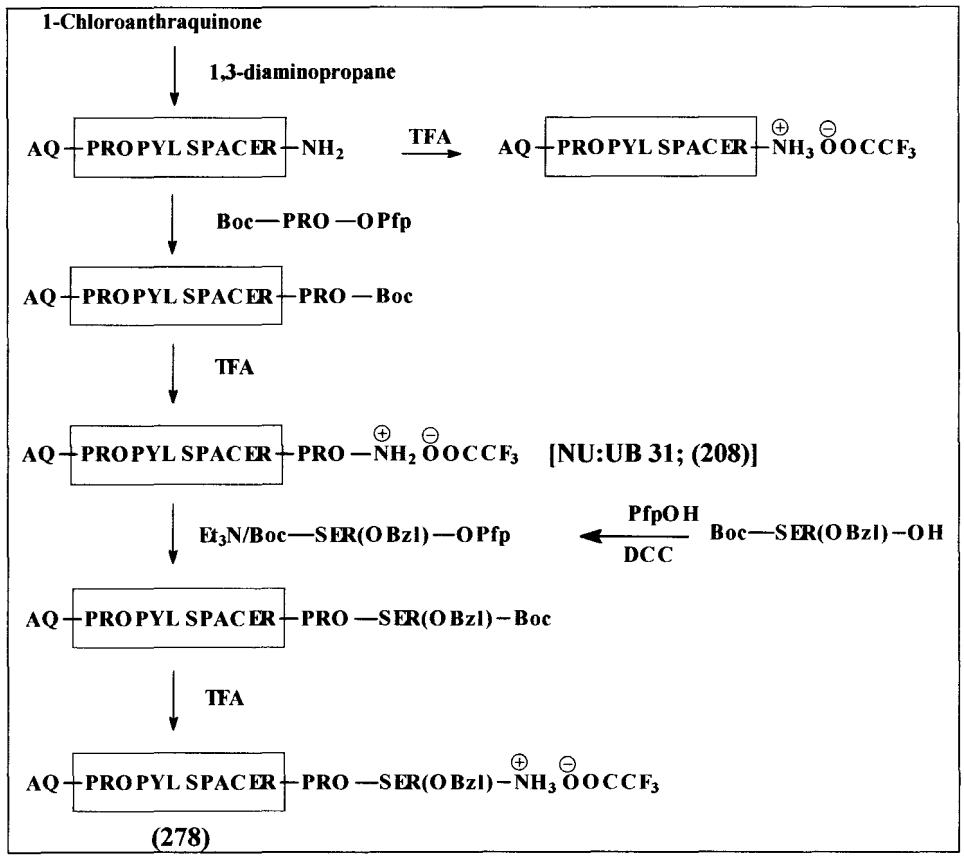
4.3 The NU:UB Library of Spacer-Linked Anthraquinone Amino Acid/Peptide Conjugates: Synthesis and Characterisation

During this research programme, greater than 100 novel spacer-linked anthraquinone amino acid/peptide conjugates (either in protected or salt forms) were prepared from some fifteen anthraquinone spacer compounds. Due to the large number of conjugates in the compound library, this section necessarily discusses the synthesis and characterisation of **selected examples**; the criteria for selection were: illustrations of the scope of the reaction chemistry, protection and deprotection methods, diversity of amino acid/peptide motif, variation in anthraquinone substitution pattern and spacer group, conjugation via amide or ester groups, physical and spectroscopic (mass and nmr) analysis. The full experimental procedures for the synthesis of all compounds in the library and complete physical and chemical characterisation data are documented in **Chapter 9** (Experimental) and are preceded in **Chapter 8** by a complete catalogue of compound structures and their descriptors.

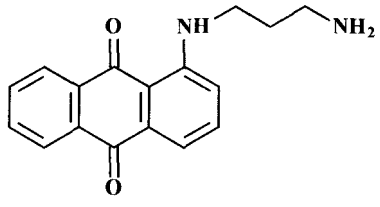
4.3.1 Example [A]: Amide-Linked (Nuclear-Unsubstituted) Aminoanthraquinone-Mono-Amino Acid and Dipeptide Conjugates

Outline syntheses of example proline and serylproline (single amino acid and dipeptide respectively) conjugates of aminoanthraquinones unsubstituted further in the nucleus and with flexible spacer groups (spacers) (Example [A]), are shown in **Scheme 16**. Notably, this category includes the propyl-spaced proline conjugate NU:UB 31 (**208**) that was subsequently shown to be a dual inhibitor of topoisomerase I and the individual isoforms of topoisomerase II and had *in vivo* antitumour activity.

Scheme 16: Outline Synthesis Example [A]



4.3.1.1 1-[(3-Aminopropyl)amino]anthraquinone (145)

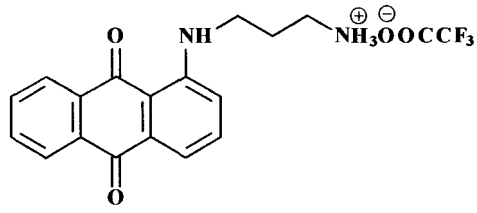


(145)

1-Chloroanthraquinone was reacted with 1,3-diaminopropane in DMSO under reflux for one hour. Cooling and addition of water gave a red precipitate of the title compound. This was used without further purification since the product formed after reaction with a ^{t}Boc protected amino acid was considerably more soluble and therefore easier to purify than the free anthraquinone-spacer compound. The crude product was characterised by

its CI (+) mass spectrum which showed a signal at m/z 281, corresponding to a relative molecular mass of 280 and by its subsequent reactions [**Scheme 16**].

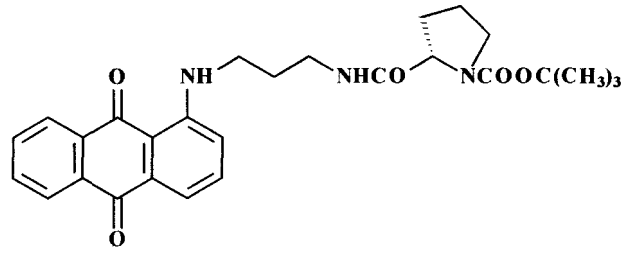
4.3.1.2 1-[(3-Aminopropyl)amino]anthraquinone trifluoroacetate (146) /(NU:UB 197)



(146)

An analytically pure sample of the spacer compound (**145**) was prepared as the water-soluble trifluoroacetate salt (**146**). Compound (**145**) (crude) was purified by column chromatography and dissolved in trifluoroacetic acid; the pure trifluoroacetate precipitated with ether to give the title compound (**146**). The electrospray (+) mass spectrum gave a signal at m/z 281 for the alkylammonium cation $(RNH_3)^+$. A signal at m/z 113 in the electrospray (–) mass spectrum confirmed the presence of the trifluoroacetate anion. This compound was required in a pure form for hplc monitoring of the stability of amide-linked conjugates of this type because the anthraquinone spacer (likely to be protonated at physiological pH) was a potential *in vivo* metabolite. Given the latter it was also desirable to measure its cytotoxicity [**Scheme 16**].

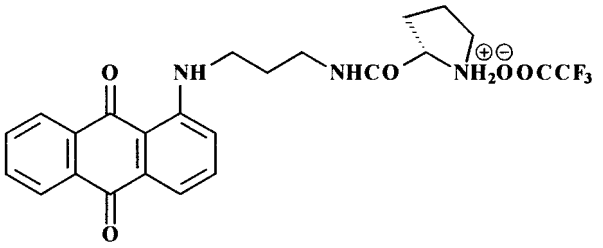
4.3.1.3 1-[3-(N-Tertiarybutoxycarbonyl-L-prolylamino)propylamino]-anthraquinone (207)



(207)

Compound (**207**), the precursor to NU:UB 31 (**208**), was prepared by the reaction of N-Boc-proline-pentafluorophenolate with 1-[(3-aminopropyl)amino]anthraquinone (**145**) in DMF. Purification of the crude product, firstly by partitioning between chloroform/ water and chloroform/ aqueous sodium bicarbonate (to remove water soluble and acidic impurities) and then column chromatography gave an analytically pure sample of the title compound [**Scheme 16**]. The structure of (**207**) was confirmed by ^{1}H nmr spectroscopy which showed, for example, the presence of the ^{t}Boc protecting group as a 9-proton singlet at 1.45ppm. The signals for 12 methylene protons were evident between 1.75 and 3.55ppm. The amino proton at C-1 of the anthraquinone gave a D_2O exchangeable signal as a triplet at 9.75ppm. All remaining protons could be fully assigned and the FAB (+) mass spectrum showed a signal at m/z 478 $(MH)^+$ corresponding to a molecular mass of 477. Furthermore, elemental analysis was consistent with that required for the title compound.

4.3.1.4 1-[3-(L-Prolylamino)propylamino]anthraquinone trifluoroacetate (208) /(NU:UB 31)

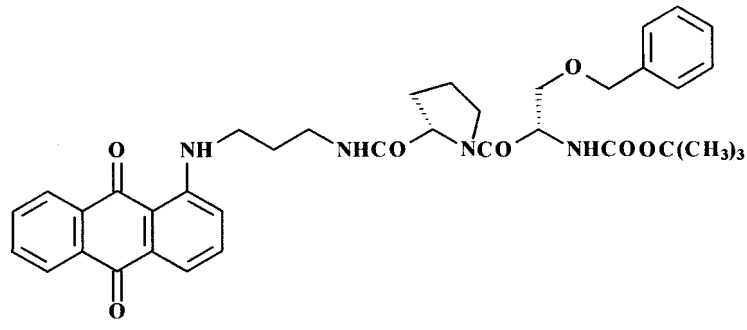


(208)

Deprotection of the N-protected proline conjugate (**207**) was carried out with trifluoroacetic acid [**Scheme 16**]. A precipitate of the resultant salt (**208**) was obtained from an ethanol/ ether solution, the structure of which was confirmed by its ^{1}H nmr spectrum (in d_6-DMSO). This showed that the ^{t}Boc protecting group had been removed by the absence of a 9-proton singlet at 1.45ppm, present in the spectrum of the protected precursor (**207**). A one-proton triplet at 4.10ppm was assigned to the α-methine proton of the proline residue. The 2 protons of the NH_2^+ group were observed as an exchange-broadened signal at 9.00ppm and the proton of the amide bond was seen as a triplet at 8.70ppm. The aromatic protons were all successfully assigned; H-2 and H-4 gave one proton doublets at 7.25 and 7.45ppm respectively and H-3 a one proton triplet at 7.65ppm. The H-6 and H-7 protons were present at 7.80 and 7.95 ppm and the H-5 and H-8 protons at 8.10-8.20 ppm. The FAB(+) mass spectrum had m/z 378 corresponding to the complex proline cation RNH_2^+.

4.3.1.5 1-[3-(N-Tertiarybutoxycarbonyl-O-benzyl-L-seryl-L-prolylamino)propyl-amino]anthraquinone (277)

Mono-amino acid conjugate salts could be extended at the N-terminus by further peptide coupling reactions, exemplified by the O-protected serylprolyl adduct (**277**), to afford elongated conjugate salts upon satisfactory deprotection.



(277)

The pentafluorophenolate ester of N-^{t}Boc-O-benzyl-L-serine was prepared from the reaction of pentafluorophenol and N-^{t}Boc-O-benzyl-L-serine in dry ethyl acetate using dicyclhexylcarbodiimide (DCC) as the coupling agent. The dipeptide conjugate (**277**) was formed by the addition of N-tertiarybutoxycarbonyl-O-benzyl-L-serine-pentafluorophenolate to a cooled stirred solution of the free base of the proline conjugate (**208**) (liberated from the trifluoroacetate by triethylamine) [**Scheme 16**]. After twelve hours reaction time, solvent extraction and a single chromatographic purification on silica gel gave the title compound (**277**) in an analytically pure form. The compound was fully characterised by spectroscopy and elemental analysis. The ^{1}H nmr spectrum showed a 9-proton signal at 1.40ppm and a 5-proton multiplet centred at 7.25ppm which were assigned to the N-^{t}Boc and Bzl protecting groups respectively. The α-methine proton was assigned to a triplet at 4.65ppm and the serine α-methine-proton to a quartet at 4.75ppm. The anthraquinone amino group gave a triplet at 9.70ppm. The methylene protons of the propyl spacer group adjacent to the anthraquinone and C-

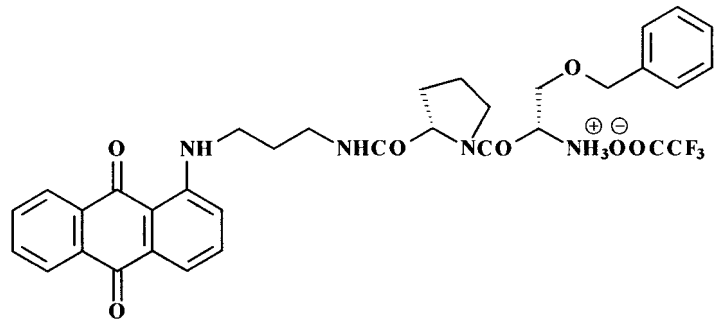
terminus of the proline residue were identifiable two-proton multiplets at 3.60 and 3.25ppm respectively; the signal for the central methylene group of the spacer, commonly a quintet, overlapped a β-hydrogen of the proline ring system. A doublet at 5.45ppm was assigned to the amide proton of the ^{t}Boc group and a triplet at 6.80ppm to the amide proton linking the spacer to the proline residue. Signals at 3.80 and 4.50ppm, each integrating for 2 protons, were assigned to the β methylene protons of serine and the methylene protons of the benzyl protecting group respectively.

Additionally, the proton-decoupled, ^{13}C nmr spectrum showed signals for all carbon environments. A DEPT experiment clearly differentiated the 14 (2 equivalent) methine CH carbons, eight methylene CH_2 carbons and three methyl CH_3 carbon atoms with the remaining 12 quaternary carbons being accounted for by difference. A signal at 28.35ppm was assigned to the 3 equivalent methyl carbons of the ^{t}Boc group with the quaternary ^{t}Boc carbon appearing at 80.18ppm. The two chiral α-carbon atoms were found (and confirmed by DEPT) at 51.31 and 60.54ppm. The low field quaternary signals at 183.79 and 184.91ppm were assigned to the two quinone carbonyl carbons. Both amide carbonyl groups were found at 170.44 and 171.04 ppm and a quaternary signal at 155.15ppm was assigned to the carbamate carbonyl of the ^{t}Boc group.

Three aromatic methine carbon signals, corresponding to the (equivalent) ortho, (equivalent) meta and para carbon atoms of the benzyl group were found in their expected positions at 128.21 and 128.65ppm and the quaternary benzyl C-1 atom was located at 136.91ppm.

Furthermore, the FAB (+) mass spectrum showed a signal at m/z 655 corresponding to MH^+ confirming the molecular mass of 654 for (**277**). Elemental analysis gave good correlation with the structure.

4.3.1.6 1-[3-(O-Benzyl-L-seryl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (278) (NU:UB 41)



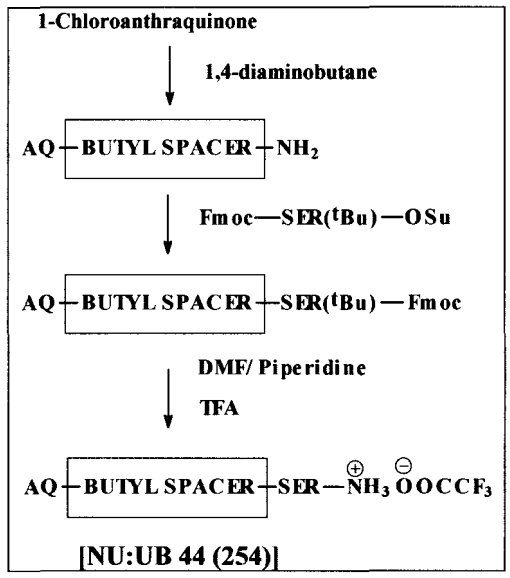
(278)

Compound (**277**) was partially deprotected, using TFA to remove the ^{t}Boc group [**Scheme 16**]. The structure of compound (**278**) was confirmed by its ^{1}H nmr spectrum which showed that the ^{t}Boc group had been successfully removed by the absence of a signal at 1.40ppm, found in the spectrum of the protected precursor (**277**), and that the Bzl protecting group was still intact by the persistence of a signal at 7.20-7.45ppm. The FAB(+) mass spectrum gave a signal at m/z 556 assigned to the anthraquinonyldipeptide cation, RNH_3^+

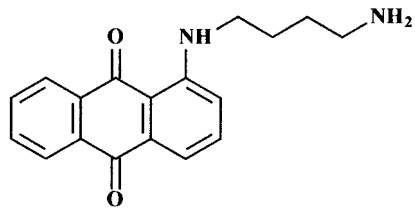
4.3.2 Example [B]: Amide-Linked (Nuclear-Unsubstituted) Aminoanthraquinone-Mono-Amino Acid Conjugates with Polar Side Chains

The butyl-spaced aminoanthraquinone conjugate (**254**), without further nuclear substitution, was prepared by coupling an active ester of O-protected serine to the N-terminus of the precursor spacer compound, followed by selective N- and O-deprotection of the serine residue of intermediate (**253**) [**Scheme 17**].

Scheme 17: Outline Synthesis Example [B]



4.3.2.1 1-[(4-Aminobutyl)amino]anthraquinone (147)

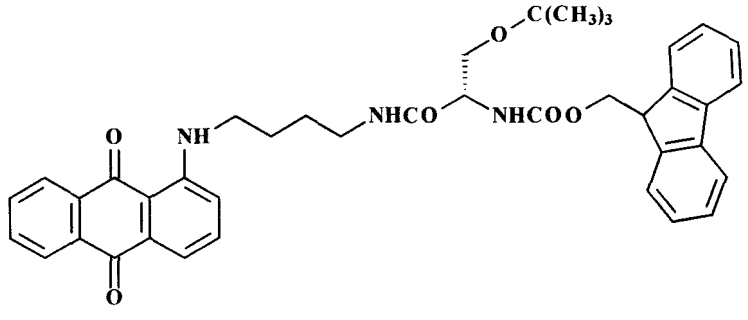


(147)

The (aminobutylamino)anthraquinone spacer compound (**147**) was prepared by the same procedure as compound (**145**) except that the amine was 1,4-diaminobutane [**Scheme 17**]. The FAB(+) mass spectrum showed a signal at m/z 295, corresponding to a molecular mass of 294. The (crude) compound was judged sufficiently pure for subsequent reactions.

4.3.2.2 1-[4-(N-α-Fluorenylmethoxycarbonyl-O-tertiarybutyl-L-serylamino)butyl-amino]anthraquinone (253)

Use of the tertiary butyl protecting group (more acid labile than benzyl) in the orthogonally-protected, activated serine derivative allowed the facile synthesis of the seryl conjugate (**254**), later shown to be antitumour active *in vivo*, via the doubly protected intermediate (**253**).

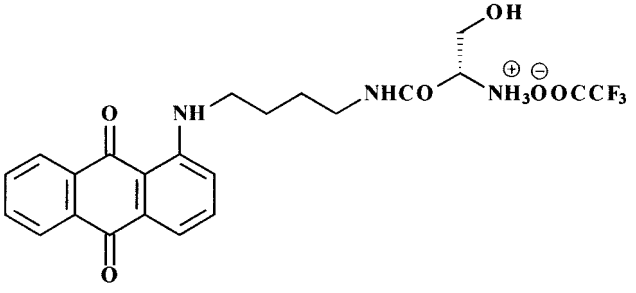


(253)

Compound (**253**) was prepared by the reaction of the butyl spacer compound (**147**) with N-α-Fmoc-O-^{t}Bu-serine-N-hydroxysuccinimide in THF [**Scheme 17**]. The structure of the compound was confirmed by its ^{1}H nmr spectrum. The tertiary butyl group gave a 9-proton singlet at 1.15ppm. Signals for the methylene protons of the butyl spacer group together with H-9 of the Fmoc group were found between 1.65 and 3.45ppm. The chiral methine proton was assigned to a quartet at 3.85ppm and the seryl methylene group gave a two-proton multiplet at 4.20ppm. A two-proton doublet at 4.40ppm (vicinal coupling, *J* value 6 Hz) was assigned to the Fmoc methylene protons. Signals for the carbamate and amide NH protons were found at 5.85 and 6.65ppm, derived from the Fmoc group and spacer-serine junction respectively. All signals in the aromatic region of the spectrum were successfully assigned. H-2 of the anthraquinone skeleton gave a one-proton double doublet at 7.05ppm. Signals for H-3 and H-4 of the anthraquinone and H-1 and H-8 of the Fmoc protecting group were found between 7.20 and 7.40ppm. Fmoc H-2, -4, -5 and -7 protons gave signals at 7.50-7.65ppm. A 4-proton multiplet was

assigned to the remaining Fmoc protons and H-3 and H-6 of the anthraquinone system. The anthraquinone H-5 and H-8 protons were found at 8.20-8.30ppm and the arylamino proton gave a characteristic one-proton triplet at 9.75ppm. The FAB(+) mass spectrum had m/z 661 $(MH)^+$ confirming a molecular mass of 660.

4.3.2.3 1-[4-(L-Serylamino)butylamino]anthraquinone trifluoroacetate/ (254) NU:UB 44



(254)

The Fmoc protecting group was removed from compound (**253**) using piperidine in DMF. The crude product was purified by solvent extraction and silica gel chromatography using a chloroform-methanol gradient. The partially deprotected compound was dissolved in TFA for 24 hours to remove the tertiarybutyl group. Conversion to the free base allowed purification by further chromatography using initially chloroform and a chloroform-ethyl acetate-methanol (16:3:1) gradient. The product was converted to the trifluoroacetate affording an analytically pure sample of the title compound (**254**) [**Scheme 17**].

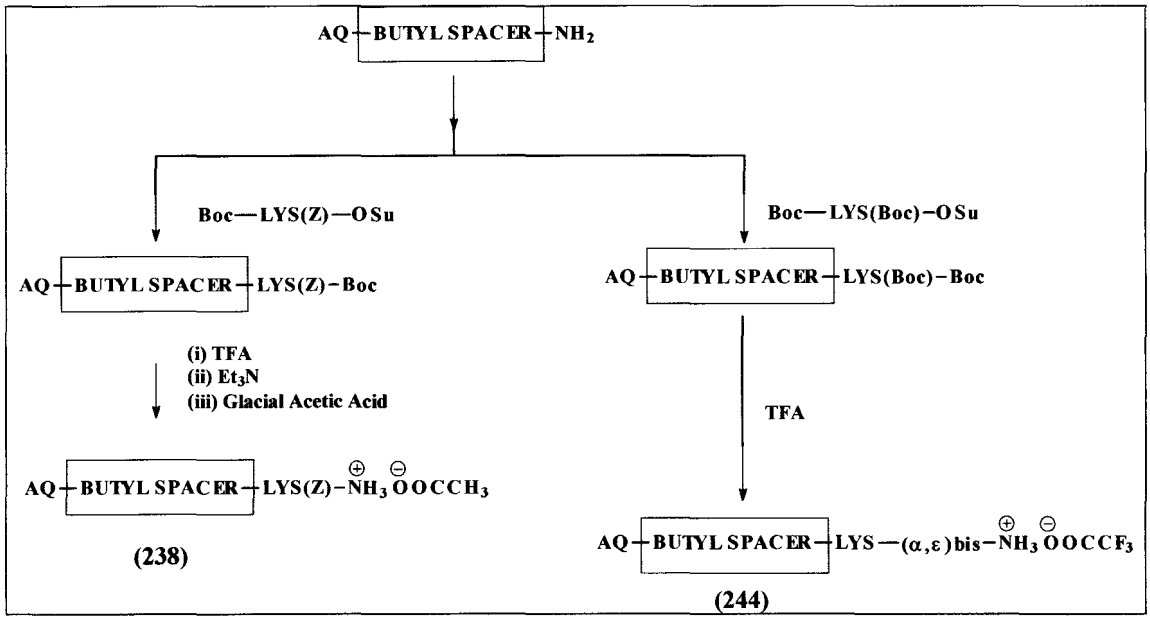
The ^1H nmr spectrum showed signals for the spacer group protons between 1.45 and 3.35ppm. The α-methine proton and the methylene protons of the hydroxymethylene group gave signals between 3.70 and 3.85ppm. Two exchange-broadened signals at 5.55ppm and 8.45ppm were assigned to the hydroxy and amide protons respectively.

The 3 protons of the NH_3^+ group were observed between 7.90 and 8.30ppm. All aromatic protons were successfully assigned. The FAB(+) mass spectrum had a signal at m/z 382 for the species RNH_3^+. Also, good correlation was obtained between the determined and theoretical elemental composition.

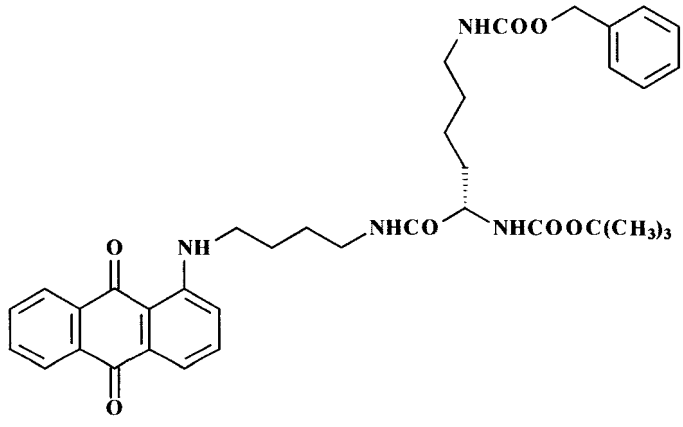
4.3.3 Example [C]: Amide-Linked (Nuclear-Unsubstituted) Aminoanthraquinone-Mono-Lysine Conjugates

The preparation of lysine conjugates was interesting from a number of points of view, including the prospect of increased aqueous solubility as a consequence of bis-salt formation on the α- and ε- amino groups. Lysine residues, in common with arginine are normally protonated in the side chains and may possess increased DNA binding potential; the lysine (K)-containing peptide motif, SPKK (without native DNA binding properties) was coupled to an anilinoacridine in the design of hybrid intercalating and groove binding agents. The SPKK unit, containing a beta-turn, was postulated to be located in the minor groove with electrostatic bonds between ε-N atoms of the lysine side chains and DNA phosphate groups. However, the intercalative process for an SPKKSPKK octamer hybrid anilinoacridine was hindered by binding of the peptide group resulting in reduced DNA binding affinity and a corresponding reduction in biological activity (Bailly 1992). Here, mono- and bis- salts of lysine (and related ornithine) conjugates were synthesised as illustrated in **Scheme 18** for lysine compounds (**238**) and (**244**).

Scheme 18: Outline Synthesis Example [C]



4.3.3.1 1-[4-(N-α-Tertiarybutoxycarbonyl-N-ε-benzyloxycarbonyl-L-lysylamino)butylamino]anthraquinone (237)



(237)

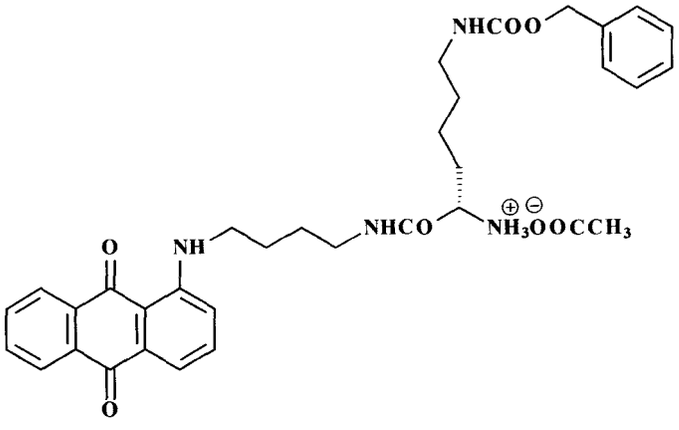
Compound (**237**) was prepared by the reaction of the butyl spacer precursor (**147**) with N-α-Boc-N-ε-Z-L-lysine-N-hydroxysuccinimide ester in DMF. Purification by solvent extraction, column chromatography and recrystallisation from absolute ethanol afforded the title compound in an analytically pure form [**Scheme 18**]. The structure was confirmed by both ^{1}H and ^{13}C nmr spectroscopy. The ^{1}H spectrum showed, for example,

a signal for a 9-proton singlet at 1.45ppm confirming the presence of the ^{t}Boc group. The amide proton was assigned to a one-proton triplet at 6.45ppm and the phenyl ring of the N-ε-Z group was seen as a 5-proton broad singlet at 7.30ppm. All other protons were successfully assigned. Furthermore, a signal in the FAB(+) mass spectrum at m/z 658 $(MH)^{+}$ corresponded to the required molecular mass of 657.

Additionally, all 37 carbon signals in the ^{13}C nmr spectrum could be accounted for. A signal at 28.32ppm was assigned to the three equivalent methyl groups of the ^{t}Boc group and the ^{t}Boc quaternary carbon gave a signal at 80.12ppm. Signals for the total of eight methylene carbons of the butyl spacer group and the lysine side chain were found (and confirmed by DEPT) at 22.56, 26.32, 27.24, 29.49, 31.85, 38.96, 40.38 and 42.43ppm The lysine α-carbon gave a signal at 54.58ppm. The amide carbonyl carbon was assigned to a peak at 172.25ppm with the ^{t}Boc and Z (carbamate) carbonyls giving signals at 156.65 and 155.90ppm. The quaternary carbon of the phenyl group was assigned to a peak at 136.58ppm and five other quaternary carbon signals were assigned to the anthraquinone skeleton of (**237**).

There was also good correlation with the expected elemental analysis.

4.3.3.2 1-[4-(N-ε-benzyloxycarbonyl-L-lysylamino)butylamino]anthraquinone acetate (238) (NU:UB 19)



(238)

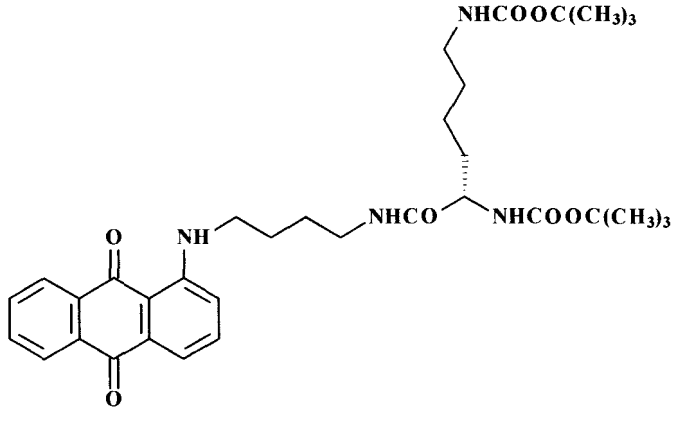
The doubly protected compound (**237**) was partially deprotected using trifluoroacetic acid to give 1-[4-(N-ε-Z-L-lysylamino)butylamino]anthraquinone trifluoroacetate as the major product. Purification of this lysine conjugate was performed on the free base; column chromatography was necessary to remove traces of the bis-deprotected compound. The pure free base was converted to an analytically homogeneous sample of the acetate salt (**238**) [**Scheme 18**]. The structure of the compound was confirmed by elemental analysis and spectroscopic techniques. The ^{1}H nmr spectrum of the compound showed, for example, a two proton quintet at 3.15ppm which was assigned to a methylene group of the butyl spacer (adjacent to NHCO). The benzylic methylene group gave a two-proton singlet at 4.95ppm and the amino proton was seen as a characteristic triplet at 9.70ppm, together with full assignment of all other proton signals.

The ^{13}C nmr spectrum of (**238**) showed signals for the eight methylene carbons of the butyl spacer group and the lysine side chain at 23.48, 26.89, 27.65, 30.21, 35.75, 38.71, 41.10 and 42.72ppm with the benzylic carbon assigned to a signal at 65.92ppm.

The electrospray (+) mass spectrum had m/z 557, corresponding to the lysyl (mono) cation RNH_3^+, and a signal at m/z 59 in the electrospray (-) spectrum confirmed the presence of the acetate anion. Additionally, the elemental analysis was consistent with theory.

4.3.3.3 1-[4-(N-(α,ε)-Di-tertiarybutoxycarbonyl-L-lysylamino)butylamino]-anthraquinone (243)

The bis-trifluoroacetate lysine conjugate (**244**) was conveniently prepared from the α,ε-bis (^{t}Boc) intermediate (**243**).



(243)

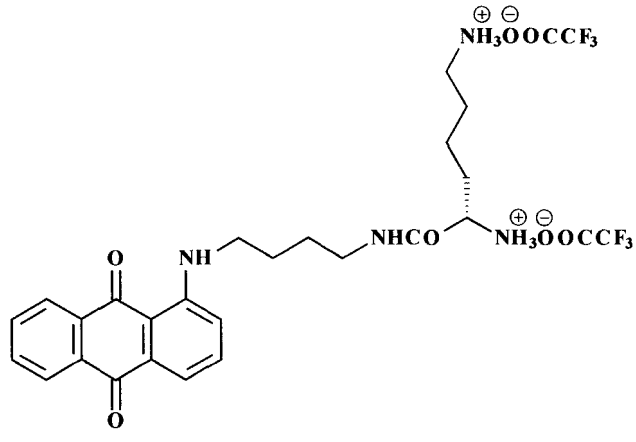
The doubly protected intermediate was prepared by the reaction of N-(α,ε)-di-^{t}Boc-L-lysine-N-hydroxysuccinimide ester with the anthraquinone-butyl spacer compound (**147**) in THF [**Scheme 18**].

The ^{1}H nmr spectrum of the compound showed unresolved signals, integrating to 20 protons, at 1.30-1.55ppm which confirmed the presence of the two ^{t}Boc protecting groups along with the γ-methylene protons of the lysine side chain. The signals for the remaining 14 methylene protons were evident between 1.60 and 3.40ppm. The chiral methine proton was assigned to a one-proton quartet at 4.05ppm. The NH protons of the

α- and ε- ^{t}Boc (carbamate) protecting groups were found as two broad singlets at 5.15 and 4.60ppm, respectively, and the amide proton was assigned to a triplet at 6.35ppm. All proton signals of the anthraquinone ring system were successfully assigned.

The FAB(+) mass spectrum had m/z 623 $(MH)^{+}$, confirming a molecular mass of 622, with fragmentation peaks at m/z 523 and 423 that corresponded to the successive loss of the ^{t}Boc protecting groups. Furthermore, elemental analysis was consistent with calculated values.

4.3.3.4 1-[4-(L-Lysylamino)butylamino]anthraquinone bis-trifluoroacetate (244) /(NU:UB 20)



(244)

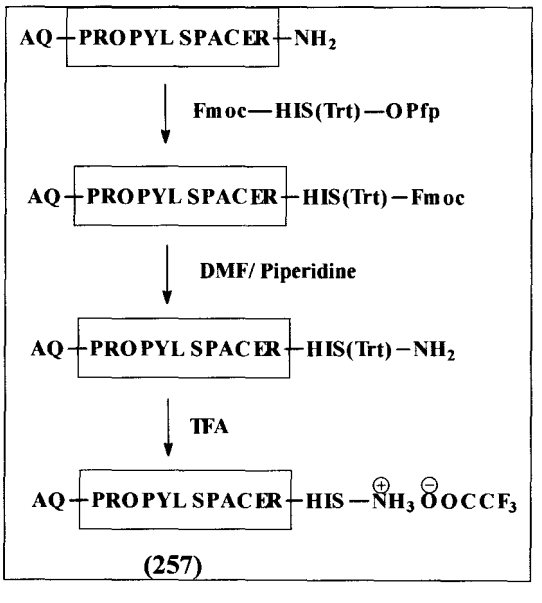
Deprotection of compound (**243**) was carried out using trifluoroacetic acid [**Scheme 18**]. The structure of the resultant salt (**244**) was confirmed by ^{1}H, ^{13}C nmr and mass spectroscopy. The ^{1}H nmr spectrum showed, for example, a signal for the amide proton at 8.55ppm and the methylene protons for the butyl spacer and the lysine side chain were evident between 1.30 and 3.40ppm. The amino proton on the anthraquinone was assigned to a one-proton triplet at 9.65ppm.

The ^{13}C nmr spectrum of the compound showed 25 different carbon signals. A DEPT experiment confirmed the presence of the eight methylene group carbons of the spacer

residue and lysine side chain. The chiral methine carbon gave a signal at 52.64ppm. The remaining seven methine carbon signals were assigned to the anthraquinone skeleton. The electrospray (+) mass spectrum gave a signal at m/z 423 corresponding to the species $[R(NH_2)NH_3]^+$. In general, bis-amine salts of lysine and analogous ornithine conjugates consistently gave rise to the mono-cation/mono-free base ions in FAB and electrospray mass spectra, rather than the doubly-charged ion. The presence of the trifluoroacetate anion was confirmed by the electrospray (-) mass spectrum which showed m/z 113.

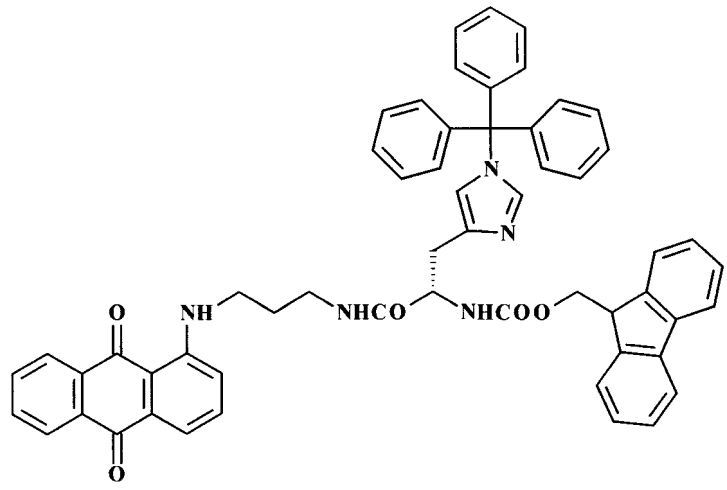
4.3.4 Example [D]: Amide-Linked (Nuclear-Unsubstituted) Aminoanthraquinone-Mono-Heteroaromatic Amino Acid [Histidine] Conjugates

Scheme 19: Outline Synthesis Example [D]



4.3.4.1 1-[3-(N-α-Fluorenylmethoxycarbonyl-N-im-trityl-L-histidylamino)propyl-amino]anthraquinone (255)

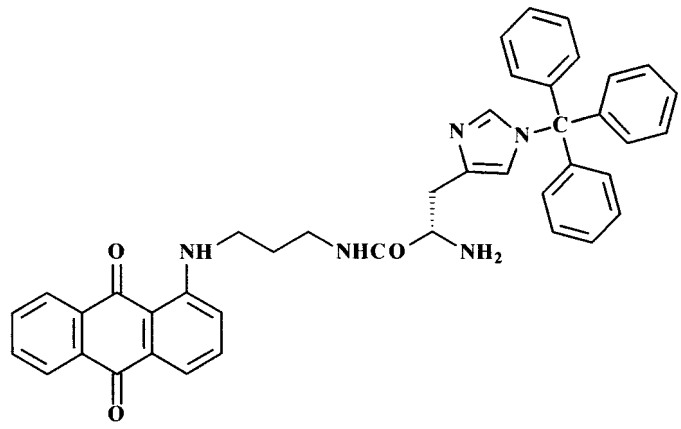
Histidine conjugates were conveniently synthesised using the acid-labile trityl group to protect the imidazole nitrogen of the heteroaromatic side chain with retention of the base-labile Fmoc group at the alpha amino group.



(255)

This orthogonally bis-protected compound was prepared by the reaction of N-α-Fmoc-N-im-trityl-L-histidine pentafluorophenolate ester with the propyl spacer compound (**145**) in THF. The crude product was purified by solvent extraction and column chromatography. Recrystallisation from ethyl acetate gave the title compound in an analytically pure form [**Scheme 19**]. The structure was confirmed by its FAB(+) mass spectrum which gave a signal at m/z 882 for $(MH)^+$ and 904 $[(M+Na)^+]$ corresponding to a molecular mass of 881. Elemental analysis was consistent with that calculated for the compound.

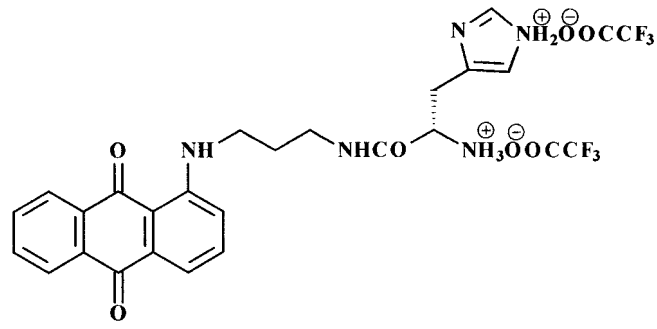
4.3.4.2 1-[3-(N-im-trityl-L-histidylamino)propylylamino]anthraquinone (256)



(256)

The foregoing bis-protected intermediate (**255**) was treated with piperidine in DMF at room temperature to remove the N-α-Fmoc protecting group. The crude product was purified by solvent extraction and silica gel column chromatography afforded an analytically pure sample of the partially deprotected histidine conjugate (**256**) [**Scheme 19**]. The compound was characterised by ^{1}H and ^{13}C nmr spectroscopy. The ^{1}H nmr spectrum gave a two proton signal at 2.10ppm for the NH_2 group confirming the successful removal of the Fmoc protecting group; full assignment of all other proton signals was possible. The ^{13}C nmr spectrum of (**256**) showed 25 different signals. Signals for four methylene carbons (propyl spacer and histidine residue) were found at 29.31, 32.92, 36.99 and 40.68ppm. The chiral α- carbon gave a signal at 55.56ppm and the quaternary trityl carbon was assigned to a signal at 75.32ppm. The FAB(+) mass spectrum had a signal at m/z 660, confirming a molecular mass of 659.

4.3.4.3 1-[3-(L-Histidylamino)propylylamino]anthraquinone trifluoroacetate (257) /(NU:UB 30)



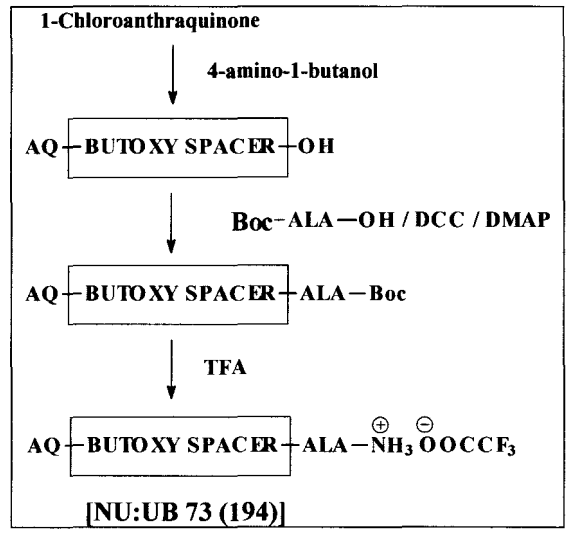
(257)

Treatment of compound (**256**) with TFA for 1 hour removed the trityl protecting group and concomitantly gave the bis-trifluoroacetate salt of the target histidine conjugate (**257**). This conjugate was purified by silica gel column chromatography on the free base (derived by treatment with trithylamine) and re-conversion to the trifluoroacetate bis-salt [**Scheme 19**]. The compound was characterised by ^{1}H nmr and mass spectroscopy. The ^{1}H nmr spectrum had, for example, signals for the 6 methylene protons of the spacer group, comprised of a two proton quintet for the central methylene protons centred at 1.80ppm and a four proton signal for the methylenes on each flank between 3.30 and 3.65ppm. The chiral methine proton and the amide proton were assigned to triplets at 4.05 and 8.60ppm respectively. All other protons were assigned. The electrospray (+) mass spectrum had a signal at m/z 418 corresponding to the species $[R(NH_2)NH_3^+]$, the mono-cation/mono-free base, rather than the doubly-charged ion, in common with the bis-lysine conjugate (**244**). Furthermore, a signal at m/z 113 in the electrospray(–) spectrum confirmed the presence of the trifluoroacetate counter anion.

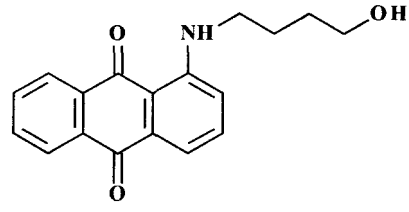
4.3.5 Example [E]: Ester-Linked (Nuclear-Unsubstituted) Aminoanthraquinone-Mono-Amino Acid Conjugates

Entry to the ester-linked examples of the NU:UB library was easily achieved via the (hydroxyalkyl)aminoanthraquinone intermediate spacer compounds, exemplified by the hydroxybutylamino compound (**150**), upon esterification with the appropriately protected amino acid derivative under DCC-mediated coupling conditions [**Scheme 20**].

Scheme 20: Outline Synthesis Example [E]



4.3.5.1 1-[(4-Hydroxybutyl)amino]anthraquinone (150)



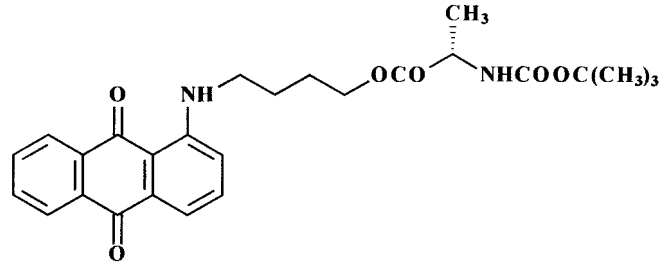
(150)

1-Chloroanthraquinone was aminated with 4-amino-1-butanol to afford the crude title compound upon precipitation with water. The crude product was partially purified by passing through a silica gel pad prepared with chloroform to remove unreacted 1-

chloroanthraquinone. The title compound, eluting with chloroform-methanol (9:1), was deemed sufficiently pure for subsequent reactions [**Scheme 20**]. The structure of the compound was confirmed by its ^{1}H nmr spectrum which had, for example, a two-proton triplet for the methylene protons of the primary alcohol and a two-proton triplet at 3.35ppm for the methylene protons adjacent to the amino group; the centrally located methylenes gave rise to an unresolved, four-proton multiplet between 1.65 and 1.95ppm. Complete assignment of the anthraquinone ring system was possible. The electron impact mass spectrum had signals at m/z 295 and m/z 277 for the molecular ion and dehydration product, respectively.

4.3.5.2 1-[4-(N-Tertiarybutoxycarbonyl-L-alanyloxy)butylamino]anthraquinone /(193)

Esterification of spacer compound (**150**) with N-tertiarybutoxycarbonyl –L-alanine gave the protected intermediate (**193**), a key step towards the synthesis of the significantly *in vivo*-active alanine conjugate NU:UB 73 (**194**) [**Scheme 20**].



(193)

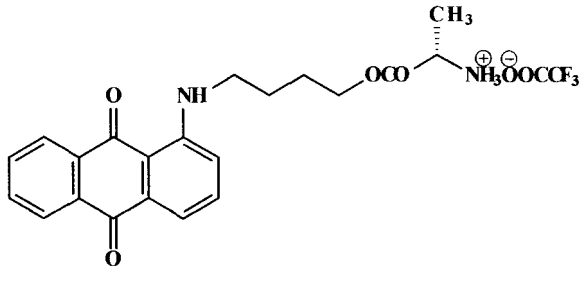
Esterification was conducted with dicyclohexylcarbodiimide (DCC) coupling, promoted by 4-dimethylaminopyridine (DMAP) in dichloromethane over twelve hours. Precipitated dicyclohexylurea was filtered off and the crude product was purified by solvent extraction and column chromatography using toluene : ethyl acetate (4:1). The structure of the compound was confirmed by its ^{1}H nmr spectrum which included a shift

in the two-proton triplet methylene protons (of the precursor hydroxymethyl group) to 4.40ppm, consistent with esterification of the hydroxy group. A twelve-proton unresolved signal between 1.35 and 1.45ppm accounted for the additional alanyl methyl group protons and those of the ^{t}Boc protecting group.

The strong signal at m/z 467 in the FAB(+) mass spectrum $(MH)^{+}$ confirmed the molecular mass of 466.

4.3.5.3 1-[4-(L-alanyloxy)butylamino]anthraquinone trifluoroacetate (194) /(NUUB 73)

Standard deprotection of the intermediate (**193**) with trifluoroacetic acid gave the water-soluble alanine conjugate NU:UB 73, that had good antitumour activity against experimental solid tumour models.



(194)

Deprotection of compound (**193**) was necessarily performed during a controlled period of no more than fifteen minutes otherwise some cleavage of the ester bond occurred [**Scheme 20**]. The structure of (**194**) was confirmed by its ^{1}H nmr spectrum. The methyl group of the alanine residue gave a 3-proton doublet at 1.35ppm (*J* 7Hz). The 2 central methylene groups of the butyl spacer were found between 1.60 and 1.80ppm. The two remaining methylene groups were assigned to signals at 3.40ppm (adjacent to N) and 4.10ppm (adjacent to O) respectively. The chiral methine proton was a one-proton multiplet at 4.20ppm. All of the aromatic protons were successfully assigned; H-2 and

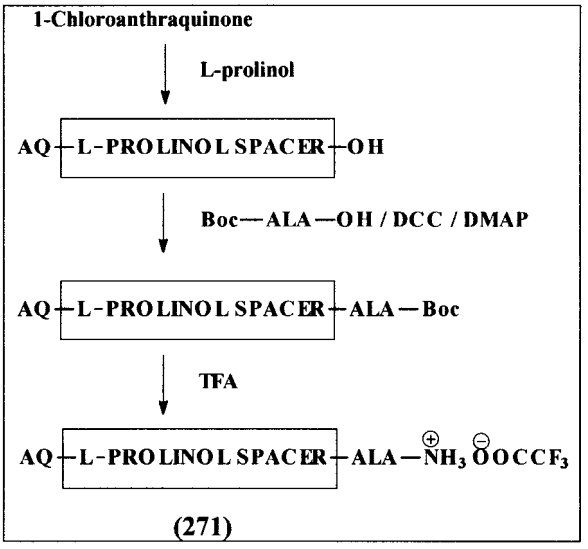
H-4 appeared as double doublets at 7.25 and 7.4ppm respectively, H-3 gave a multiplet at 7.60ppm, H-6 and H-7 were found between 7.75 and 7.85ppm and H-5 and H-8 at 8.25-8.35ppm. A broad singlet centred at 8.10ppm was assigned to the NH_3^+ group. The arylamino proton was found as a familiar triplet at 9.70ppm.

The structure of the compound was also confirmed by FAB mass spectroscopy. A signal at m/z 367 corresponded to the mono-cation $(RNH_3)^+$. Elemental analysis gave good agreement with theoretical values.

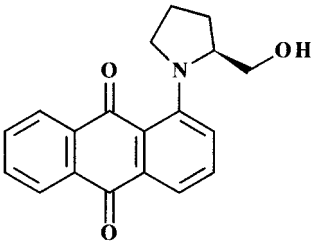
4.3.6 Example [F]: Ester-Linked (Nuclear-Unsubstituted) Aminoanthraquinone-Mono-Amino Acid Conjugates with Chiral Spacer Groups

As part of widening the scope of the syntheses and systematic variation of the nature of the spacer group, cyclic secondary amines were used in the nucleophilic amination of 1-chloroanthraquinone. Structurally hindered examples included the chiral prolinol-derived spacer (**155**), the precursor of the ester linked alanyl conjugate (271) [**Scheme 21**].

Scheme 21: Outline Synthesis Example [F]



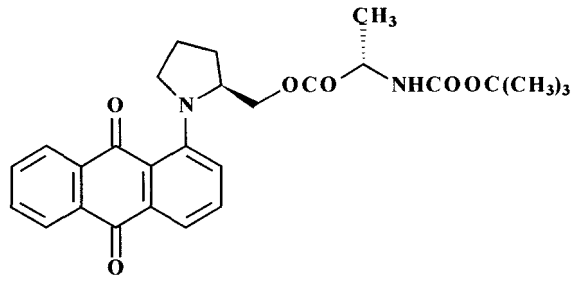
4.3.6.1 (2S)-1-[(2-Hydroxymethyl)pyrrolidinyl]anthraquinone (155)



(155)

When the relatively expensive L-prolinol was used as the amine in reaction with 1-chloroanthraquinone, less than the usually large excess of amine was used; it was found that yields of the desired product were boosted by addition of pyridine when heating in DMSO. Precipitation with water gave the crude title compound which was used without further purification [**Scheme 21**]. Its structure was confirmed by the FAB(+) mass spectrum wherein the base peak at m/z 282 for the ion MH^+ corresponded to the molecular mass of 281 Daltons.

4.3.6.2 (2S)-2-[(9,10-Dioxoanthryl)pyrrolidin-2-yl]methyl (2S)-2-[(tertiarybutoxy)-carbonylamino]propanoate (270)

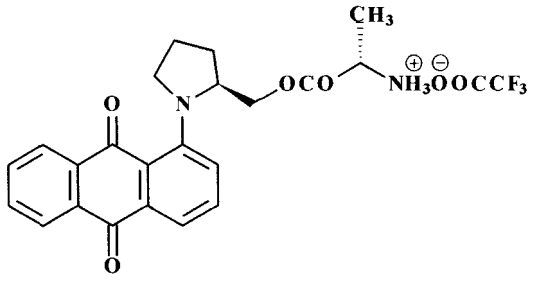


(270)

(2S)-1-[(2-Hydroxymethyl)pyrrolidinyl]anthraquinone (**155**) was coupled to N-^{t}Boc-L-alanine, using DCC and DMAP in dichloromethane [**Scheme 21**]. The protected intermediate (**270**) was isolated in a pure form by solvent extraction and chromatography by an identical procedure (above) to that used for the related alanine intermediate (**193**). The structure of (**270**) was confirmed by its ^{1}H nmr spectrum which included a 3-proton doublet at 1.25ppm for the methyl group of the alanine residue. A 9-proton singlet at 1.45ppm and a one-proton doublet at 5.40ppm (assigned to the carbamate NH proton) confirmed the presence of the N-^{t}Boc protecting group. All methylene protons of the spacer pyrrolidine ring aromatic protons were resolved and the anthraquinone system was successfully assigned.

The base peak in the FAB(+) mass spectrum at m/z 479 for the ion $(MH)^{+}$ confirmed the molecular mass of 478 Daltons. Elemental analysis figures were consistent with theory.

4.3.6.3 (2S)-2-[(9,10-Dioxoanthryl)pyrrolidin-2-yl]methyl (2S)-2-aminopropanoate trifluoroacetate (271) (NU:UB 171)



(271)

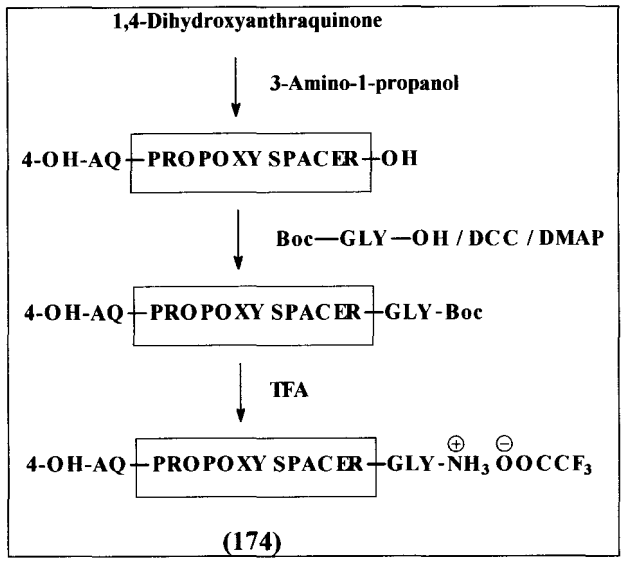
Deprotection of intermediate (**270**) with trifluoroacetic acid [**Scheme 21**] gave the title compound (**271**) whose structure was confirmed by its ^{1}H nmr spectrum which showed that the N-^{t}Boc protecting group had been removed (by the absence of the 9-proton singlet in the spectrum of the protected precursor). The spectrum showed, for example, a 3-proton doublet at 1.35ppm for the methyl group of the alanine residue in addition to a two-proton multiplet at 4.30ppm for the methylene group adjacent to the ester bond. The aromatic protons were all evident between 7.65 and 8.20ppm. Full assignment of all other proton signals was possible in confirmation of the structure. The electrospray (+) mass spectrum of the compound showed a peak at m/z 379 for the species RNH_3^+.

The base peak in the electrospray (–) mass spectrum at m/z 113 confirmed the presence of the trifluoroacetate anion. Elemental analysis data was consistent with that required for the title compound.

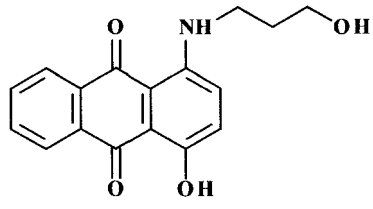
4.3.7 Example [G]: Ester-Linked (Nuclear-Mono-Hydroxylated) Aminoanthraquinone-Mono-Amino Acid Conjugates

Access to the 4-hydroxy-1-aminoanthraquinone system was achieved by controlled amination of 1,4-dihydroxyanthraquinone (quinizarin), as illustrated with 3-aminopropanol [**Scheme 22**].

Scheme 22: Outline Synthesis Example [G]



4.3.7.1 4-Hydroxy-1-[(3-hydroxypropyl)amino]anthraquinone (156) (NU:UB 58)

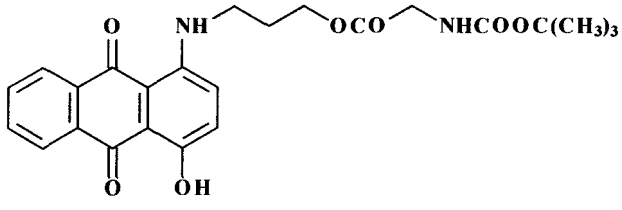


(156)

1,4-Dihydroxyanthraquinone and 3-amino-1-propanol were heated in ethanol and THF (1:1) over a steam bath for 1.75h with close monitoring by t.l.c., which showed that a major purple product had formed, together with the emergence of the blue (presumed) 1,4-bis-substituted compound, at which point the reaction was stopped and immediately subjected to silica gel chromatography to prevent further reaction [**Scheme 22**]. The major product was eluted using toluene-ethyl acetate (4:1) and recrystallised from ethanol to give the title compound (**156**). The structure of the compound was confirmed by ^{1}H nmr and FAB(+) mass spectroscopy. A two-proton signal (quintet) in the ^{1}H nmr spectrum at 1.50ppm was assigned to the central methylene group of the propyl spacer.

The two remaining methylene groups of the spacer gave signals at 3.45ppm (CH_2 adjacent to N) and 3.55ppm (CH_2 adjacent to OH). The spacer hydroxy proton was a triplet at 4.70ppm and the aryl hydroxy proton was a singlet at 13.65ppm. The aromatic protons were all successfully assigned; H-2 and H-3 gave doublets at 7.30 and 7.45ppm respectively, H-6 and H-7 protons were found between 7.80 and 7.90ppm and H-5 and H-8 at 8.15-8.25ppm. The arylamino proton gave a triplet at 10.70ppm. The base peak in the FAB(+) mass spectrum at m/z 298 for the ion $(MH)^+$ confirmed a molecular mass of 297 Daltons.

4.3.7.2 4-Hydroxy-1-[3-(N-tertiarybutoxycarbonylglycyloxy)propylamino]-anthraquinone (173)

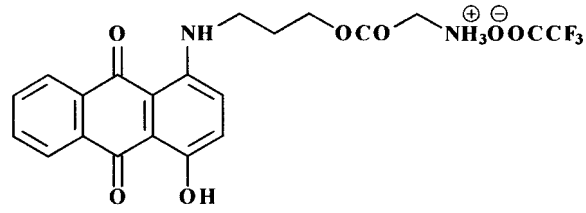


(173)

Esterification of 4-hydroxy-1-[(3-hydroxypropyl)amino]anthraquinone (**156**) with N-^{t}Boc-glycine, by the same procedure adopted for (foregoing) protected conjugates (**193**) and (**270**), afforded the title compound (**173**) which was analytically pure after chromatography and precipitation from an ethyl acetate-pentane mixture (1:50) [**Scheme 22**]. The structure of the compound was confirmed by its ^{1}H nmr spectrum. A 9-proton singlet at 1.45ppm together with a 1 proton broad singlet at 5.20ppm confirmed the presence of the N-^{t}Boc methyl and carbamate NH protons. The methylene group of the glycine residue was assigned to a 2-proton doublet at 4.00ppm (J 5Hz). Signals for the 3 methylene groups of the propyl spacer were found at 2.10ppm (central CH_2), 3.50ppm (adjacent to N) and 4.35ppm (adjacent to O). A one-proton triplet at 10.30ppm and a

one-proton singlet at 13.60ppm were assigned to the arylamino and hydroxy protons respectively. The anthraquinone protons were fully assigned. The base peak in the FAB(+) mass spectrum at m/z 455 for ion $(MH)^+$ confirmed a molecular mass of 454. The compound was further characterised by a satisfactory elemental analysis.

4.3.7.3 4-Hydroxy-1-[3-(glycyloxy)propylamino]anthraquinone trifluoroacetate (174) (NU:UB 165)



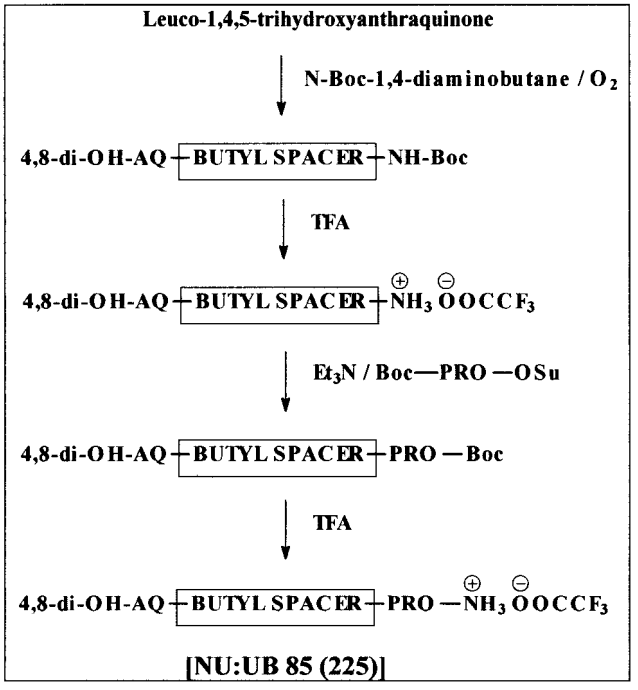
(174)

Compound (**173**) was deprotected using trifluoroacetic acid [**Scheme 22**]. The structure of the trifluoroacetate (**174**) was confirmed by nmr and mass spectroscopy and elemental analysis. The ^1H nmr spectrum of the compound showed, for example, a two-proton singlet at 3.90ppm for the methylene group of the glycine residue and a triplet at 10.30ppm, integrating to 1 proton, which was assigned to the arylamino proton. All other signals were successfully assigned. A signal in the electrospray(+) mass spectrum at m/z 355 was assigned to the RNH_3^+ cation. The base peak in the electrospray(–) mass spectrum at m/z 113 corresponded to the trifluoroacetate anion, confirming a molecular mass of 468 Daltons. Finally, there was good correlation with the expected elemental analysis.

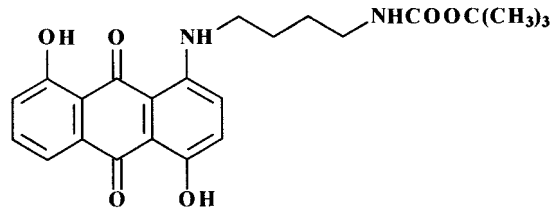
4.3.8 Example [H]: Amide-Linked (Nuclear-Dihydroxylated) Aminoanthraquinone-Mono-Amino Acid Conjugates

Regiospecific amination of leuco-1,4,5-trihydroxyanthraquinone with primary diamines gave access to nuclear-dihydroxylated aminoanthraquinone spacer compounds with a terminal amino group for amino acid conjugation. The sequence is illustrated in **Scheme 23** using the mono-protected ^{t}Boc-diaminobutane to give the intermediate spacer compound and the derived proline conjugate NU:UB 85 (**225**).

Scheme 23: Outline Synthesis Example [H]



4.3.8.1 4,8-Dihydroxy-1-[4-(N-tertiarybutoxycarbonylamino)butylamino]-anthraquinone (159)

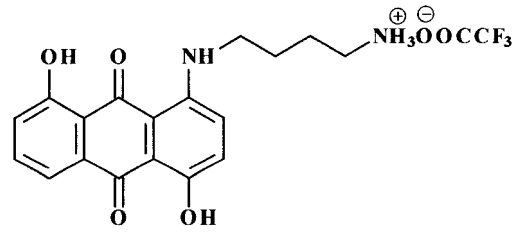


(159)

A solution of N-^{t}Boc-1,4-diaminobutane in dichloromethane was stirred with a suspension of leuco-1,4,5-tyrihydroxyanthraquinone for 6 hours at room temperature. After aeration the crude product was purified by silica gel chromatography using initially dichloromethane then a dichloromethane-ethyl acetate (4:1) gradient [**Scheme 23**].

The CI mass spectrum of the major product (**159**) had a signal at m/z 427 $(MH)^{+}$ confirming the molecular mass of 426 Daltons.

4.3.8.2 4,8-Dihydroxy-1-[(4-aminopropyl)amino]anthraquinone trifluoroacetate(160)

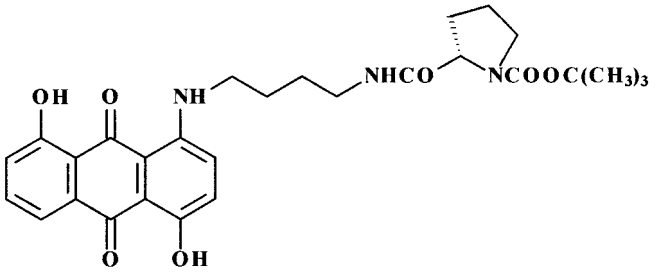


(160)

Standard deprotection of compound (**159**) with trifluoroacetic acid [**Scheme 23**] gave the resultant salt (**160**) whose structure was confirmed by its ^{1}H nmr spectrum. A signal centred at 1.65ppm, integrating to 4 protons, was assigned to the 2 central methylene groups of the butyl spacer. A triplet at 2.85ppm was assigned to the methylene group adjacent to RNH_3^{+}. The remaining spacer methylene group gave a 2-proton quartet at

3.45ppm. The aromatic protons were fully assigned; H-2 and H-3 to a 2-proton multiplet between 7.20 and 7.35ppm, H-7 was seen as a doublet at 7.50ppm (*J* 10Hz) and H-5 and H-6 gave a multiplet between 7.65 and 7.70ppm. A one-proton triplet at 9.85ppm was assigned to the arylamino proton (*J* 4Hz). Further confirmation of the structure was given by the FAB(+) mass spectrum which had a signal at m/z 327 corresponding to the RNH_3^+ cation.

4.3.8.3 4,8-Dihydroxy-1-[4-(N-tertiarybutoxycarbonylprolylamino)butylamino]-anthraquinone (224)

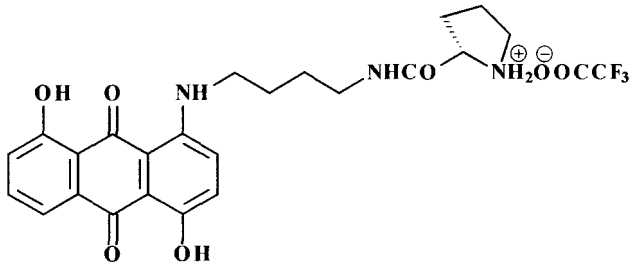


(224)

4,8-Dihyroxy-1-[(4-aminobutyl)amino]anthraquinone trifluoroacetate (**160**) was coupled with N-^{t}Boc-L-proline-N-hydroxysuccinimide ester in the presence of triethylamine in THF at 0°C [**Scheme 23**]. Silica gel column chromatography and recrystallisation from ethyl acetate gave the title compound (**224**) which was characterised by nmr and mass spectroscopy. The ^{1}H nmr spectrum had, for example, a 9 proton singlet at 1.45ppm and a one-proton broad singlet centred at 7.05ppm which were assigned to the tertiarybutyl and carbamate protons respectively, confirming the presence of the N-^{t}Boc protecting group. The chiral methine proton gave a one-proton broad singlet at 4.25ppm. The aromatic protons were fully assigned; H-2 and H-3 gave a 2-proton multiplet between 7.20 and 7.30ppm, H-6 was a triplet at 7.60ppm and H-5 a doublet at 7.80ppm. A triplet at 9.90ppm was assigned to the arylamino proton. The two one-proton singlets 13.25

and 13.85ppm were assigned to the 4-hydroxy and 8-hydroxy protons. The FAB(+) mass spectrum had a signal at m/z 524 for the ion $(MH)^+$ confirming the molecular mass of 523 Daltons.

4.3.8.4 4,8-Dihydroxy-1-[4-(prolylamino)butylamino]anthraquinone trifluoroacetate (225) NU:UB 85



(225)

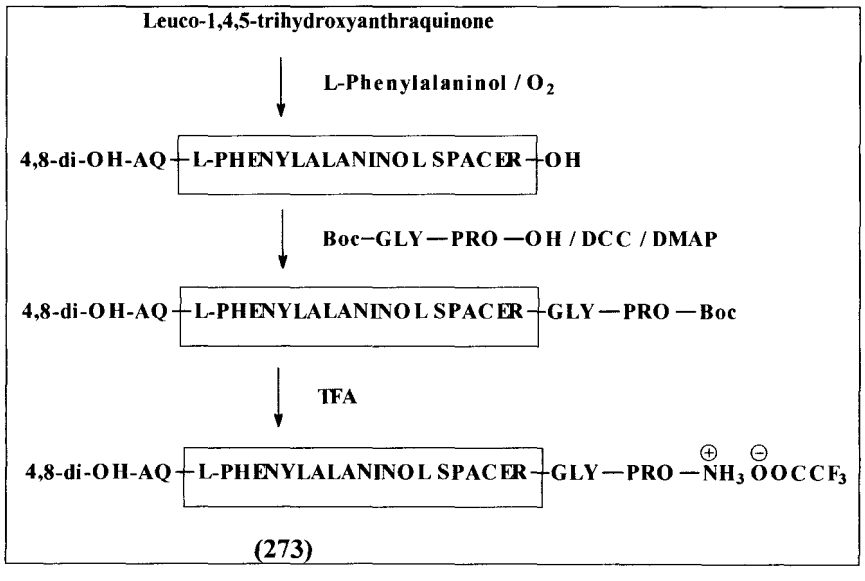
Compound (**224**) was deprotected using trifluoroacetic acid [**Scheme 23**] and the resultant salt (**225**) was purified by silica gel chromatography using chloroform-ethanol (2:1).

The ^1H nmr spectrum of (**225**) showed that the N-^{t}Boc protecting group had been successfully removed. A multiplet at 4.15ppm was assigned to the chiral methine proton. The amide proton was a one-proton triplet at 8.60ppm and the arylamino proton was found as a triplet at 9.85ppm. The base peak at m/z 424 in the electrospray(+) mass spectrum corresponded to the species RNH_3^+. There was a signal at m/z 113 in the electrospray(–) mass spectrum for the trifluoroacetate anion, confirming the molecular mass of 537.

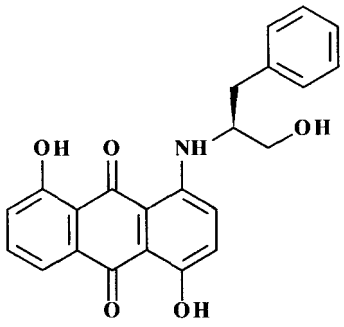
4.3.9 Example [I]: Mixed Ester/Amide-Linked (Nuclear-Dihydroxylated) Aminoanthraquinone-Peptide Conjugates

Additional amino acids were reliably added to the N-terminus of anthraquinone-amino acid conjugates in the linear synthesis of peptide derivatives of either amide- or ester-linked examples. Alternatively, preformed peptide fragments (suitably protected) could be coupled directly to give elongated peptide motifs, illustrated for the complex deriative (**273**) which contained a mixed ester and amide backbone [**Scheme 24**].

Scheme 24: Outline Synthesis Example [I]



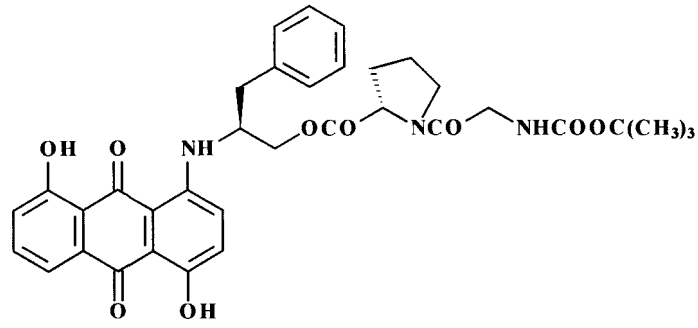
4.3.9.1 4,8-Dihydroxy-1-{[(S)-2-hydroxy-1-benzylethyl]amino}anthraquinone /(162)



(162)

The chiral spacer compound (**162**) was derived from L-phenylalaninol and leuco-1,4,5-trihydroxyanthraquinone [**Scheme 24**] by a procedure analogous to that used for (**159**) above. The structure of the compound was confirmed by its ^1H nmr spectrum. A one-proton triplet at 2.35ppm was assigned to the hydoxy group of the phenylalaninol residue. Signals for the 2 methylene groups were found at 3.05ppm (adjacent to C_6H_5) and 3.85ppm (adjacent to OH) and the chiral methine proton gave a 1 proton multiplet at 4.05ppm. The protons of the anthraquinone chromophore were successfully assigned; for example, H-6 gave a doublet at 7.55ppm (*J* 8Hz). A one-proton doublet at 10.20ppm was assigned to the arylamino proton. Two one-proton singlets at 13.20ppm and 13.80ppm confirmed the presence of the 4- and 8- hydroxy groups respectively. The base peak in the FAB(+) mass spectrum at m/z 390 for the ion $(MH)^+$ confirmed the molecular mass of 389.

4.3.9.2 (2S)-2-[(4,8-Dihydroxy-9,10-dioxoanthryl)amino]-3-phenylpropyl (2S)-1{2-[(tertiarybutoxy)carbonylamino]acetyl}pyrrolidine-2-carboxylate (272)

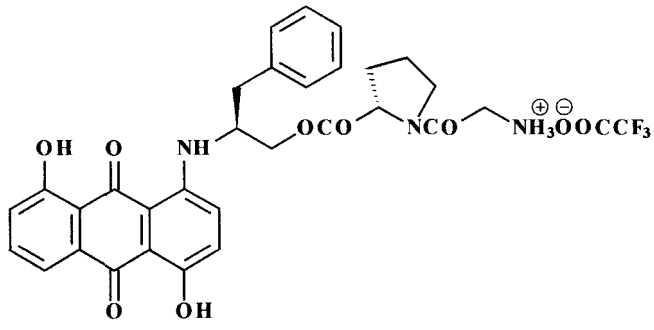


(272)

Esterification of 4,8-dihydroxy-1-{[(S)-2-hydroxy-1-benzylethyl]amino}anthraquinone (**162**) with the protected dipeptide N-^{t}Boc-glycyl-L-proline using standard DCC coupling gave the N-protected intermediate (**272**) after solvent extraction and column chromatography using toluene-ethyl acetate (4:1) [**Scheme 24**]. The title compound was precipitated from an ethyl acetate-hexane solution to give a purple solid which was characterised by nmr and mass spectroscopy and elemental analysis. For example, a 9-proton singlet at 1.40ppm in the ^{1}H nmr spectrum confirmed the presence of the ^{t}Boc protecting group. Signals for the 3 methylene groups of the proline residue were found as an unresolved 4-proton multiplet between 1.90 and 2.25ppm (β and γ) and a 2-proton multiplet at 3.60ppm (δ). A two-proton doublet at 3.95ppm was assigned to the methylene group of the glycine residue. The aromatic protons were all assigned to their corresponding signals. H-2, H-3 and H-7 protons were seen together with the phenyl protons between 7.15 and 7.35ppm. The H-6 proton gave a multiplet at 7.60ppm and H-5 was seen as a double doublet at 7.85ppm. The amino proton gave a doublet at 10.15ppm and the 2 aryl hydroxy groups were assigned to singlets at 13.20 and 13.85ppm. In the FAB(+) mass spectrum a signal at m/z 644 was assigned to the ion

$(MH)^+$ confirming the molecular mass of 643. Good correlation with elemental analysis additionally confirmed the structure.

4.3.9.3 (2S)-2-[(4,8-Dihydroxy-9,10-dioxoanthryl)amino]-3-phenylpropyl (2S)-1-(2-aminoacetyl)pyrrolidine-2-carboxylate trifluoroacetate (273)



(273)

The ^{t}Boc compound (**272**) was deprotected using trifluoroacetic acid to give a purple solid of the title compound [**Scheme 24**]. The ^{1}H nmr spectrum showed, for example, a 2 proton singlet at 3.85ppm for the methylene group of the glycine residue. Two multiplets at 3.00 and 3.50ppm, each integrating to 2 protons, were assigned to the methylene protons of the phenylalaninol side chain and the δ protons of the proline residue respectively. An unresolved 7 proton multiplet in the aromatic region of the spectrum was assigned to H-2 and H-3 of the anthraquinone skeleton and the 5 phenyl protons. The H-7 proton gave a one-proton doublet at 7.60ppm and H-5 and H-6 were seen as a 2-proton multiplet at 7.75ppm. A one-proton doublet at 10.15ppm was assigned to the amino proton. The structure of the compound was further confirmed by mass spectroscopy. A signal at m/z 544 in the electrospray (+) mass spectrum corresponded to the RNH_3^+ cation. The base peak at m/z 113 in the electrospray(–) mass spectrum confirmed the presence of the trifluoroacetate anion.

4.4 Biological and Biochemical Evaluation

The majority of spacer-linked anthraquinone amino acid/ peptide conjugates prepared in this research programme were evaluated for their growth inhibitory and cell killing effects on a panel of human and animal tumour cell lines, either conducted in the host or collaborating laboratory; selected conjugates were additionally screened in the National Cancer Institute (NCI) 60 cell line *in vitro* assay (Monks *et al* 1991). Selected conjugates were investigated for their ability to interact with DNA topoisomerase enzymes, in particular to identify catalytic inhibitory properties or poisoning action, and for limited DNA-binding studies. Based upon their *in vitro* profile, a number of candidates were selected for pre-clinical development in collaborating laboratories and progressed to *in vivo* antitumour studies.

Within the scope of this thesis, the key data on a necessarily representative selection only of leading candidate compounds is discussed. NU:UB 31 (**208**), NU:UB 51 (**176**) and NU:UB73 (**194**), examples of nuclear-unsubstituted/ amide-linked, nuclear-dihydroxylated/ amide-linked and nuclear-unsubstituted/ ester-linked aminoanthraquinone classes of conjugate have emerged as significant lead compounds, the emphasis in the ensuing discussion will be placed upon the promising proline conjugate NU:UB 31 (**208**).

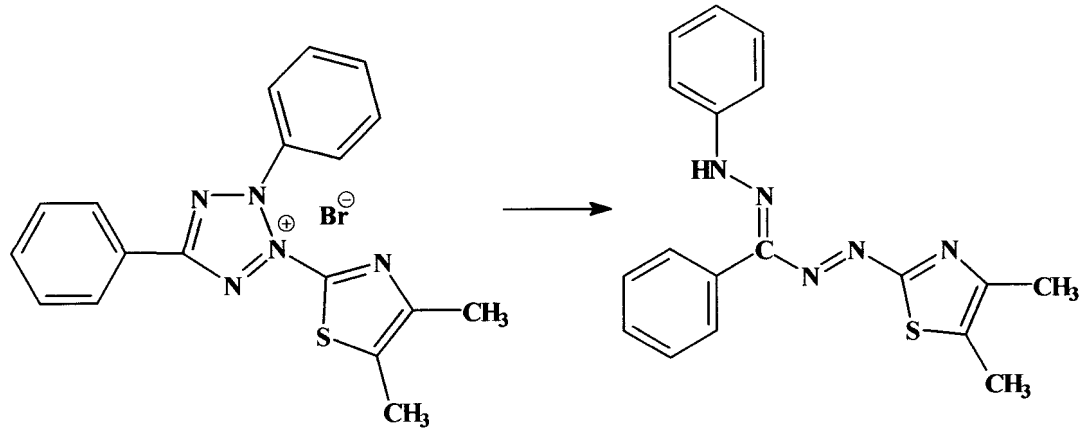
4.4.1 *In Vitro* Chemosensitivity

Chemosensitivity was determined using the MTT assay. This was derived from the original MTT assay method (Mosmann 1983) and adapted for cancer research by other investigators (Plumb *et al* 1989). Some modifications were made because some cell lines used were non-adherent.

This assay, a measure of cell survival, is based on the cleavage of the yellow tetrazolium salt, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] into a

purple formazan crystalline product by metabolically active cells. This cellular reduction is carried out by NADH and NADPH cofactors of the mitochondrial enzyme succinate-dehydrogenase. The formazan crystals formed are solubilised and the resulting colour is quantified spectrophotometrically at a wavelength of 580nm. The amount of formazan produced is proportional to the number of viable cells present. The full assay protocol is detailed in the experimental section [**Chapter 9**].

Figure 6: Metabolism of MTT to a formazan salt by viable cells



4.4.2 Murine MAC15A Colon Adenocarcinoma Cell Line Sensitivity

The murine (MAC15A) colon cancer cell line (Double and de Castro 1978) was chosen as a 'front-line' screen for evaluation of the cytotoxicity of spacer-linked aminoanthraquinone amino acid/peptide conjugates because, in common with human clinical disease, it is refractory to standard clinical agents, including topoisomerase inhibitors, as the transplantable *in vivo* experimental tumour model (Laws *et al* 1995). Potency in this model (*in vitro* and *in vivo*) was considered a stringent criterion for compound selection. Cytotoxic potency against this cell line was expressed as IC_{50} values, the concentration of compound to effect 50% growth inhibition compared to untreated control cells, using data from the MTT assay following a 96 hour exposure as standard.

4.4.2.1 Amide-Linked (Nuclear-Unsubstituted) Aminoanthraquinone Mono-Amino Acid and Dipeptide Conjugates/ MAC15A Chemosensitivity

Data against the MAC15A cell line for these conjugates are presented in **Table 1**.

Table 1: *In vitro* chemosensitivity of Amide-Linked (Nuclear-Unsubstituted) Aminoanthraquinone Mono-Amino Acid and Dipeptide Conjugates against the MAC15A Adenocarcinoma Cell Line*

NU:UB Code	Structure Number	Spacer Type	Peptide Motif	$IC_{50}\mu M^{\#}$
1	**(163)**	PROPYL	Gly-Boc	278
2	**(164)**	PROPYL	Gly-TFA	24
4	**(182)**	PROPYL	Ala-TFA	5.3
8	**(227)**	PROPYL	Lys(ε-Z)α-TFA	37
16	**(234)**	PROPYL	Lys(α,ε)-bis-TFA	18
18	**(166)**	BUTYL	Gly-TFA	22
19	**(238)**	BUTYL	Lys(ε-Z)α-ACE	12
20	**(244)**	BUTYL	Lys(α,ε)-bis-TFA	22
21	**(184)**	PROPYL	D-Ala-TFA	3.5
22	**(252)**	PROPYL	Ser(OBzl)-ACE	18
23	**(276)**	PROPYL	Gly-Lys(α,ε)-bis-TFA	30
24	**(236)**	PROPYL	Orn(α,δ)-bis-TFA	26
30	**(257)**	PROPYL	His-bis-TFA	32
31	**(208)**	PROPYL	Pro-TFA	2.5
33	**(186)**	BUTYL	Ala-TFA	5.5
41	**(278)**	PROPYL	Pro-Ser(OBzl)-TFA	19.5
42	**(242)**	BUTYL	Lys(ε-ACE)α-Z	19
43	**(215)**	BUTYL	Pro-TFA	4.5
44	**(254)**	BUTYL	Ser-TFA	3
45	**(233)**	PROPYL	Lys(ε-Boc)α-ACE	9.5
46	**(210)**	PROPYL	D-Pro-TFA	3.5
47	**(229)**	PROPYL	Orn(δ-Z)α-ACE	24
48	**(240)**	BUTYL	Orn(δ-Z)α-ACE	23
49	**(250)**	PROPYL	Asn-TFA	21
50	**(213)**	PROPYL	Hyp-TFA	15
72	**(188)**	BUTYL	D-Ala-TFA	3.2
99	**(246)**	BUTYL	Orn(α,δ)-bis-TFA	29
197	**(146)**	PROPYL	Nil-TFA	25

* Chemosensitivity (cytotoxicity) as determined by MTT assay, 96 hour exposure.
# Expressed as IC_{50} values [the concentration (μM) of compound to effect 50% growth inhibition] and represents the mean of (at least) three separate experiments (standard deviations were ± 5-20%); the complete protocol may be found in **Chapter 9**.

Potency data for conjugates that retained the N-terminal protecting group and were not in a salt form were relatively inactive, for example the Boc-protected glycine conjugate NU:UB 1 (**163**) that had an IC_{50} value of 278μM; other conjugates of this type behaved similarly (data not shown). Lack of potency was not simply the result of decreased aqueous solubility since these conjugates were adequately solubilized in DMSO/ tissue culture medium.

The most active conjugates had IC_{50} values in the low micromolar range and were conjugates with small aliphatic relatively hydrophobic α-side chains in the amino acid residue including alanines, prolines and (the somewhat more polar) serines. IC_{50} values ranged from 2.5 to 6μM. No significant differences were observed either for differently spaced (C-3 *vs* C-4) conjugates of the same amino acid, or for substitution of the unnatural D-isomer of a given amino acid.

Within a sub-series of lysine and analogous ornithine conjugates, IC_{50} values were consistently an order of magnitude greater than the most active compounds whether or not these were partially protected on either the α- amino or side chain amino groups or were the fully deprotected bis-salts. Furthermore, the histidine conjugate bis-salt NU:UB 30 (**257**) was similarly lacking in potency. Strongly cationic side chains in aminoanthraquinones had been found by Gatto *et al* (1996) to reduce cytotoxic potency in a series of lysine containing agents which were inactive below 100μM in a number of cell lines; these compounds, exemplified by (**106**) and (**107**) [**Chapter 3**], were, however, symmetrically bis-substituted in contrast to the mono-substituted NU:UB agents (average IC_{50} values of 20-30μM) which suggested that the greater the number of charged residues, the greater the reduction in cytotoxic potency within the anthraquinone series and underlines the lack of correlation between cytotoxic potency and strong DNA binding.

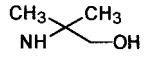
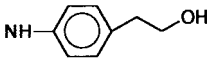
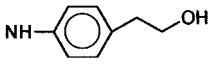
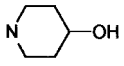
NU:UB 21 (**184**), NU:UB 31 (**208**), NU:UB 43 (**215**) and NU:UB 44 (**254**) were all later shown to possess notable antitumour activity *in vivo* against MAC15A experimental colon cancer.

4.4.2.2 Ester-Linked (Nuclear-Unsubstituted) Aminoanthraquinone Mono-Amino Acid and Dipeptide Conjugates/ MAC15A Chemosensitivity

Data against the MAC15A cell line for these conjugates are presented in **Table 2**.

In common with their amide-linked counterparts, the majority of ester conjugates were active in the MAC15A cell line at low micromolar concentrations. The most active conjugates again had small aliphatic relatively hydrophobic α-side chains in the amino acid residue including alanines, prolines, α and γ aminobutyric acids, aminoisobutyric acid and glycines. The least active compounds were sterically hindered at N-1 of the anthraquinone, (**158**) and (**271**), or were branched in the spacer group (**128**). The propoxy proline conjugate (**219**) was notably 4-fold more potent than its shorter ethoxy homologue (**217**). The propoxy spaced dipeptide conjugates (**281**) and (**283**) were notably amongst the most potent conjugates in the ester-linked category. The alanine conjugate NU:UB 73 (**194**) and its D-isomer NU:UB 76 (**196**) were later shown to have significant antitumour activity against MAC15A tumours *in vivo*.

Table 2: *In vitro* chemosensitivity of Ester-Linked (Nuclear-Unsubstituted) Aminoanthraquinone Mono-Amino Acid and Dipeptide Conjugates against the MAC15A Adenocarcinoma Cell Line*

NU:UB CODE	Structure Number	SPACER TYPE	PEPTIDE MOTIF	$IC_{50}\mu M$ MAC15A#
73	**(194)**	BUTOXY	Ala-TFA	2.6
76	**(196)**	BUTOXY	D-Ala-TFA	2.9
107	**(192)**	PROPOXY	D-Ala-TFA	6.9
108	**(190)**	PROPOXY	Ala-TFA	9.7
109	**(170)**	PROPOXY	Gly-TFA	10.6
110	**(172)**	BUTOXY	Gly-TFA	9.4
111	**(219)**	PROPOXY	Pro-TFA	8.3
112	**(221)**	PROPOXY	D-Pro-TFA	10.2
115	**(217)**	ETHOXY	Pro-TFA	33.5
116	**(281)**	PROPOXY	Pro-Gly-TFA	2.5
117	**(168)**	ETHOXY	Gly-TFA	3.5
120	**(231)**	PROPOXY	Orn(δ-Z)-α-TFA	2.4
127	**(283)**	PROPOXY	Gly-Leu-TFA	1.2
128	**(204)**		Ala-TFA	35
156	**(200)**		Ala-TFA	4.7
157	**(202)**		D-Ala-TFA	3.4
158	**(198)**		Ala-TFA	14.0
163	**(259)**	BUTOXY	Sar-TFA	7.2
166	**(261)**	BUTOXY	MeAla-TFA	4.8
167	**(263)**	BUTOXY	Abu-TFA	3.0
168	**(265)**	BUTOXY	γ- Abu-TFA	4.5
169	**(267)**	BUTOXY	Aib-TFA	3.2
170	**(269)**	L-Alaninol	Ala-TFA	5.5
171	**(271)**	L-Prolinol	Ala-TFA	22

* Chemosensitivity (cytotoxicity) as determined by MTT assay, 96 hour exposure.

Expressed as IC_{50} values [the concentration (μM) of compound to effect 50% growth inhibition] and represents the mean of (at least) three separate experiments (standard deviations were ± 5-20%); the complete protocol may be found in **Chapter 9**.

4.4.2.3 Amide/Ester-Linked (Nuclear-Hydroxylated) Aminoanthraquinone Mono-Amino Acid and Dipeptide Conjugates/ MAC15A Chemosensitivity

Data against the MAC15A cell line for these conjugates are presented in **Table 3**.

Table 3: *In vitro* chemosensitivity of Amide/Ester-Linked (Nuclear-Hydroxylated) Aminoanthraquinone Mono-Amino Acid and Dipeptide Conjugates against the MAC15A Adenocarcinoma Cell Line*

NU:UB CODE	Structure Number	SPACER TYPE	Linkage♦	PEPTIDE MOTIF	$IC_{50}\mu M$ MAC15A#
51	**(176)**	PROPYL(AQ 4,8-di-OH)	A	Gly-TFA	2
83	**(223)**	PROPYL(AQ 4,8-di-OH)	A	Pro-TFA	0.8
118	**(206)**	PROPYL(AQ 4,8-di-OH)	A	D-Ala-TFA	1.7
61	**(178)**	BUTYL(AQ 4,8-di-OH)	A	Gly-TFA	2.8
85	**(225)**	BUTYL(AQ 4,8-di-OH)	A	Pro-TFA	2.1
129	**(180)**	PROPOXY(AQ 4,8-di-OH)	E	Gly-TFA	1.3
159	**(273)**	L-Phenylalaninol (AQ-4,8-di-OH)	E	Pro-Gly-TFA	2.5
58	**(156)**	Hydroxypropyl (AQ-4-OH)	E	NIL	>100
165	**(174)**	PROPOXY (AQ-4-OH)	E	Gly-TFA	0.3

* Chemosensitivity (cytotoxicity) as determined by MTT assay, 96 hour exposure.
♦ Where A and E are amide and ester-linked conjugates respectively
Expressed as IC_{50} values [the concentration (μM) of compound to effect 50% growth inhibition] and represents the mean of (at least) three separate experiments (standard deviations were ± 5-20%); the complete protocol may be found in **Chapter 9**.

All of the mono-amino acid and example dipeptide conjugates, whether amide- or ester-linked were significantly cytotoxic below 3μM and were amongst the most potent compounds in the NU:UB library. It should be noted that based on activity relationships of the non-hydroxylated series, the (smaller) sub-set of hydroxylated conjugates had amino acid/ peptide motifs drawn from proline, alanine and glycine. Notably, it was later shown that the di-hydroxylated glycine conjugates (**176**) [NU:UB 51] and its homologue (**178**) [NU:UB 61], and the propyl- and butyl- spaced proline homologues (**223**) and (**225**) [NU:UB 83 and NU:UB 85 respectively] had significant *in vivo* activity.

4.4.2.4 MAC15A Chemosensitivity Summary

Potency in the single amino acid conjugates required their salt forms and was determined more so by the nature of the α- side chain residue than differences in stereochemistry or spacer length. In the MAC15A cell line, ester-linked conjugates had comparable potency to amide-linked analogues, although this relationship did not hold in panels of human tumour cell lines [**Tables 5** and **6**]. In contrast to amide-linked dipeptides, ester-linked dipeptide conjugates retained activity comparable to single amino acid conjugates.

It was anticipated that the hydroxylated conjugates might be more cytotoxic than non-hydroxylated compounds and indeed all examples in this class were at least as potent or more potent than any in the non-hydroxylated categories, however, orders of magnitude differences observed in mitoxantrone (**7**) over non-hydroxylated derivatives were not reflected in the NU:UB library of aminoanthraquinones.

4.4.3 Chemosensitivity in Resistant Cell Lines

Since resistance to current chemotherapeutics and especially topoisomerase inhibitors, frequently develops as a result of active drug efflux mechanisms (P-glycoprotein or Pgp) and/or altered topoisomerase resistance (at-MDR), it was considered important to determine the cytotoxicity and pattern of cell kill by selected structurally diverse NU:UB conjugates in cell lines with known levels of Pgp expression and known differential topoisomerase expression. Suitable cell lines were considered to be the MCF-7 breast cancer cell line and a doxorubicin resistant variant, and wild-type and resistant variants of human (A2780 and A2780/ADR) and Chinese hamster (CHO K-1 and CHO/ADR-r) ovarian cells.

4.4.3.1 Cell Lines with Differential Topoisomerase Expression

Down-regulation of topoisomerases, or (compensating) up-regulation of a topoisomerase enzyme that is not targeted by the drug, are mechanisms with implications for the efficacy of topoisomerase inhibitors (Larsen and Skladonowski 1998; Pommier 1999; Whitaker *et al* 1997).

The highly Pgp-expressing, mutated Chinese Hamster Ovarian cell line, CHO/ADR-r [14-fold resistant to doxorubicin (Hoban *et al* 1992)] showed 2.7-fold hypersensitivity [**Table 4**] to NU:UB 31 (**208**) when compared to the wild-type CHO- K1 parent (Pgp-expression was measured by flow cytometry as the percentage of cells staining positive with MRK-16 anti-Pgp antibody (21% and 91% for wild-type and resistant variant, respectively). The data may suggest that NU:UB 31 is a poor substrate for the Pgp protein efflux pump and given that its hydrophobic cationic properties permit cellular uptake (Lampidis *et al* 1997), may facilitate early mechanisms of cell death induction, in contrast to anthracyclines like doxorubicin, which although intrinsically more potent, have slower onsets of cell death.

Additionally, the observed cell line hypersensitivity to NU:UB 31 may be correlated to the topoisomerase protein expression in this highly resistant CHO/ADR cell line, which, when compared with the wild-type was characterised as having a two-fold decrease in topo IIα levels and concomitant increases (2.5-fold and 2-fold, respectively) in topoisomerase IIβ and topoisomerase I proteins. The hypersensitivity may thus reflect the demonstration that NU:UB 31 has here been shown to inhibit all three topoisomerases [**Section 4.4.5**] (at least *in vitro*) whereas doxorubicin and other anti-topoisomerase II agents have been considered largely to target the alpha isoform, that is down-regulated in this and other resistant cell lines.

Furthermore, the resistant CHO/ADR-r cell line showed similar hypersensitivity (2.8-fold) to NU:UB 43 (**215**), the butyl-spaced homologue of NU:UB 31, when compared to the wild-type CHO-K1 parent. The dihydroxylated conjugates NU:UB 51 (**176**), NU:UB 83 (**223**) and NU:UB 85 (**225**) were the most potent in both the wild-type and resistant cell lines, with the proline conjugates also being completely non cross-resistant to doxorubicin. The greatest level of cell line hypersensitivity was observed with the ester-linked conjugate NU:UB 73 (**194**) which was approximately 8-fold more potent against ADR-r than the parental cell line. Cell line hypersensitivity may be directly related to the observation that NU:UB 73 selectively inhibited topoisomerase I and the β-isoform of topoisomerase II but had no effect on the α-isoform of topoisomerase II, *in vitro* [**Section 4.4.5**]; the pattern of cell kill was thus consistent with cellular protein expression.

The A2780/ADR human ovarian cancer cell line is widely considered to possess the classic MDR phenotype (90% staining for Pgp) compared to the wild-type (negative for Pgp). The cell line has 4-fold down-regulation of topo IIα expression, normal topo IIβ levels and 1.5-fold overexpression of topo I. The cell line thus displays resistance properties, characteristic not only of MDR but also non-classical at-MDR (Cummings *et al* 1996)

The (150-fold) doxorubicin-resistant A2780/ADR ovarian cell line was found, in this laboratory, to be only 4.8-fold resistant to NU:UB 31 (**208**) when resistance levels were compared to the wild-type A2780 parent cell line [**Table 4**].

Similarly, other NU:UB conjugates were only nominally cross-resistant to doxorubicin; NU:UB 43 and NU:UB 51 were significantly cytotoxic below 2μM in the wild-type cell line and retained low micromolar activity in the resistant variant [**Table 4**].

Table 4: Summary of *In Vitro* Chemosensitivity Data for Selected Compounds in Wild Type and Doxorubicin Resistant Ovarian Cell Lines

NU:UB Code	Structure Number	Spacer Type	Peptide Motif	A2780 $IC_{50}\mu M$	A2780/ ADR $IC_{50}\mu M$	CHO- K1 $IC_{50}\mu M$	CHO- ADR-r $IC_{50}\mu M$
21	**(184)**	Propyl	D-Ala-TFA	7.4	18.7	N/D	N/D
24	**(236)**	Propyl	Orn-bis-TFA	17.5	32.0	N/D	N/D
31	**(208)**	Propyl	Pro-TFA	3.1	14.9	27.0	10.0
43	**(215)**	Butyl	Pro-TFA	1.6	2.2	50.4	18.1
51	**(176)**	Propyl(4,8-di-OH)	Gly-TFA	0.6	1.1	2.7	5.4
61	**(178)**	Butyl(4,8-di-OH)	Gly-TFA	N/D	N/D	16.2	18.0
73	**(194)**	Butoxy	Ala-TFA	N/D	N/D	54.0	6.6
83	**(223)**	Propyl(4,8-di-OH)	Pro-TFA	N/D	N/D	6.6	6.3
85	**(225)**	Butyl(4,8-di-OH)	Pro-TFA	N/D	N/D	4.1	4.1
DOXORUBICIN				N/D	N/D	0.1	1.4

4.4.3.2 Doxorubicin-Resistant Cell Lines

One major contributor to low potency of cytotoxic agents is drug resistance commonly manifested through the expression of the MDR1 gene product, P-glycoprotein, Pgp (classical multidrug resistance phenotypes); furthermore, many standard topoisomerase inhibitors are susceptible to Pgp- or the related MRP-mediated protein efflux mechanisms (Larsen and Skladonowski 1998, Ueda *et al* 1999). As most known P-glycoprotein drug substrates are weakly basic and positively charged at physiological pH it would perhaps be expected that anthraquinone-peptide conjugates would be targets for this resistance mechanism (Germann 1996). The anthraquinone NU:UB 31 (**208**), however, was shown to be active in a number of cell lines that are classically resistant to

topoisomerase inhibitors [**Table 5**]; in the NCI screen, for example, NU:UB 31 showed only 1.3-fold

Table 5: *In vitro* cytotoxicity of selected spacer-linked anthraquinone-peptide conjugates against MCF7 wild type and Doxorubicin resistant breast cell lines

NU:UB CODE	Structure Number	SPACER TYPE	PEPTIDE MOTIF	MCF7 $GI_{50}\mu M$	NCI/ ADR-res $GI_{50}\mu M$	Fold Resistance (R) /Hyper-sensitivity (H)
20	**(244)**	BUTYL	Lys-bis-TFA	11.8	13.5	1.1 (R)
24	**(236)**	PROPYL	Orn-bis-TFA	11.2	14.3	1.3 (R)
31	**(208)**	PROPYL	Pro-TFA	6.17	7.76	1.3 (R)
43	**(215)**	BUTYL	Pro-TFA	4.84	14.1	2.9 (R)
44	**(254)**	BUTYL	Ser-TFA	3.63	12.9	3.6 (R)
73	**(194)**	BUTOXY	Ala-TFA	17.2	16.6	1.1 (H)
99	**(246)**	BUTYL	Orn-bis-TFA	13.4	16.6	1.2 (R)
107	**(192)**	PROPOXY	D-Ala-TFA	16.1	19.1	1.2 (R)
108	**(190)**	PROPOXY	Ala-TFA	16.6	20.1	1.2 (R)
110	**(172)**	BUTOXY	Gly-TFA	18.3	14.9	1.2 (H)
159	**(273)**	L-Phenylalaninol (AQ-4,8-di-OH)	Pro-Gly-TFA	9.38	2.24	4.2 (H)
171	**(271)**	L-Prolinol	Ala-TFA	60.2	12.9	4.7 (H)
DOXORUBICIN (**1**)				0.020	12.6	630 (R)
MITOXANTRONE (**7**)				0.004	4.0	1000 (R)
CAMPTOTHECIN (**12**)				0.013	0.013	1
TAXOL				0.003	3.0	1000 (R)
CISPLATIN				3.2	5.0	1.6 (R)
5-FLUOROURACIL				1.6	40.0	25 (R)

resistance in the (doxorubicin-resistant) high Pgp-expressing NCI/ADR-res cell line when compared to the wild type MCF7 counterpart, whereas the anthraquinones, doxorubicin and mitoxantrone were 630- and 1000-fold resistant, respectively.

Similarly, NCI/ADR-res cell line hypersensitivity to other NU:UB conjugates [**Table 5**] including the ester conjugates NU:UB 73 (**194**), NU:UB 110 (**172**), NU:UB 159 (**273**) and NU:UB 171 (**271**) was observed, whilst nominal resistance patterns were observed for several others.

4.4.4 Activity of NU:UB Conjugates in the NCI Anticancer Drug Screen

The National Cancer Institute (NCI) of the USA has been at the forefront of cancer drug discovery and development since 1955. The current *in vitro* anticancer drug screen, which has been operational since April 1990, consists of 60 human tumour cell lines representative of common adult malignancies grouped into disease sub panels (Monks *et al* 1991).

The *in vitro* testing results generated GI_{50}, TGI and LC_{50} values [a measurement of growth inhibitory, cytostatic and cytotoxic (cell-kill) effects respectively] for each compound against each cell line. Selected results are summarised in **Table 6**. Presentation of the data in a mean graph format, where the behaviour of a particular cell line is displayed graphically as a deflection from the calculated mean of the whole cell line panel (to the right for sensitive cells and to the left for resistant cells) allows visual comparison of the pattern of response of all cell lines to different anti-tumour agents. The mean graphs and *in vitro* test data for selected NU:UB conjugates at the GI_{50}, TGI and LC_{50} levels are given in **Appendix 2**. This sensitivity profile or 'fingerprint' can be quantified using the COMPARE programme. The COMPARE programme ranks every

compound from one of several databases (i.e. standard agents, synthetic molecules etc.) in order of the similarity of its *in vitro* cell growth pattern to the *in vitro* cell growth pattern of a selected seed or probe compound. The similarity of the pattern to that of the seed is expressed quantitatively as a pairwise correlation coefficient (PCC). Compounds with the highest PCC are most similar to the seed and may possess a similar mechanism of action.

The data generated for the NU:UB conjugates in the 60 cell line screen was used (by the NCI) to generate a COMPARE calculation, using the Standard Anticancer Agent database, at the GI_{50}, TGI and LC_{50} concentration parameters. Example data at the LC_{50} level is given for the *in vivo* active, dual topoisomerase inhibiting [**Section 4.4.5**] proline conjugates NU:UB 31 (**208**) and NU:UB 43 (**215**) in **Tables 7** and **8**.

Table 6: Mean GI_{50}, TGI and LC_{50} values (60 cell line panel)

NU:UB CODE	Structure Number	SPACER TYPE	PEPTIDE MOTIF	MEAN GI_{50} μM	MEAN TGI μM	MEAN LC_{50} μM
20	**(244)**	BUTYL	Lys(α,ε)-bis-TFA	11.5	26.3	55.0
24	**(236)**	PROPYL	Orn(α,δ)-bis-TFA	11.5	26.3	56.2
31	**(208)**	PROPYL	Pro-TFA	5.37	16.2	46.7
43	**(215)**	BUTYL	Pro-TFA	3.80	12.3	29.5
44	**(254)**	BUTYL	Ser-TFA	5.37	16.2	45.7
73	**(194)**	BUTOXY	Ala-TFA	14.4	31.6	67.6
99	**(246)**	BUTYL	Orn(α,δ)-bis-TFA	14.1	31.6	64.6
107	**(192)**	PROPOXY	D-Ala-TFA	18.2	41.7	81.3
108	**(190)**	PROPOXY	Ala-TFA	18.6	39.8	75.9
110	**(172)**	BUTOXY	Gly-TFA	13.8	31.6	69.2
159	**(273)**	L-Phenylalaninol (AQ-4,8-di-OH)	Pro-Gly-TFA	6.17	28.8	79.4
171	**(271)**	L-Prolinol	Ala-TFA	31.6	83.2	>100

There was broad agreement between the data obtained from the NCI panel and the 'in-house' MAC15A frontline screen for the most potent conjugates. Low micromolar IC_{50}

values in the latter equated well with the GI_{50} values (the most comparable parameter), allowing for differences in exposure time and assay procedures. NU:UB 31 (**208**), NU:UB 43 (**215**) and NU:UB 44 (**254**) were again the most potent and were approximately 2-fold more active than examples of conjugate bis-salts of lysine and ornithine [NU:UB 20 (**244**), NU:UB 24 (**236**) and NU:UB 99 (**246**)].

Whereas ester-linked conjugates had comparable activity to amide-linked analogues in MAC15A cells, in the NCI screen esters were consistently 2- to 3-fold less potent than corresponding amides, unless their potency was boosted by anthraquinone nuclear hydroxylation [NU:UB 159 (**273**)]. Furthermore, steric hindrance at the anthraquinone-spacer [NU:UB 171 (**271**)] junction led to considerably reduced cytotoxic potency [**Table 6**].

COMPARE has proved valuable in providing insights into potential mechanisms of action which may not have otherwise been considered. It is interesting to note that at concentrations required to effect cell kill, the COMPARE analysis for NU:UB 31 (**208**) gave correlations which included topoisomerase (I or II) inhibitors [correlation numbers 5 and 9, **Table 7**] but also a number of structurally diverse, yet mechanistically related antimetabolites [correlation numbers 2 and 3, **Table 7**]. The butyl homologue NU:UB 43 (**215**) correlated well with the dual topoisomerase I and II inhibitor aclacinomycin A (**6**) and other topoisomerase inhibitors [correlation numbers 1, 3 and 9, **Table 8**]. In common with NU:UB 31, correlations with a common family of antimetabolites were observed for NU:UB 43 [correlation numbers 4 and 10, **Table 8**]; This family (using brequinar as a seed) is known to inhibit *de novo* pyrimidine biosynthesis at the fourth enzyme, dihydroorotate dehydrogenase (McLean *et al* 2001). Inhibition of this mitochondrial enzyme leads to a block in the formation of uridine monophosphate; investigation of the sub-cellular localisation of the NU:UB agents and further work

would clearly be necessary to confirm potential antimetabolite contribution to the cytotoxic activity for these compounds.

Table 7: COMPARE Correlation (LC_{50}) for NU:UB 31

CORRELATION NUMBERS	CHEMICAL NAME	P.C.C.
1	BLEOMYCIN	0.509
2	CYCLOPENTYL CYTOSINE	0.507
3	(DUP 785) BREQUINAR	0.486
4	PHOSPHOTRIENIN	0.473
5	N,N-DIBENZYLDAUNOMYCIN	0.472
6	ANGUIDINE	0.469
7	CCNU	0.452
8	METHYL CCNU	0.451
9	TOPOTECAN	0.439
10	D-TETRANIDINE	0.433

Table 8 : COMPARE Correlation (LC_{50}) for NU:UB 43

CORRELATION NUMBERS	CHEMICAL NAME	P.C.C.
1	ACLACINOMYCIN A	0.663
2	MITRAMYCIN	0.659
3	DEOXYDOXORUBICIN	0.653
4	5-AZACYTIDINE	0.645
5	CYTEMBENA	0.619
6	ANGUIDINE	0.612
7	BACTOBOLIN	0.607
8	PANCRATISTATIN	0.604
9	DAUNOMYCIN	0.598
10	(DUP 785) BREQUINAR	0.590

4.4.4.1 NCI Cancer Cell Line Sub-Panel Sensitivity

A major goal of the NCI anti-cancer screening programme is to identify compounds with disease sub-panel specificity. This means that a compound is significantly more toxic to cells in 1, 2 or 3 particular histological sub-categories (e.g. colon, melanoma

etc.) than it is to the remainder of the tumour cell panel. A compound with significant sub-panel specific toxicity may be of interest without regard to its mechanism of action. Its novelty may even be more interesting if it does not share a mechanism of action with any known agent as evaluated by COMPARE. The sensitivity of the colon and melanoma sub-panels towards NU:UB 31 (**208**) and NU:UB 43 (**215**) are illustrated in **Figures 6 (a), 6 (b)** and **Figures 7 (a), 6 (b)** respectively.

Figure 6(a): Comparison of Delta Values for the NCI Colon Cancer Sub-Panel (plotted in log mean graph format)for NU:UB 31 (208)

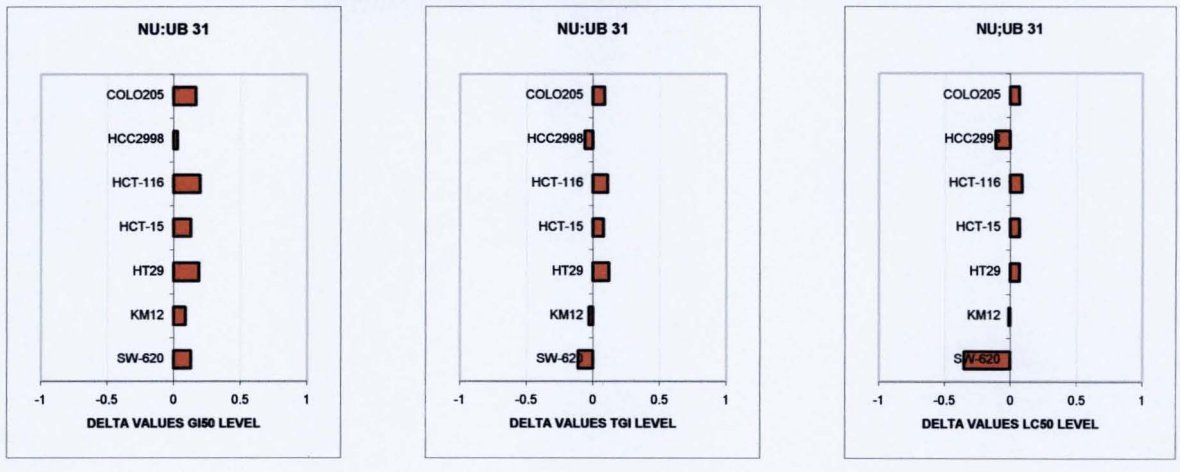


Figure 6(b): Comparison of Delta Values for the NCI Melanoma Cancer Sub-Panel (plotted in log mean graph format)for NU:UB 31 (208)

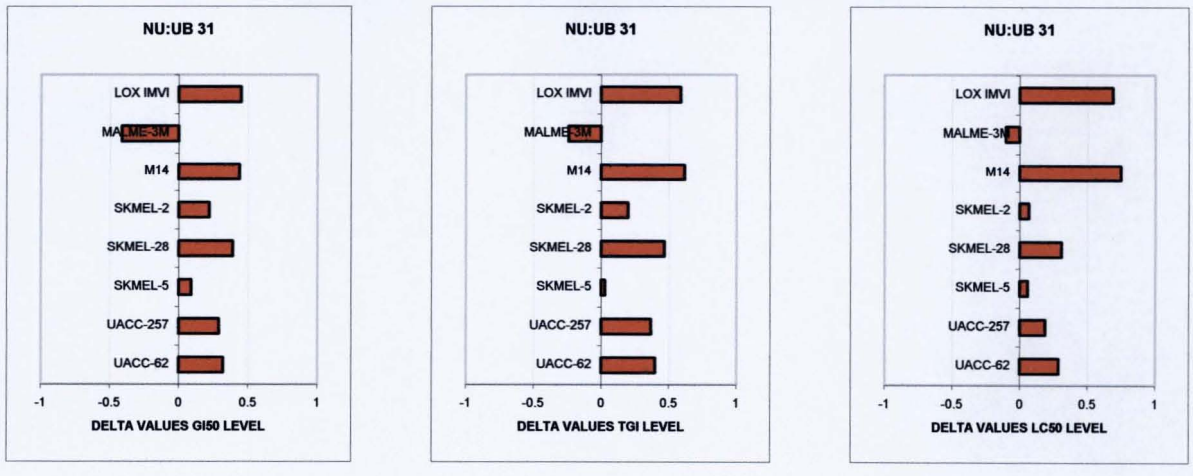


Figure 7(a): Comparison of Delta Values for the NCI Colon Cancer Sub-Panel (plotted in log mean graph format)for NU:UB 43 (215)

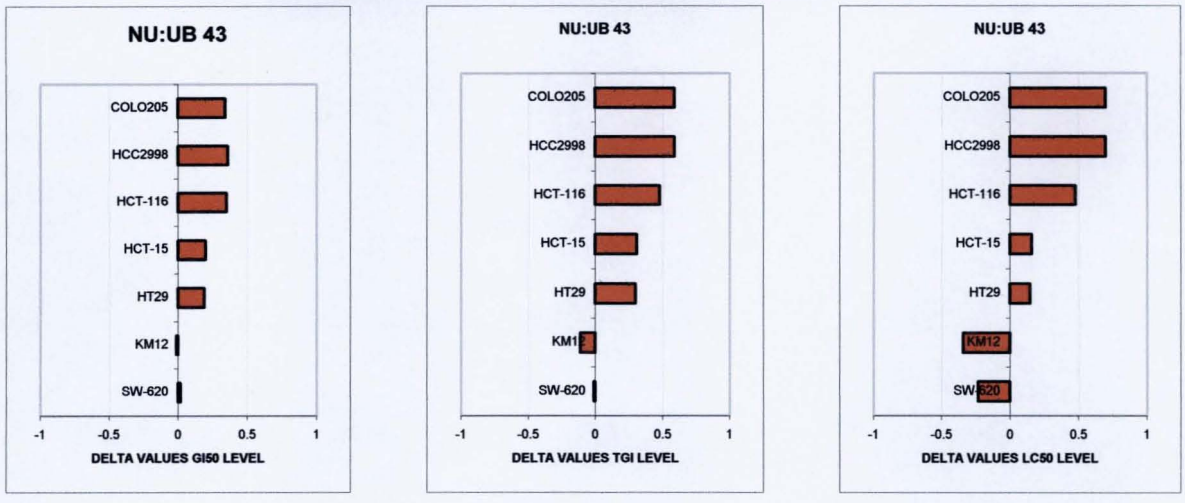


Figure 7(b): Comparison of Delta Values for the NCI Melanoma Cancer Sub-Panel (plotted in log mean graph format)for NU:UB 43 (215)

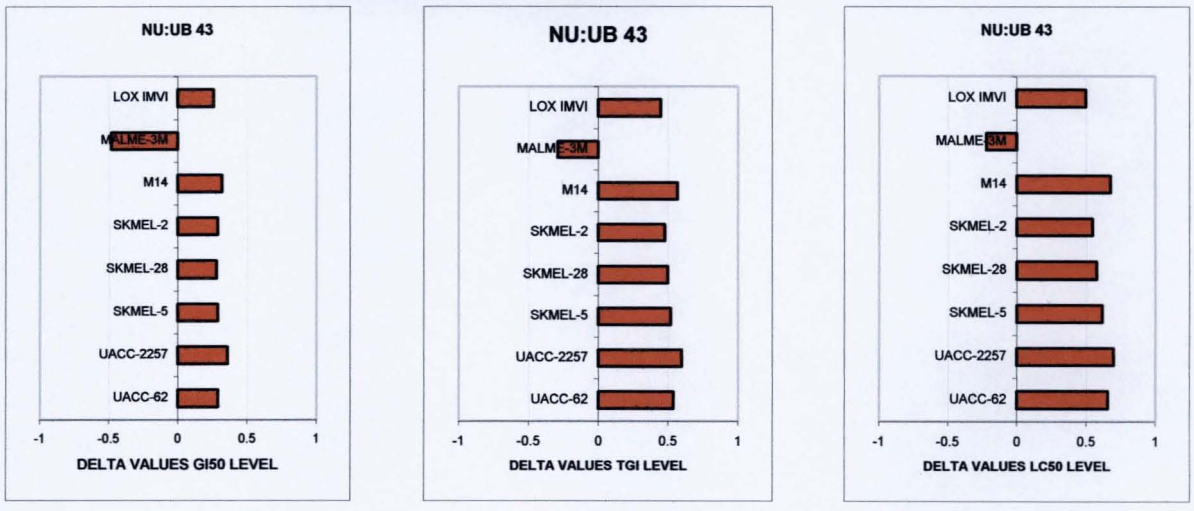


Figure 8(a): Comparison of Delta Values for the NCI Colon Cancer Sub-Panel (plotted in log mean graph format) for the standard topoisomerase II inhibitors, doxorubicin and mitoxantrone, and the topoisomerase I poison camptothecin

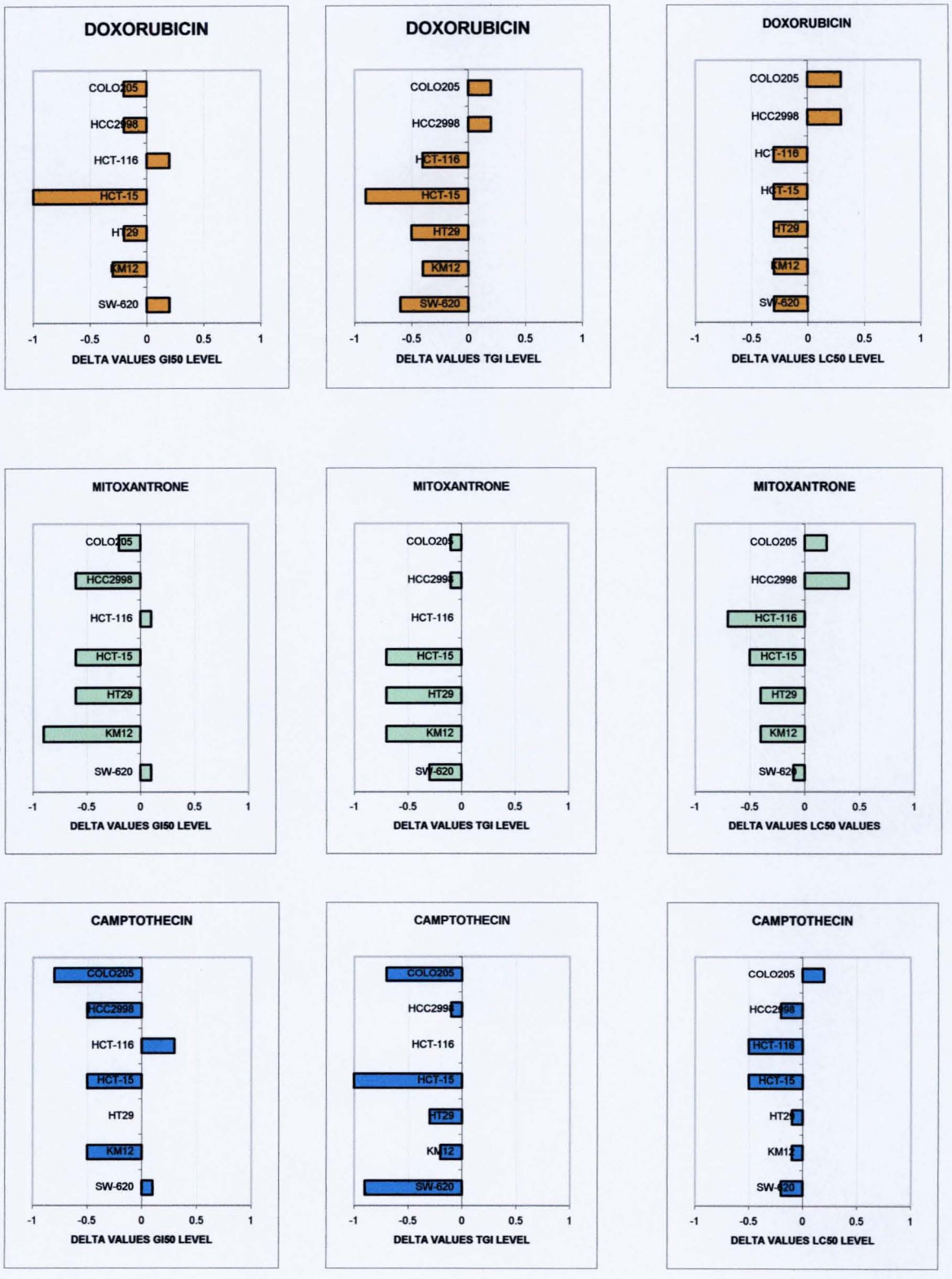
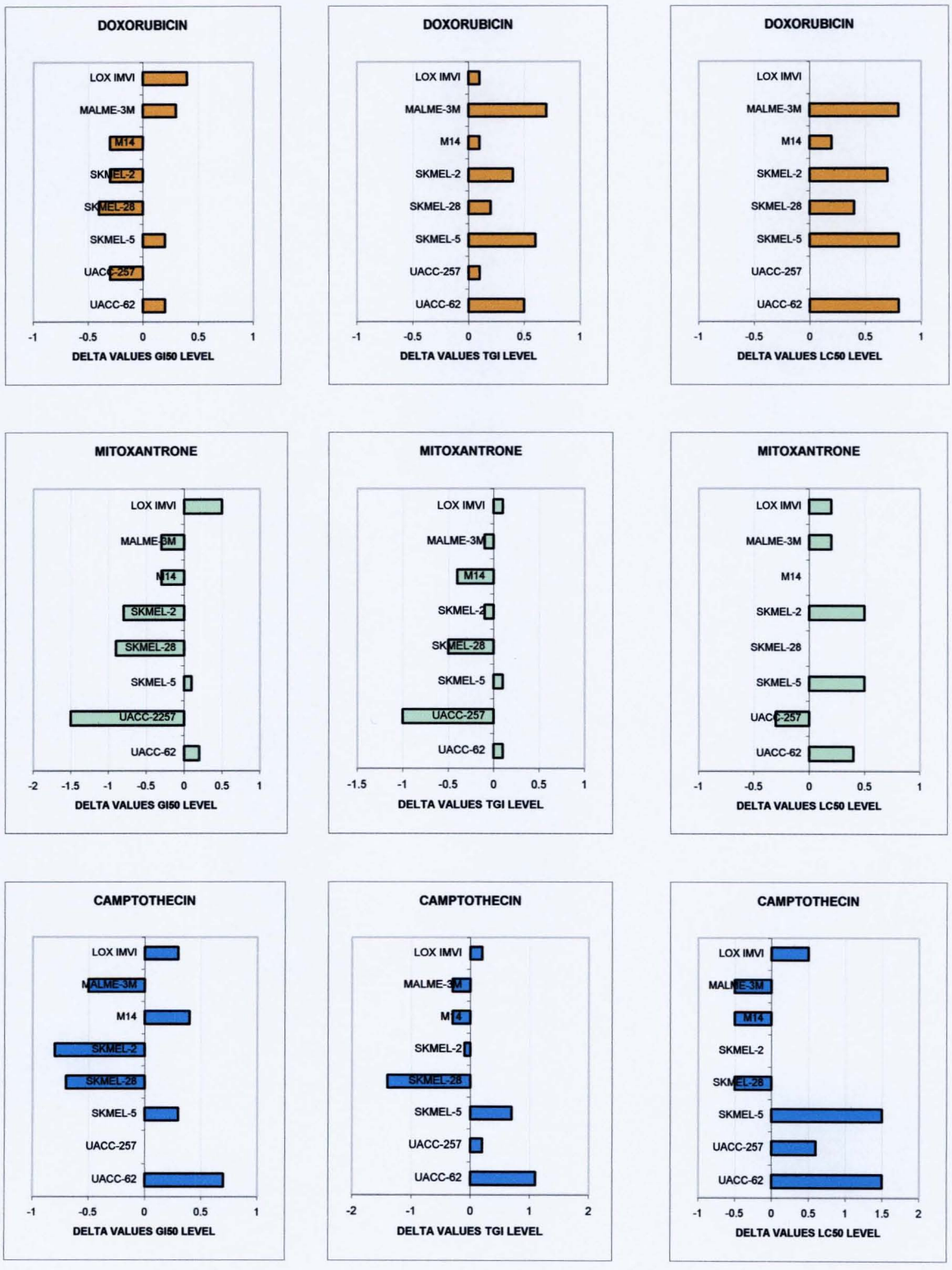


Figure 8(b): Comparison of Delta Values for the NCI Melanoma Cancer Sub-Panel (plotted in log mean graph format) for the standard topoisomerase II inhibitors, doxorubicin and mitoxantrone, and the topoisomerase I poison camptothecin



The mean graph presentations for the propyl-spaced proline conjugate NU:UB 31 (**208**) and the homologous, butyl-spaced proline conjugate NU:UB 43 (**215**) at the GI_{50} level clearly showed significant disease panel specificity for colon and melanoma cell lines. Breast cells (for both conjugates), and (for NU:UB 43) ovarian and non-small cell lung cancer cell lines were particularly resistant [**Appendix 2**]. The very pronounced sub-panel specificity displayed by NU:UB 43 is relatively rare for compounds tested in the NCI *in vitro* screen.

A quinocarmycin analogue DX-52-1 (NSC S607097) was found to be selective for 8 out of 9 melanoma cell lines at the LC_{50} level (mean LC_{50} for 60 cell line panel 49 μM; mean for melanoma sub-panel 7.3 μM) (Plowman *et al* 1995). The COMPARE algorithm at the LC_{50} level for DX-52-1 indicated that the pattern of differential cytotoxicity most closely resembled actinomycin D, mithramycin and doxorubicin and its analogues which would perhaps imply some form of topoisomerase mediated cytotoxic mechanism. DX-52-1 was one of the first compounds to be selected for preclinical development based on disease-panel sensitivity discovered in the NCI cancer drug screen (Monks *et al* 1997).

The colon sub-panel sensitivity profiles for the two NU:UB conjugates contrasts starkly with those of comparator, standard topoisomerase inhibitors, including the anthraquinone topoisomerase II inhibitors, doxorubicin and mitoxantrone, and the topo I poison camptothecin, across all three parameters [**Table 8(a)].** Differential melanoma sensitivity is also substantially different [**Table 8(b)]** to comparator agents at the GI_{50} level; NU:UB 43 is exceptional for the sensitivity across all three parameters in this panel of cell lines. Furthermore, the patterns of cell line sensitivity for the NU:UB conjugates was markedly different to other diverse compounds in the topoisomerase II (etoposide and mAMSA) or dual (intoplicine) categories [**Appendix 3**].

4.4.5 Biochemistry of DNA Topoisomerase Inhibition by NU:UB conjugates

The ability of topoisomerases to uncoil supercoiled plasmids provides the basis for DNA gel electrophoresis methods to identify topoisomerase interacting compounds. By monitoring changes in the electrophoretic mobility of supercoiled pBR322 DNA plasmid, in the presence of topoisomerase I or II, with or without a candidate drug, it is possible to identify inhibitors of the catalytic activity of either enzyme. Using an appropriate range of concentrations, measurement of the ability of a compound to inhibit enzyme-mediated relaxation of supercoiled plasmid may be made and referenced to comparator compounds. Furthermore, use of higher (usually 5 to 10-fold) concentrations of enzyme (necessary to effect discernible levels of cleavable complex formation between DNA and enzyme) can identify poisoning activity as evidenced by the formation of increased levels of open-circular (nicked) or linear DNA either with topoisomerase I or II, respectively [full details of the electrophoresis protocols are given in **Appendix 1**].

Additionally, a diagnostic test of the ability of a compound to inhibit topoisomerase II relaxation activity is inhibition of decatenation (separation into 'mini-circles') of kinetoplast (k-DNA) which is a process uniquely mediated by topoisomerase II (both isoforms).

Summary data on relaxation, cleavage and decatenation effects by selected NU:UB conjugates are presented in **Tables 9** and **10**.

Table 9: Inhibitory Effects of NU:UB Conjugates on Topoisomerase Relaxation and Decatenation Activities

NU:UB CODE	Structure Number	I RELAX.	II α RELAX.	II β RELAX.	II α DECAT.	II β DECAT.
21	**(184)**	++	++	++	N/D	N/D
24	**(236)**	+++	++	+	N/D	N/D
31	**(208)**	++	++	+++	++	++
43	**(215)**	+	+++	++	++	++
44	**(254)**	+	+	+	+	+
51	**(176)**	++	++	+++	+++	+++
61	**(178)**	+++	+++	+++	++	++
73	**(194)**	++	—	++	—	N/D
83	**(223)**	+++	+++	+++	++	++
85	**(225)**	+++	+++	+++	++	++

— Inactive
\+ $25\mu M < IC_{50} \leq 50\mu M$
++ $10\mu M < IC_{50} \leq 25\mu M$
+++ $IC_{50} \leq 10\mu M$
N/D Not Determined
α and β refer to the individual isoforms of human (recombinant) topoisomerase II

Table 9 shows the relative abilities of selected NU:UB conjugates to inhibit the catalytic activity of the enzymes, topoisomerase I or topoisomerase II (individual isoforms). The selection was made on the basis that this group had shown not only potent cytotoxicity *in vitro* against cancer cell lines, but also *in vivo* antitumour activity against experimental colon cancer [see **Section 4.4.8**]

Unequivocal evidence was obtained to assert that conjugates, NU:UB 31, 43, 44, 51, 61, 83 and 85 were catalytic inhibitors of the alpha and beta isoforms of human topo II as

demonstrated by their ability to inhibit enzyme-mediated decatenation of (the highly catenated network in) kinetoplast DNA, whereas the ester-linked conjugate NU:UB 73 was shown to inhibit the beta-isoform only. Of this series, NU:UB 51 (**176**) and NU:UB 44 (**254**) were the most and least potent agents, respectively.

With the exception of NU:UB 73 (**194**) that inhibited the catalytic activity of the beta isoform only of topoisomerase II to unwind supercoiled plasmid (relaxation), all conjugates showed (dual) inhibitory activity against all three topo enzymes. The most potent agents were the dihydroxylated, glycine conjugates [NU:UB 51 (**176**) and NU:UB 61 (**178**)] and the homologous proline conjugates [NU:UB 83 (223) and NU:UB 85 (225)] that efficiently inhibited the relaxation activity of topo I, IIα and IIβ.

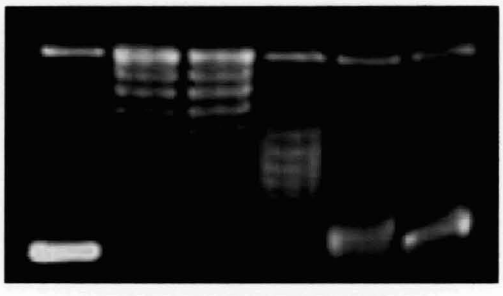
Table 10: Stimulation of Topisomerase (I-, IIα,β-) Cleavable Complex Formation by Selected NU:UB Conjugates

NU:UB CODE	Structure Number	I	II α	II β
21	**(184)**	Not Active	Not Active	Not Active
24	**(236)**	Not Active	N/D	N/D
31	**(208)**	Optimum 5 μM	Optimum 25 μM	Optimum 25 μM
43	**(215)**	Optimum 50 μM	Optimum 50 μM	Optimum 50 μM
44	**(254)**	Not Active	Not Active	Not Active
51	**(176)**	Optimum 50 μM	Optimum 25 μM	Optimum 25 μM
61	**(178)**	Optimum 50 μM	Optimum 25 μM	Not Active
73	**(194)**	Optimum 50 μM.	Not Active	Optimum 25 μM
85	**(225)**	N/D	Not Active	Not Active

Table 10 shows the data on the propensity of *in vivo*-active NU:UB conjugates to stimulate topoisomerase I or II (α- and β-) mediated cleavage of supercoiled plasmid DNA as evidence of cleavable complex stabilisation or poisoning activity.

Poisoning activity was demonstrated for conjugates, NU:UB 31, 43, 51, 61 and 73 [(208), (215), (176), (178) and (194) respectively]. The most potent conjugate was NU:UB 31 (208), judged by the maximum increases in the formation of either the open-cicular (topo I) or linear (topo II) DNA bands on gel electrophoresis. Representative DNA gel electrophoresis data is exemplified by **Figures: 9** (inhibition of topo I relaxation), **10** (topo I cleavage) and **11** [(a) and (b)] [inhibition of topo II (α and β respectively) relaxation], by reference to the lead compound NU:UB 31 (**208**) only.

Figure 9: Inhibition of DNA-topoisomerase I-mediated relaxation of supercoiled pBR322 plasmid DNA by NU:UB 31 (208).



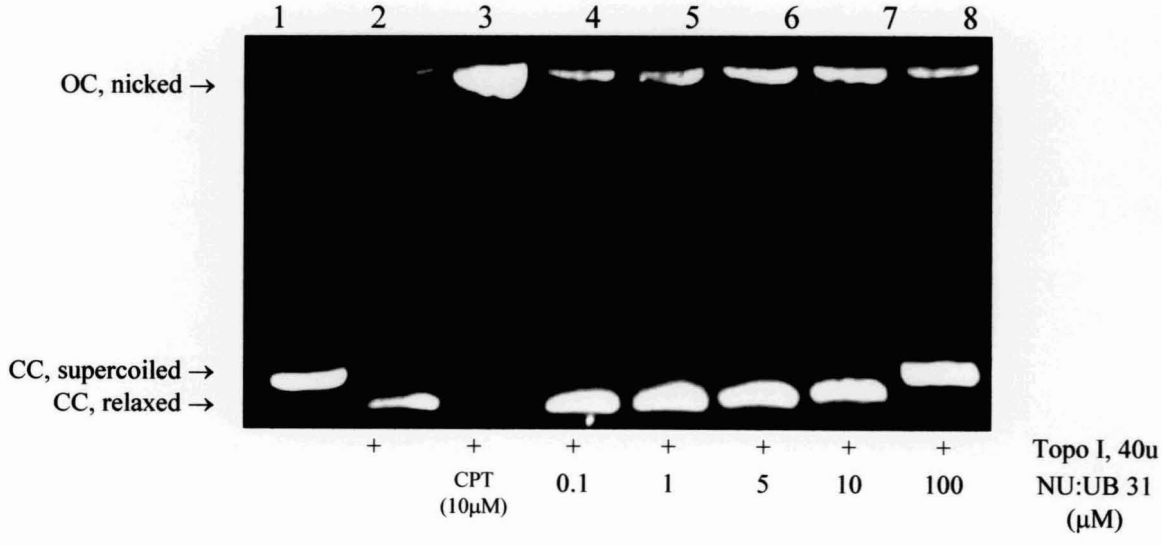
1 2 3 4 5 6

Lane 1 – pBR322 DNA (200 ng)
Lane 2 – pBR322 DNA + topo I (3 units)
Lane 3 – pBR322 DNA + topo I + 5 μM NU:UB 31
Lane 4 – pBR322 DNA + topo I + 10 μM NU:UB 31
Lane 5 – pBR322 DNA + topo I + 25 μM NU:UB 31
Lane 6 – pBR322 DNA + topo I + 50 μM NU:UB 31

The capacity of NU:UB 31 (**208**) to inhibit topoisomerase I-mediated relaxation of DNA was determined. When the plasmid DNA was treated with topoisomerase I

enzyme, supercoils were removed, resulting in a more relaxed (conformationally flexible) structure, which was retarded on the gel compared to the supercoiled DNA. **Figure 9** shows an assay of inhibition of plasmid relaxation by NU:UB 31. In lane 1 (DNA) the main band is the supercoiled plasmid DNA. In lane 2 (DNA + topoisomerase I) relaxed DNA bands appeared, and the supercoiled DNA band disappeared. This meant that topoisomerase I had converted the previously supercoiled DNA into relaxed topoisomers, which were retarded to various degrees on the agarose gel. Treatment with 5μM or 10μM NU:UB 31 resulted in partial inhibition of the topoisomerase I mediated DNA relaxation (lane 3 and 4). At concentrations of 25μM and 50μM NU:UB 31, complete inhibition of topoisomerase I-mediated relaxation was observed. (lanes 5 and 6).

Figure 10: Stimulation of Topoisomerase I-Mediated Cleavage of pBR322 Plasmid DNA by NU:UB 31 (208)



The formation of drug-stabilised topoisomerase I–DNA cleavable complexes were investigated with the topoisomerase I cleavage assay. **Figure 10** shows a topoisomerase I cleavage assay of NU:UB 31 using camptothecin as a positive control. Upon

visualisation, supercoiled plasmid DNA appeared as one band, travelling far down the gel due to its compact size (lane 1). For this assay, more topoisomerase was used than in the relaxation assay in order to induce a high degree of cleavable complex formation. In contrast to relaxation gels, the conditions for running the DNA gels under cleavage conditions required the use of ethidium bromide in the gel matrix in addition to the running buffer; in this way, the fully relaxed plasmid topoisomers migrated as a single band ahead of the supercoiled band, thereby allowing resolution of the retarded nicked plasmid from relaxed forms. Topoisomerase I–DNA complexes are trapped and stabilized by anti-topoisomerase drugs, including the anti-topoisomerase I standard agent camptothecin. Treatment with camptothecin resulted in increased levels of cleavable complex formation, resulting in corresponding increases in intensity of open-circular (or nicked) DNA plasmids that were severely retarded on the gel (lane 3) compared to the background cleavage with topoisomerase I treated DNA (lane 2). Camptothecin increased the intensity of the open circular plasmid form by 3.9-fold, compared to the background cleavage, which is equivalent to a complete conversion of the pBR322-DNA. NU:UB 31 gave a 2.2-fold effect, equivalent to capturing more than 50% of the plasmid DNA in topo I-complexed form at 10μM [lane 7]. At low concentrations (0.1μM, 1μM, 5μM and 10μM; lanes 4-7) NU:UB 31 showed stimulation of topoisomerase I induced cleavage (optimum at 5 μM), whereas at the higher concentration (100μM) NU:UB 31 appeared to antagonise its own cleavage reaction (lane 8).

The observation that NU:UB 31 antagonised its own topoisomerase I cleavage reaction (i.e. 'self inhibition') with increased concentrations may be consistent with its properties of being a catalytic inhibitor and poison vested in the same molecule. Precedent exists for this type of behaviour and has notably been observed for the dual topoisomerase

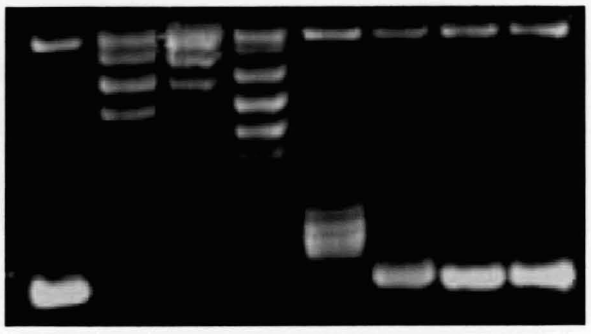
inhibitor, DACA (**18**) that like NU:UB 31 possesses a planar (acridine) chromophore with a single, positively charged aminoalkyl side chain substituent (Bridewell *et al* 1999).

Nevertheless, NU:UB 31 significantly stabilised DNA-topoisomerase I cleavable complexes *in vitro* thus, in common with camptothecin, NU:UB 31 produces irreversible lesions in DNA. In contrast, NU:UB 31 does not suffer from the disadvantage of the structural lability of the camptothecins.

In an analogous manner to the action of topoisomerase I, the individual isoforms (α and β) of human topoisomerase II are capable of converting supercoiled plasmid DNA into relaxed topoisomers. Inhibition of topoisomerase IIα or topoisomerase IIβ mediated relaxation of supercoiled plasmid DNA was investigated following NU:UB 31 (**208**) treatments. **Figure 11 (a)** is representative of the assays of topoisomerase IIα-mediated relaxation of plasmid DNA by NU:UB 31. A NU:UB 31 concentration of 10μM was sufficient to partially inhibit topoisomerase IIα-mediated DNA relaxation (lane 5). At the higher concentrations 25μM and 50μM, complete inhibition of topoisomerase IIα was evident (lane 6-7). Similarly, inhibition of topoisomerase IIβ-mediated DNA relaxation was also observed following 10μM NU:UB 31 treatment (lane 4), with the higher NU:UB 31 concentrations resulting in complete inhibition (lane 5-7) of topoisomerase IIβ catalytic activity [**Figure 11 (b)**]. NU:UB 31 alone did not effect plasmid unwinding (enzyme free lane 8). Thus, NU:UB 31 is a dual inhibitor of the catalytic activity of each of the isoforms of topoisomerase II in addition to topoisomerase I.

Figure 11:

(a) Inhibition of DNA topoisomerase IIα-mediated relaxation of supercoiled pBR322 plasmid by NU:UB 31 (208).



1 2 3 4 5 6 7 8

Lane 1 – pBR322 DNA (200 ng)

Lane 2 – pBR322 + topo IIα (5 units)

Lane 3 – pBR322 + topo IIα + 1.0 μM NU:UB 31

Lane 4 – pBR322 + topo IIα + 5.0 μM NU:UB 31

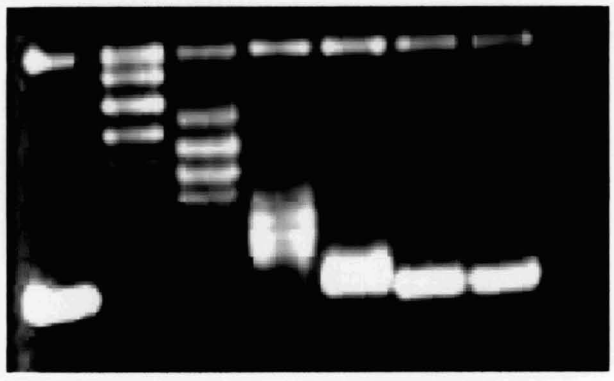
Lane 5 – pBR322 + topo IIα + 10.0 μM NU:UB 31

Lane 6 – pBR322 + topo IIα + 25.0 μM NU:UB 31

Lane 7 – pBR322 + topo II α+ 50.0 μM NU:UB 31

Lane 8 – pBR322 + 50.0 μM NU:UB 31

(b)Inhibition of DNA topoisomerase IIβ-mediated relaxation of supercoiled pBR322 plasmid by NU:UB 31.



Lane 1 – pBR322 (200 ng)

Lane 2 – pBR322 + topo IIβ (5 units)

Lane 3 - pBR322 + topo IIβ + 5 μM NU:UB 31

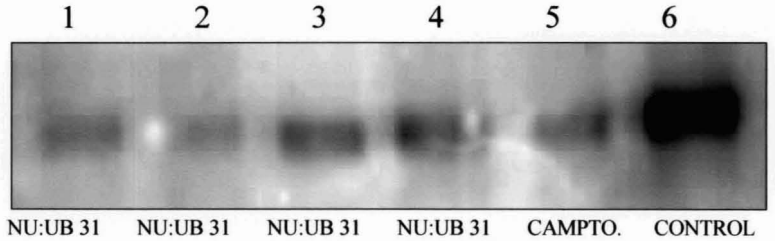
Lane 4 – pBR322 + topo IIβ + 10 μM NU:UB 31

Lane 5 – pBR322 + topo II β+ 15 μM NU:UB 31

Lane 6 – pBR322 + topo IIβ + 20 μM NU:UB 31

Lane 7 – pBR322 + 50 μM NU:UB 31

Figure 12: Immunoband depletion of Topoisomerase I in human HL-60 cells: Evidence for drug-stabilised covalent Topo I-DNA complexes in intact cells



(Control Mr × 10^{-3} 112-97)

The inhibition of the religation step during the processing of DNA topoisomerases is believed to be the molecular basis of the anti-tumour activity of topoisomerase I poisons (Pommier 1999) including camptothecin (**12**) and its derivatives irinotecan (**14**) (CPT 11) and topotecan (**13**). Inhibition of religation can be detected in drug-treated cells by immunoband depletion assays. Whereas topoisomerase will migrate at the molecular mass of the topoisomerase molecule on SDS-polyacrylamide gels, the covalent topoisomerase-DNA complexes are larger in size and will exhibit a lower mobility. In untreated cells, there are few and probably short-lived covalent topoisomerase-DNA complexes. Drug treatment of cells may however increase the number of covalent topoisomerase-DNA complexes. Thus, the degree of topoisomerase I immunoband depletion will reflect the drugs' capacity to stabilise topoisomerase I–DNA cleavable complexes by depleting the Western blot (100kDa) topoisomerase I signal. HL60 cells were treated with 30μM and 300μM NU:UB 31 (**208**) for 45 minutes [essentially according to the method of Boege *et al* (1996)]. In the topoisomerase I band depletion assay [**Figure 12**], 20μM camptothecin treatment (positive control) resulted in a weaker topoisomerase I signal because the topoisomerase I enzymes became covalently trapped by the drug. The extent of immunoband depletion by NU:UB 31 at 30μM was similar to that obtained by camptothecin at 20μM, providing evidence for drug stabilised cleavable complex formation in intact cancer cells, in accord with the cell free topo I cleavage assay data [**Figure 10**] [full protocol for immunoband depletion given in **Appendix 1**].

4.4.6 DNA Binding Properties of NU:UB Conjugates

The DNA-binding properties of drug molecules are important factors that can contribute to cytotoxic potency, mutagenicity, or the ability to interact with DNA-processing

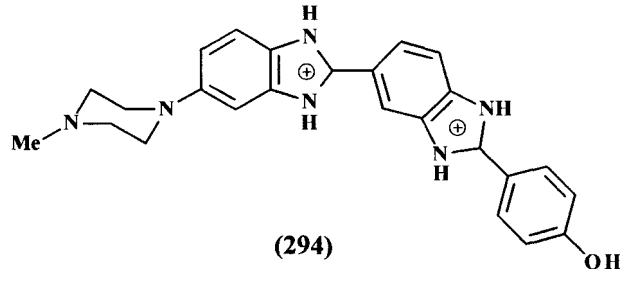
enzymes including topoisomerases. Indeed, direct targeting of DNA or DNA-associated proteins have received great attention in the past and still figure prominently as viable approaches in cancer therapy strategies (Hurley 2002).

DNA binding studies were not conducted as a component of this research programme but rather formed a major part of parallel projects conducted by other research workers in this and collaborating laboratories, therefore, brief inclusion only is made on NU:UB 31 in relation to its interaction with the DNA processing topoisomerases.

Reversible drug interactions with DNA take place in three primary ways: non-specific interactions, involving electrostatic binding along the exterior of the helix; specific groove binding, involving interactions with the edges of base pairs in the major or minor grooves; DNA intercalation that relies on insertion of a planar or approximately planar (aromatic) ring system between base pairs (Neidle 2002).

Strong correlations exist between chemical structure and DNA-binding properties.

Groove-binding molecules are generally crescent-shaped and incorporate an aromatic ring such as benzene, or heteroaromatic ring such as pyrrole, that is able to twist into the helical curve of the groove; netropsin (**76**), distamycin (**77**) and Hoechst dye 33258 (**294**) are typical minor groove binding molecules.



(294)

DNA intercalators typically display an aromatic ring system that is planar. Nucleic acid conformation is changed when intercalation takes place, which favours insertion of the

flat molecules into DNA. Mitoxantrone (**7**) and ethidium bromide are typical intercalating molecules.

Competitive DNA-ethidium (or Hoechst dye) fluorescence quenching is a well established technique that has been applied to structurally diverse DNA-binding ligands to give a measure of the relative strengths of binding of small molecules to DNA. (McConnaughie and Jenkins 1996). The methods used to probe the DNA binding characteristics of NU:UB 31 in relation to mitoxantrone and netropsin, as comparator molecules for intercalation and groove-binding, respectively, were essentially adaptations of the procedures according to Bailly *et al* (1989).

The mode of DNA binding by NU:UB 31 (or comparative agent) was quantified by determining the reduction in fluorescence of the reporter fluorophore upon treatment with a given concentration of the analysed compound. A graph for each drug (NU:UB 31, mitoxantrone and netropsin) with each dye was plotted from the mean value (n=3) of fluorescence intensity at each drug concentration. The measure of the ability to bind to DNA was then expressed as Q_{50} values: the concentration required to reduce the fluorescence intensity of the DNA-bound ethidium bromide (QE_{50}) or Hoechst stain (QH_{50}) complexes by 50%. The competitive displacement graphs were plotted as the mean values of at least 3 separate experiments.

Mitoxantrone was determined to have a mean QE_{50} value of 0.5μM whereas NU:UB 31 was found to have a mean value of 0.79μM that confirmed that an intercalative component contributed to the process of DNA-binding by NU:UB 31 although it does not bind so tightly as the comparator compound [**Table 11**]. This observation is consistent with NU:UB 31 possessing a single cationic charge in contrast to the doubly cationic mitoxantrone which through its two side chains has additional stabilisation of the intercalated complex. It is proposed that NU:UB 31 has a mixed-modal (part

intercalative, part groove-binding) mechanism of binding to DNA, given the potent groove binding properties shown by the low mean QH_{50} value of 0.42μM compared to the groove-binding comparator netropsin which had a mean QH_{50} value of 0.67μM.

Table 11: DNA Binding by NU:UB 31 (208) and comparator compounds

Compound	DNA Binding Assay Q_{50} Values* (μM)
Mitoxantrone **(7)**	QE_{50} ~ 0.50
Netropsin **(76)**	QH_{50} ~ 0.67
NU:UB 31 **(208)**	QE_{50} ~ 0.79
NU:UB 31 **(208)**	QH_{50} ~ 0.42

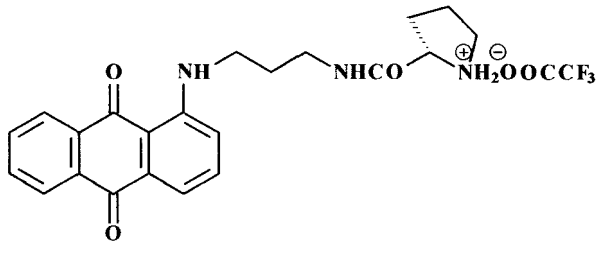
* Q_{50} Values are the concentration of compound to diminish the initial fluorescence of DNA-bound reporter complexes by 50%

Molecules with a planar chromophore (that can insert into the hydrophobic space between base pairs in DNA) combined with positively charged side chains of appropriate length and conformation have been shown to have groove-binding contributions to the DNA-bound intercalation complex in which the charges act as anchor points to the negatively charged phosphodiester backbone in the nucleic acid, effectively slowing the dissociation kinetics and tethering the molecule firmly to DNA. Empirical observations have noted that intercalating compounds, including the anthracyclines and mitoxantrone, generally favour interaction with DNA topoisomerase II and often function as poisons, whereas crescent-shaped, groove-binding molecules, including the camptothecins and Hoechst 33258 (pibenzimol), interact with topoisomerase I, also usually functioning as enzyme poisons. The mixed-modal DNA binding behaviour of NU:UB 31 is thus consistent with its dual action on each of topoisomerase I and II.

[Independently, evidence to support the DNA binding properties of NU:UB 31 have been provided by uv thermal melt analysis of thermal denaturation of DNA, wherein NU:UB 31 produced concentration dependent increases in the melting temperature of DNA and ΔTm values, in poly dA-dT DNA and calf thymus DNA, of 25.3°C and 11.3°C respectively, were recorded at equimolar (DNA/drug = 1) ratios (C. Bailly, L, Bouvier, DJ Mincher, unpublished data)].

4.4.7 Summary of Dual Topoisomerase Inhibition by NU:UB Conjugates

As part of the present study, it has been shown that members of a series of spacer-linked anthraquinone amino acid/peptide conjugates are topoisomerase inhibitors that target topo I and the individual (α and β) isoforms of human topo II, typified by NU:UB 31 (**208**). Across the NU:UB library, a spectrum of inhibitory action has been found, ranging from pure catalytic inhibitors to poisons and examples which share each of these properties. Broad agreement has been found between their topoisomerase poisoning activity and enzyme levels in cancer cell lines with known expression.

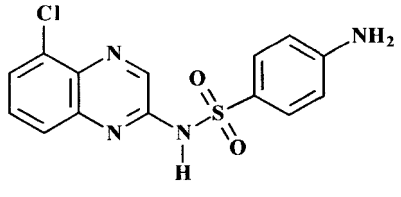


NU:UB 31 (**208**)

Since altered topo expression may confer drug resistance independently to type I or type II targeting drugs, it has been postulated that dual (topo I and topo II) inhibitors have the potential to escape cross-resistance mechanisms to agents that target solely one type of enzyme, although this remains to be proven clinically. Insufficient evidence has been accumulated regarding the value of dual inhibition vested in the same molecule; opinion

is divided, given that co-administration of topo I and II poisons has been shown to be variously antagonistic whilst the demonstration of advantages of sequential administration of type I and type II agents has enjoyed tumour type-dependent success (Kancherla *et al* 2001).

The majority of topo II poisons, including doxorubicin, are believed to target principally the α-isoform of the enzyme; topo IIα levels are tightly linked to the proliferative state of cells and transcriptional downregulation of the α-isoform correlates with resistance to anti-topo II agents in many cell lines and tumours. In contrast, topo II-β concentrations are relatively constant over cell and growth cycles. Even though the physiological role of topo II-β is less clearly defined, it was demonstrated that topo II-β, in addition to the α- isoform, is an *in vivo* target for etoposide, mitoxantrone and mAMSA (Austin *et al* 1995). A chloroquinoxaline sulphonamide (**295**) with solid tumour activity was shown recently shown to be a poison of both topo IIα and IIβ (Gao *et al* 2000). It is interesting to note that poisoning activity was only detected by using chaotropic protein denaturants (such as guanidinium chloride) whereas the commonly used SDS denaturant failed to uncover cleavable complex formation; this leads one to speculate how many topo poisons have gone undetected in drug screening assays, if detection is so dependent on the protein denaturant.



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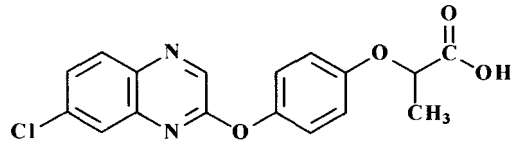
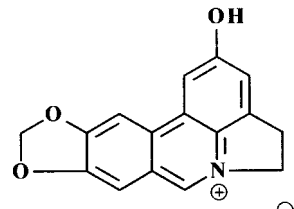


(296)

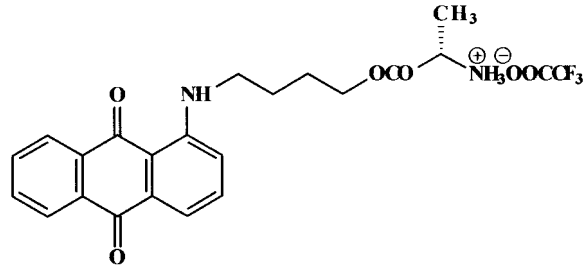
Earlier (Gao *et al* 1999), a structurally similar topo II-β targeting quinoxaline XK469 (**296**) and the more potent phenanthridine alkaloid lycobetaine (**297**) (Barthelmes *et al*

2001) were reported for which topo I and II-α were not significant contributors to cytotoxicity.



(297)

The ester-linked, L-alanyl anthraquinone conjugate [NU:UB 73] (**194**) unequivocally inhibited the β- (but not α) isoform of topo II and topo I *in vitro*.



[NU:UB 73] (**194**)

Co-overexpression of topo I and topo IIβ has been reported in ovarian tumour tissue taken from clinical samples (Fukuoka *et al* 1992). It may be speculated that compounds which co-target topo I and IIβ may find clinical application in cases of development of drug resistance in response to treatment with (principally) IIα-targeting clinical agents. Solid tumours frequently have large populations of cells in the G_1 and G_0 phases of the cell cycle in which levels of the non-proliferation dependent enzymes topo I and IIβ are high and levels of topo IIα are low; this may, in part, explain the solid tumour activity of NU:UB 73 (**194**). The novel anti-topoisomerase inhibitory profile *in vitro* and antitumour activity of (**194**) [NU:UB 73] justify the ongoing further evaluation against a broader range of tumour types in order to fully establish targets *in vivo*; similar studies on NU:UB 31 (**208**) have been instigated.

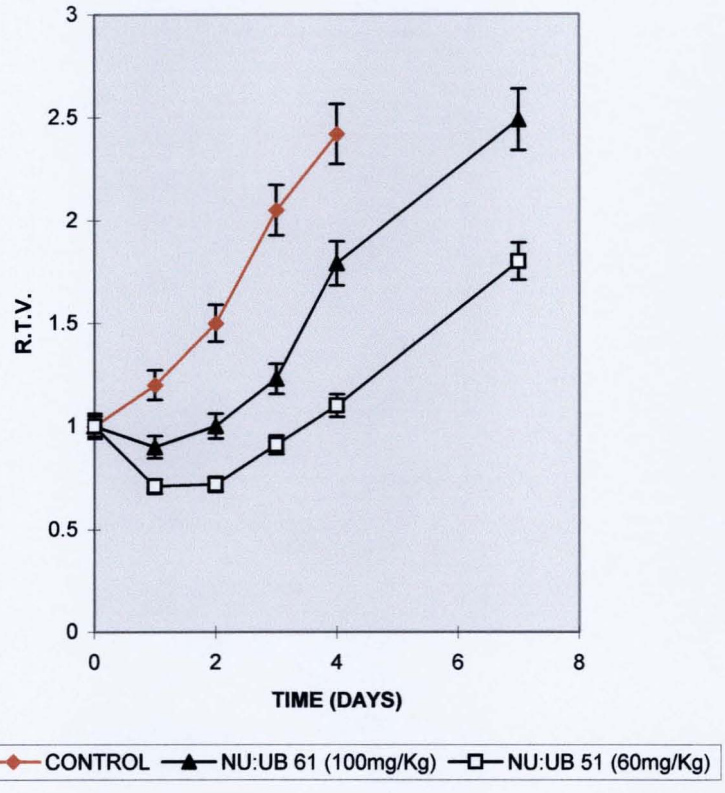
4.4.8 *In-Vivo* Chemosensitivity

Several spacer-linked anthraquinone-amino acid conjugates from the NU:UB library were selected for *in-vivo* evaluation (at the Clinical Oncology Unit, University of Bradford) against subcutaneously implanted, refractory MAC15A murine adenocarcinoma of the colon tumours [protocol is given in **Appendix 1**].

Very significant statistical differences were observed between treated and control groups (in all cases $p<0.01$) for each of the NU:UB compounds tested. In each case treatment was given as a single dose of drug (i.p.) at its MTD (maximum tolerated dose).

The 4,8-dihydroxylated propyl and butyl spaced glycine conjugates, NU:UB 51 and 61 respectively were chosen for *in-vivo* evaluation on the basis of low *in-vitro* cytotoxic potency against the MAC15A cell line and their dual topoisomerase I and II inhibitory profile. Comparison of these compounds (**Figure 13**) clearly shows that lengthening of the spacer between the anthraquinone and peptide motifs from propyl to butyl resulted in a reduction in *in-vivo* potency at MTD; greater tumour volume reduction and growth delay were achieved with NU:UB 51 at equitoxic doses.

Figure 13: *In Vivo* Chemosensitivity against MAC15A colon adenocarcinonoma: Comparison of NU:UB 51 (176) and NU:UB 61 (178)



Four structurally related proline conjugates (differing only in spacer length and the extent of anthraquinone hydroxylation) were selected for *in vivo* evaluation. Surprisingly, the non-hydroxylated compounds NU:UB 31 (**208**) and 43 (**215**) (which were marginally less active than NU:UB 83 and 85 *in vitro*) displayed better *in vivo* profiles than their respective 4,8-dihydroxylated analogues. It is encouraging that the level of tumour volume reduction given by NU:UB 31 and 43 can be achieved without introduction of hydroxyl substituents into the anthraquinone ring system. The latter may have been predicted to result in greater potency, but the potential for stronger DNA binding (with an increased risk of associated mutagenic effects) and greater potential for free radical production, are desirably avoided. The maximum growth delay at equitoxic doses was observed with NU:UB 31.

Figure 14:
***In Vivo* Chemosensitivity against MAC15A colon adenocarcinonoma: Comparison of NU:UB 31 (208), NU:UB 43 (215) NU:UB 83 (223) and NU:UB 85 (225).**

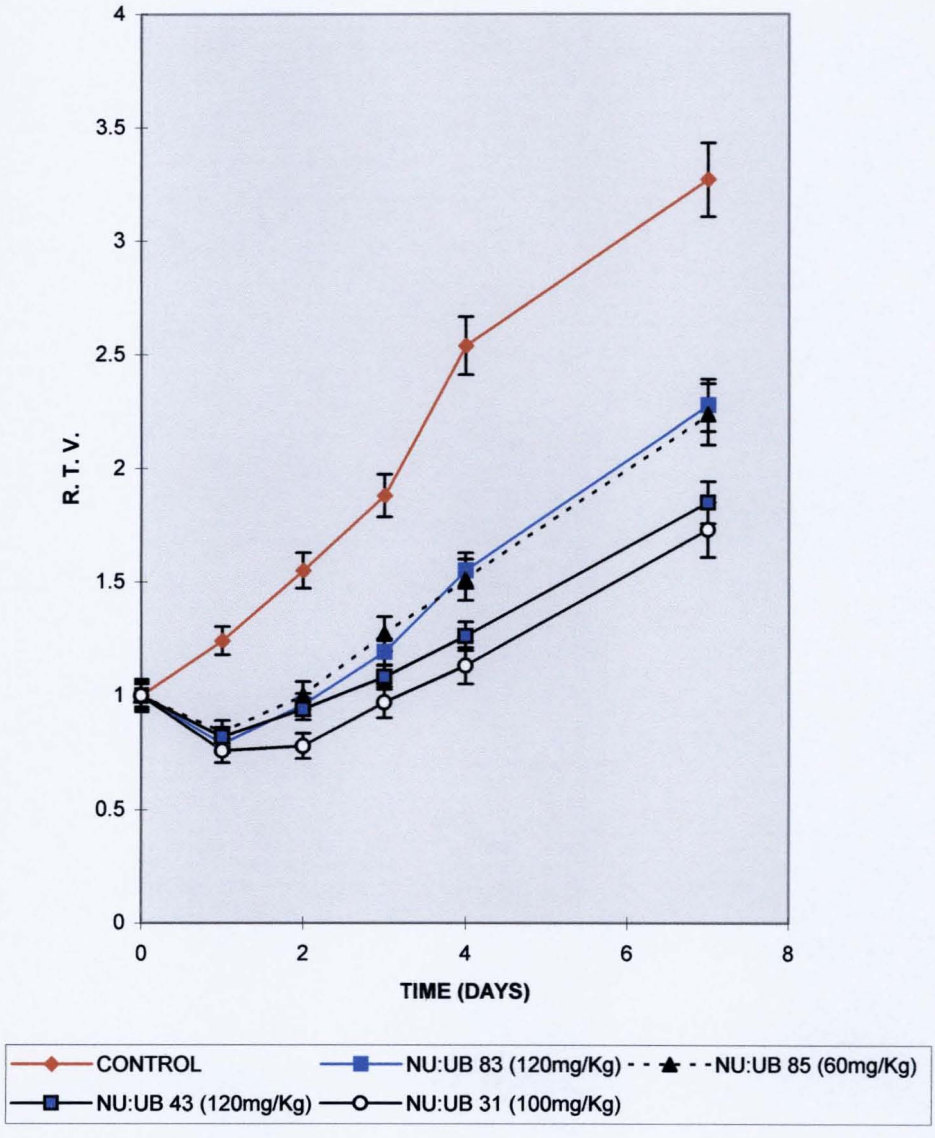
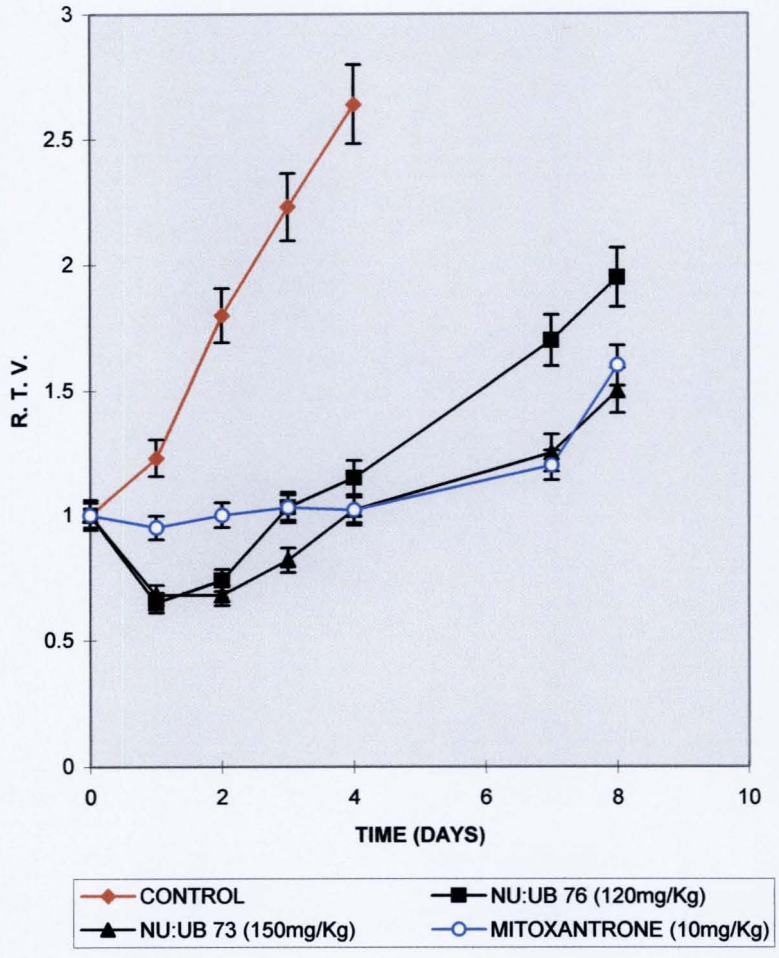
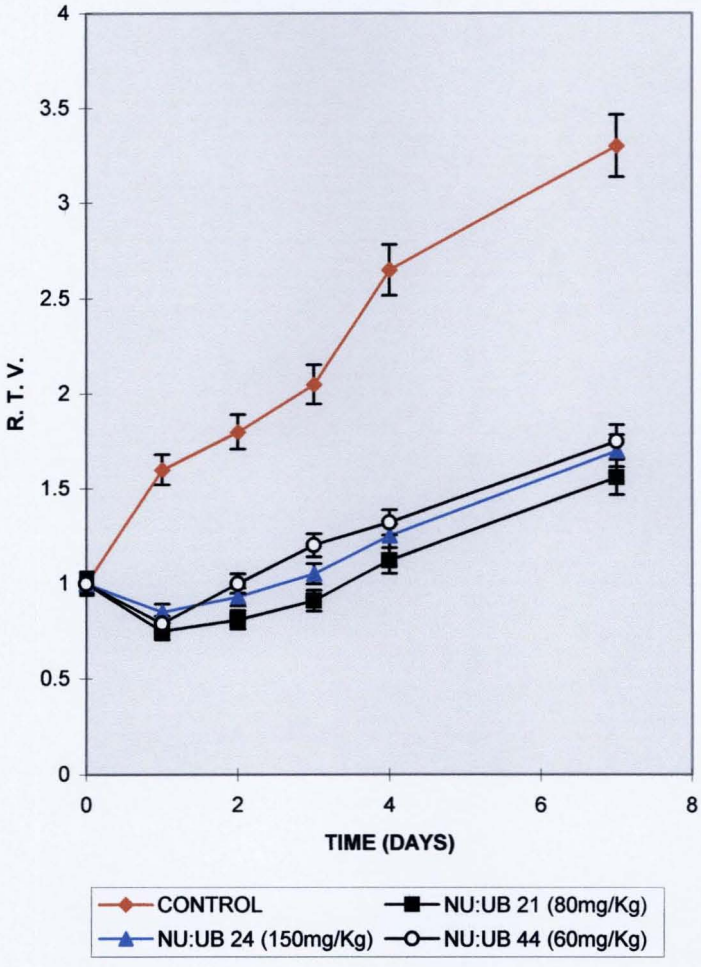


Figure 15:
***In Vivo* Chemosensitivity against MAC15A colon adenocarcinonoma: Comparison of NU:UB 73 (194), NU:UB 76 (196) and Mitoxantrone (7).**



Single (MTD) doses of the isomeric ester-linked L-alanine [NU:UB 73 (**194**)] and D-alanine [NU:UB 76 (**196**)] conjugates produced the greatest initial tumour volume reductions of any of the conjugates tested at equitoxic doses and a marginally greater growth delay was sustained with the natural isomer. Data for the comparator compound mitoxantrone has arbitrarily been plotted in **Figure 15**; a single dose at MTD was utilised in each of the NU:UB conjugate anti-tumour evaluations. It is noteworthy that mitoxantrone produces growth delay but does not shrink tumours in contrast to the effects of the NU:UB conjugates.

Figure 16: *In Vivo* Chemosensitivity against MAC15A colon adenocarcinonoma: Comparison of NU:UB 21 (184), NU:UB 24 (236) and NU:UB 44 (254)



Additionally, the amide-linked conjugates of D-alanine, ornithine and serine gave approximately comparable tumour volume reductions and growth delays at equitoxic doses.

All NU:UB conjugates were generally very well tolerated with few toxic deaths and on the basis of the *in vivo* antitumour effects at single dose, merit dose scheduling and extension to alternative tumour types and human xenograft studies.

CHAPTER FIVE

[PART B]

MATRIX METALLOPROTEINASES AND CANCER: PERSPECTIVES AND A NEW APPROACH

PART B

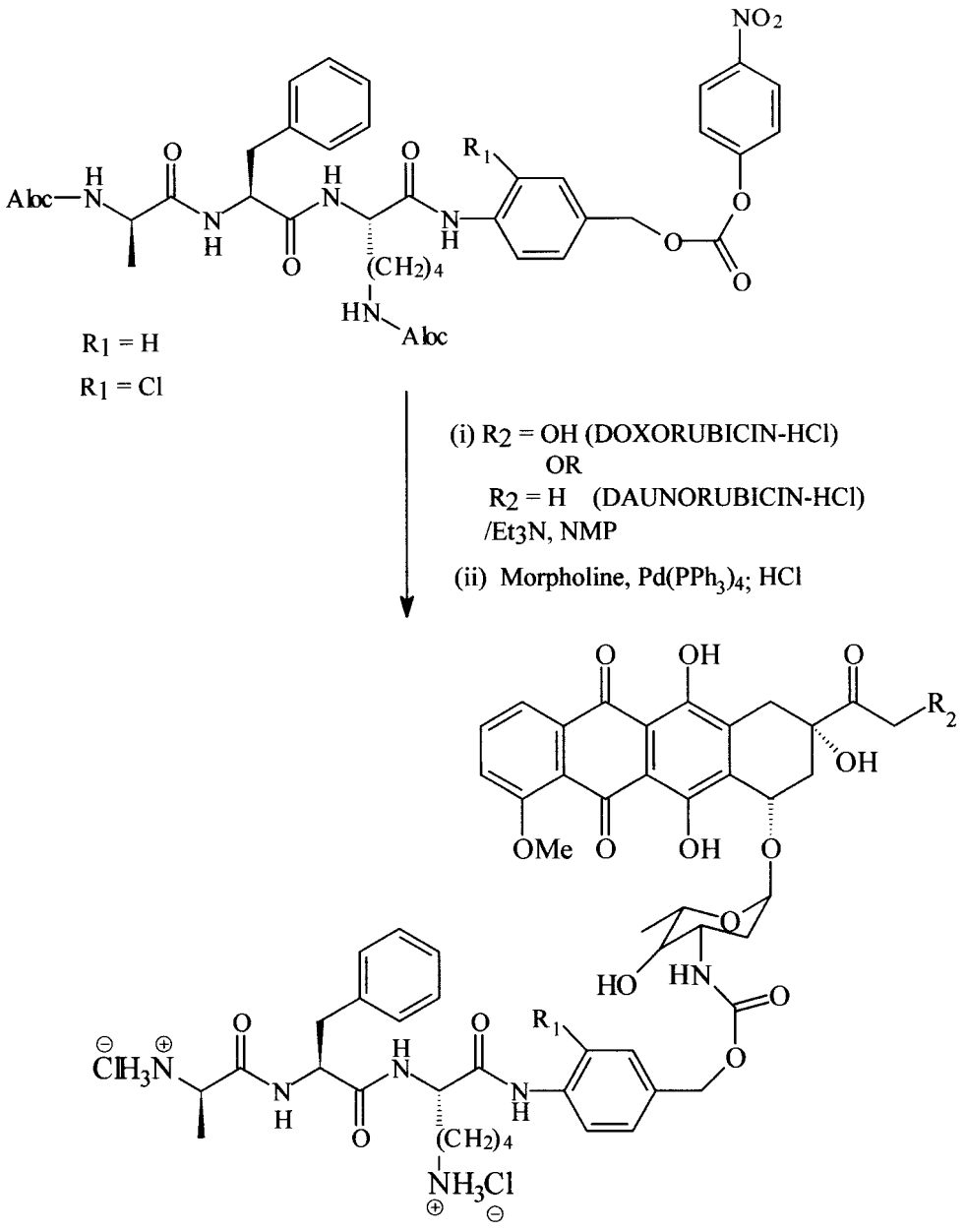
5.1 Introduction

The clinical usefulness of many low molecular mass anticancer compounds is limited because of their narrow therapeutic index. Rational drug design of new cancer chemotherapeutics against identifiable molecular targets (that ideally are causal factors in the pathogenesis of disease) has to date been relatively unproductive in affording cures for most malignancies; this approach is further hampered by incomplete identification of all the molecular targets responsible for the majority of these cancers.

An alternative approach is to capitalise on phenotypic rather than genetic differences between tumour cells and normal cycling tissues (Mincher 2002a). Lack of selectivity of chemotherapeutic agents is a major problem in cancer treatment; because highly toxic compounds are used in chemotherapy, it is typically associated with severe side effects. Drug concentrations that would completely eradicate the tumour cannot be reached due to dose-limiting effects such as gastrointestinal tract and bone marrow toxicity. In modern drug development the targeting of cytotoxic drugs selectively to the tumour site can be considered one of the primary goals. In principle, the use of a prodrug (a non-toxic derivative of the cytotoxic drug) offers a promising approach to overcome side effects and achieve a more tumour selective cancer treatment provided the prodrug can be selectively activated to regenerate the toxic parent at the site of the tumour. Early examples of prodrugs of cytotoxins failed to meet the criteria for phenotypic targeting due to non-specific activation mechanisms, however, recent advances in the development of tumour-activated prodrug therapies have shown substantial improvements in antitumour activity; consequential improvements in therapeutic index require demonstration in the clinical setting. Multiple targeting strategies for cancer therapy have been comprehensively reviewed by Dubowchik (1999) and Schally

(1999). A central feature of tumour activated prodrug therapy is the requirement for properties that distinguish neoplastic from normal cells and differences in physiological conditions (for example, pH and hypoxia), the presence of tumour specific receptors and antigens, and the presence of tumour associated enzymes have received attention (de Groot *et al* 2001). Merck (Research Laboratories) have reported a method of targeting antitumour drugs to prostate tumours which utilises prostate-specific antigen (PSA), a protease enzyme that is produced in elevated amounts in these tumours; although systemic PSA levels are raised in the majority of prostate cancer patients, the circulating form is protein-bound and catalytically inactive. Peptide-linked doxorubicin prodrugs have been designed as PSA substrates with the objective of releasing the drug in prostate tissue. While the prodrug provided selective delivery of doxorubicin to tumour tissue, there was substantial non-PSA-specific formation of the drug in laboratory animals, a factor that would limit the therapeutic gain of the prodrug (Wong *et al* 2001). In another approach, the enzyme plasmin (a serine protease) that has been shown to play a key role in tumour invasion and metastasis, has been the intended target of anthracycline prodrugs (de Groot *et al* 1999) and later, prodrugs of paclitaxel (de Groot *et al* 2000) which contained peptide residues designed to function as plasmin substrates. The essential synthetic features of the construction of the anthracycline conjugates are outlined in **Scheme 25** A spacer-containing, activated (as the p-nitrophenolate) peptide carrier was coupled to the amino group in the daunosaminyl sugar residue of doxorubicin or daunorubicin, in the presence of triethylamine in N-methylpyrrolidine. The prodrugs were obtained after N-deprotection of the allyloxycarbonyl (aloc) protected D-ala and lys residues using mild conditions with tetrakis(triphenylphosphine)palladium(0).

Scheme 25: Putative tumour associated plasmin-activated anthracycline prodrugs



The proteolytically active form of plasmin is confined to the region of the tumour because it is formed from an inactive proform plasminogen by urokinase-type plasminogen activator produced by the cancerous and/or surrounding stromal tissue (Hewitt and Dano 1996). Active plasmin is rapidly inhibited by endogenous inhibitors such as α_2-antiplasmin in the blood, and thus plasmin is a promising target to exploit in tumour-specific prodrug monotherapy.

The focus of the present study is the application of synthetic organic-peptide conjugation chemistry to the design of tumour activated prodrugs that transport and release the active agents by exploiting over-expressed matrix metalloproteinase enzymes (MMPs) in the tumour environment.

5.2 Hypothesis

It was hypothesised that effective prodrugs of anticancer agents could be developed by inverting the conventional thinking on targeting matrix metalloproteinases with inhibitors, alternatively subverting their proteolytic capacity to cleave an active agent from a suitable oligopeptide carrier. It was predicted that it should be feasible to design-in favourable MMP sensitive cleavage sites ('hotspots') in the peptide carrier and it was anticipated that the prodrugs may show differential cleavage sensitivity in tumour versus normal tissue, as a consequence of the differential expression of MMP proteins.

It was further supposed that following tissue-selective MMP-mediated cleavage at the intended hotspot the active agent would be released from any residual peptide carrier effected by non-specific protease action.

5.3 Aims

The long-term objective of the research is to develop non-toxic prodrugs of potent anticancer agents that exploit the proteolytic capacity of over-expressed matrix metalloproteinase proteins in the tumour environment to selectively release an active and potent agent thereby reducing systemic toxicity and increasing the therapeutic index.

The principal aims were:

1. To extend the chemistry of spacer-linked anthraquinone-amino acid conjugates (the NU:UB series) to oligopeptide conjugates (the PL series) designed as putative MMP substrates and model prodrug systems.
2. To utilize the *in vivo*-active anticancer candidate NU:UB 31, developed in this study, as the latent active agent ('warhead').
3. To incorporate MMP-sensitive cleavage sequences ('hotspots') in the peptide motif focusing on MMP-9.
4. To evaluate the biochemical properties of the prototype prodrug candidates in comparison with the active agent.

5.4 Matrix Metalloproteinases and Metastasis

The majority of cancer-related deaths are caused by the ability of cancer cells to metastasise to critical organs in the body. The matrix metalloproteinases (MMPs) are a group of zinc atom-dependent endopeptidases, which appear to play a major role in this metastatic process (Stetler-Stevenson *et al* 1996, Westermarck and Kahari 1999). MMPs degrade collagen and other components of the extracellular matrix (ECM) allowing for tissue remodelling or cell migration; excessive breakdown of the ECM has been associated with multiple pathologies including arthritis, cardiovascular disease and cancer.

MMP activity is known to function at multiple stages of tumour progression affecting tumour establishment, growth, neovascularization, intravasation/ extravasation and metastasis (McCawley and Matrisian 2000).

There are currently at least 26 members of the MMP family which have been classified into different subfamilies according to their substrate specificity and cellular location. These are the interstitial collagenases, the stromelysins, the gelatinases and membrane-type MMPs. That the enzymes were placed in arbitrary groups that originally arose

from considerations of the substrates cleaved, was not a very sound basis because there is insufficient information about the natural substrates of many of these enzymes. The enzymes reported earlier (MMP-4, -5, -6) were later found to correspond to known enzymes, so these three numbers have been discontinued and remain vacant; further confusion arose as individual authors assigned numbers they believed came next (Cossins *et al* 1996). The relationship of MMPs (or matrixins) to the broad class of metalloproteinases, of which there are >200 examples (almost all dependent on zinc at the active site for catalytic function), has recently been discussed (Woessner 1998). The MMP numbers are convenient as shorthand when speaking or writing but their use is diminished by the large number of protein species that are coming to light. The first attempt to rationalise nomenclature was made in 1992 (Nagase *et al* 1992). It has been difficult to define what an MMP is; an early definition was that an MMP was blocked by chelators, had a latent form activated by organomercurials, was inhibited by TIMP and acted upon at least one component of the ECM. The first criterion is too broad and the second and fourth are not valid for all MMPs so only inhibition by TIMP remains. Later criteria, including an extracellular site of function and possession of a cysteine switch are still not sufficiently specific and inhibition by synthetic inhibitors (such as hydroxamates) is no longer useful because, for example, the ADAMs (a disintegrin and metalloproteinase) such as tumour necrosis factor-α (TNF-α) are effectively inhibited by the same compounds (Moss *et al* 1997). The best current criterion would be sequence similarity to collagenase (MMP-1), establishing an evolutionary relationship (Woessner 1998).

Figure 17: Arrangement of the domain structures of MMPs (matrixins)

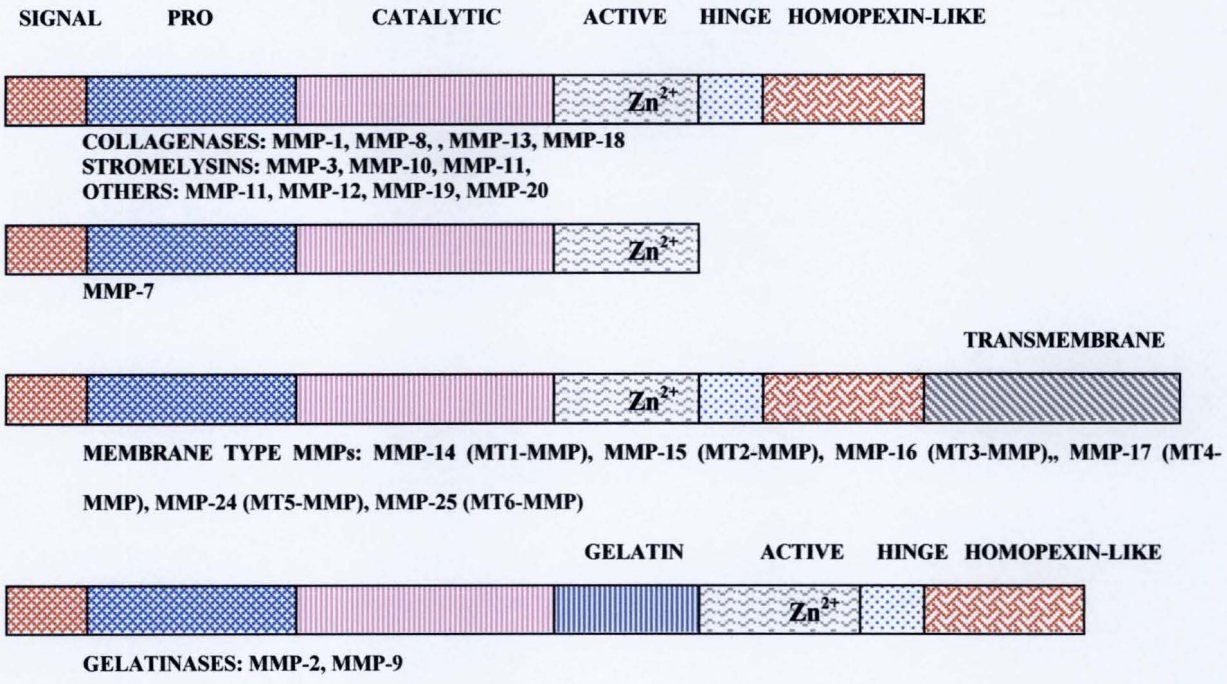


Figure 17 outlines the domain structure of matrix metalloproteinases, with MMPs having similar domain structures grouped together. All MMPs sequenced to date have at least three domains: a pro-domain containing a conserved cysteine residue, a catalytic domain and a highly conserved zinc-binding active site. The two gelatinases, MMP-2 and MMP-9, each have a gelatin-binding domain inserted between the catalytic domain and the active site domain and the six membrane-type MMPs, MMP-14, -15, -16, -17, -24, and -25, have C-terminal transmembrane domains (Woessner and Nagase 2000)

MMPs are highly regulated with expression, secretion and activity levels kept under tight control (Nagase and Woessner 1999). These enzymes are secreted in their inactive latent forms (proforms or zymogens) that are activated upon cleavage of the prodomain by a range of other proteases such as serine proteases and urokinase-type plasminogen activator in an activation cascade (Van den Steen *et al* 2001). MMP activity is also tightly controlled by a group of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The ratio of MMP activity to TIMP expression is crucial in

the metastatic process with low TIMP expression correlating with the metastatic potential of murine and human tumour cell lines (Ponton *et al* 1991).

Although studies to determine the relative contribution of individual MMP members to the multiple stages of tumour progression is still at an early stage there have been many reports on the over-expression of most MMPs in human tumours and adjacent stromal cells (De Clerck *et al* 1994).

Over-expression of the gelatinases, MMP-2 and MMP-9, in many malignant tissues has been well documented. Levels of active MMPs (MMP-2 and MMP-9) and total MMP activity (MMP-1, MMP-3 and TIMP-1) have been found to be significantly greater in tumour tissue than in normal colon with over-expression of MMP-1 correlating with advanced cancer progression (Baker *et al* 2000). Over-expression of MMP-2 and MMP-9 in primary pancreatic and colorectal carcinomas has also been associated with liver metastasis; MMP-2 and MMP-9 activities were found to be significantly higher in carcinomas with metastases than without (Matsuyama *et al* 2002).

Increased expression of MMP-1, MMP-2 and MMP-9 in human melanoma cell lines has been correlated with a highly invasive phenotype (Hofmann *et al* 2000).

More recently it has been recognised that interstitial collagenases, a sub family of MMPs comprised of MMP-1, MMP-13 and the membrane bound MMPs MMP-14, -15, -16, -17, -24 and -25, may also contribute substantially to the later stages of tumour dissemination with their over-expression being associated with more aggressive tumours (Brinckerhoff *et al* 2000). For example, MT1-MMP, MT2-MMP and MT3-MMP mRNA expression was significantly higher in clinical specimens of renal cell carcinoma than in normal renal tissues with increased levels of MT1-MMP in particular correlating with the later stages of tumour invasion (Kitagawa *et al* 1999). Highly invasive MDA-MB-231 human breast cancer cells over-express MMP-1, MMP-3,

MMP-9 and MMP-13 whereas slightly invasive T47D, MCF-7 and BT-20 human breast cancer cells do not (Balduyck *et al* 2000).

MMPs levels are also increased in haematological malignancies. MMP-2 and / or MMP-9 were found to be expressed in acute myelogenous leukaemia bone marrow samples and HEL, HL-60, K-562 and KG-1 leukaemia cell lines whereas normal (immature) bone marrow did not express either enzyme (Janowska-Wieczorek *et al* 1999).

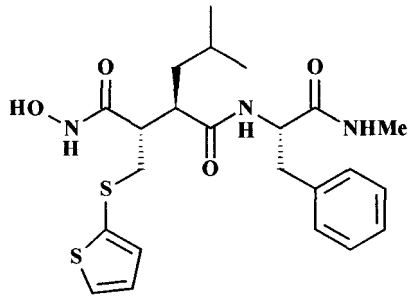
5.5 Development of MMP Inhibitors as Cytostatic Agents

The current conventional approach to controlling metastatic disease has been to use synthetic low-molecular weight inhibitors (MMPIs) to inhibit the activity of these proteinases (reviewed in Whittaker *et al* 1999). The essential structural requirements for a molecule to be an effective MMP inhibitor are

- The presence of a functional group capable of chelating the active-site zinc (II) ion (e.g. carboxylic acid or hydroxamic acid).
- At least one functional group available to hydrogen bond with the enzyme backbone.
- One or more side chains which can undergo effective van der Waals interactions with the enzyme subsites.

Two such inhibitors using MMPs as a therapeutic target are batimastat (**298**) (Wang *et al* 1994b) and marimastat (**299**)(Steward 1999). They have both been shown to inhibit the metastatic spread of tumours in experimental models. The compounds are broad-spectrum inhibitors of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-14 with IC_{50} values in the low nanomolar range and function by chelating the zinc ion present at the active site of each MMP enzyme.

Despite the considerable attention received from the pharmaceutical industry, MMPIs have been very disappointing in the clinic. Marimastat (**299**) has undergone extensive clinical investigation, however, phase III trials in glioblastoma, breast, ovarian, and small and non-small cell lung cancer have all been discontinued due to the failure of marimastat to show superior efficacy over either standard chemotherapy or placebo (Drugs in Research and Development 2003).



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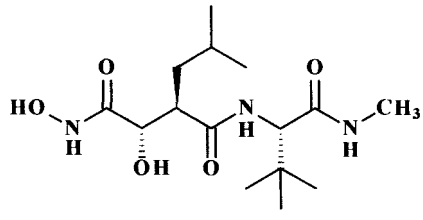


(299)

Although it is unclear whether targeting individual MMPs would be advantageous over broad spectum inhibition, the most serious side effect in human clinical trials of MMP inhibitors has been severe joint pain. It has been speculated that this side effect could be related to inhibitory activity against one (unspecified) subset of MMP family members (McCawley and Matrisian 2000).

5.6 Substrate Specificity of Matrix Metalloproteinases

Studies on the sequence dependence of MMP-mediated cleavage of natural and synthetic protein substrates gives clear evidence of differences in individual enzyme substrate preferences; this specificity is exploitable in the design of new substrates.

Nagase *et al* (1994) characterised a fluorogenic substrate, selectively hydrolysed by MMP-3, which participates in the activation of several MMP zymogens and has broad

substrate specificity. For example the substrate: mca-arg-pro-lys-pro-val-glu~nva-trp-arg-lys(dnp)-NH_2 was hydrolysed rapidly by MMP-3 and very slowly by MMP-9.

An alternative substrate: mca-arg-pro-lys-pro-tyr-ala~nva-trp-met-lys(dnp)-NH_2 was hydrolysed 60 times more rapidly by MMP-3 (and by MMP-9) than MMP-1. [mca = (7-methoxycoumarin-4-yl)acetyl].

Other studies also indicate that it is likely that substrates may be designed which are specific for individual MMPs. For example MMP-19, a novel MMP, proposed to represent the first member of a new MMP sub-family has recently been reported to hydrolyse not only the general MMP hexapeptide substrate mca-pro-leu-gly-dpa-ala-arg-NH_2, but also the heptapeptide substrate mca-pro-leu-ala-nva-dpa-ala-arg-NH_2 with unique specificity and rate enhancement (Stracke *et al* 2000).

Kridel *et al* (2001) have recently carried out an extensive study into the substrate recognition specificity of MMP-9 using a phage-displayed peptide library of random hexamers. MMP-9 was used to cleave substrates at any position within the hexamer, allowing information on the substrate specificity on both sides of the scissile bond to be obtained. Three substrate families were identified, the largest of which contained a **Pro-X-X-↓-Hy-Ser/Thr** motif (where X = an amino acid residue and Hy is a hydrophobic residue) in positions P_3-P_2'. This corresponds to a general motif cleaved by a number of MMPs and believed to represent a collagen-like substrate. However, certain substrates within this group showed considerable selectivity for MMP-9 compared to MMP-13 or MMP-7. Substrate selectivity by MMP-9 was conferred by subsite interactions outside of the dominant P_3 and P_1' positions; MMP-9 has a unique preference for arg at both P_2 and P_1 and a preference for ser/thr at P_2'.

Similar studies were also carried out to determine substrate sequences selectively cleaved by MMP-2. In particular, substrates containing consensus sequences **L/I-X-X-**

↓-Hy, Hy-S-X-↓-L and **H-X-X-↓-Hy** were between 8- to 200-fold more selective for MMP-2 than MMP-9. Sequences containing the **L/I-X-X-↓-Hy** motif were up to 350-fold more selective for MMP-2 than MMP-7. The P_2 residue was found to be crucial in conferring selectivity for MMP-2 (Chen *et al* 2002).

The membrane type-1 matrix metalloproteinase (MT1-MMP) has been reported to mediate the activation of pro MMP-2, associated with tumour invasion and metastasis and is also known to have an ability to digest extracellular matrix components. Substrate sequences have recently been identified, again, using a hexamer substrate phage library consisting of a large number of randomized amino acid sequences. (Ohkubo *et al* 1999) Consensus substrate sequences were deduced from the selected clones and gave the preferred sequence **pro-X-gly/pro-↓-leu** at the P_3-P_1' tetrapeptide subsite.

This result is a departure from the usual requirement for gly in the P_1 position; in this case tolerance and indeed enhancement of cleavage rates with proline in the P_1 position allows a distinction to be made between membrane and non-membrane targets (shown for MMP-2 and MT1-MMP). Additional studies by Kridel *et al* (2002) also showed a unique substrate recognition profile for MT1-MMP. In contrast to the findings of Ohkubo et al, proline in the P_3 position was not required for selectivity rather, the presence of arg in position P_4 was found to be essential for both efficient hydrolysis and selectivity. For example, a peptide containing an **R-I-G-F-↓-L-R** sequence was cleaved almost 40 times faster by MT1-MMP than MMP-9; the extent of hydrolysis, relative to non-treated controls, was far greater by MT1-MMP (88%) than MMP-9 (18%).

CHAPTER SIX

[PART B]

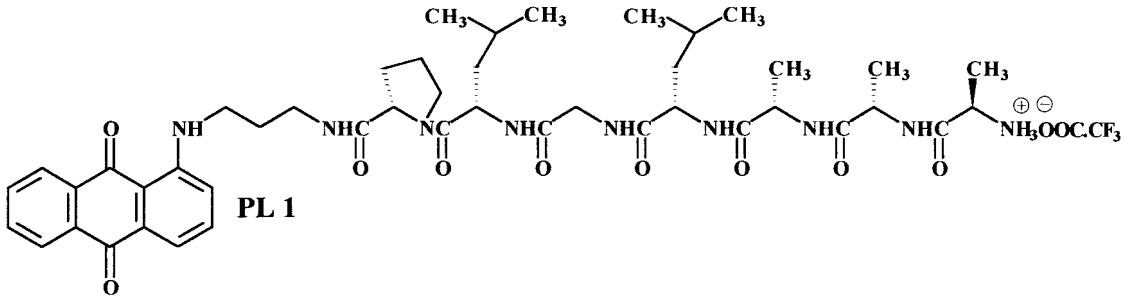
DESIGN AND SYNTHESIS OF MMP-ACTIVATED OLIGOPEPTIDE PRODRUGS: RESULTS AND DISCUSSION

6.1 Design of PL 1

The design of the prototype MMP-substrate prodrug (PL 1) was based upon the chemistry developed in this research programme for the synthesis of spacer-linked conjugates of anthraquinones and amino acids. It was envisaged that expedient access to oligopeptide prodrugs could be achieved by linear extension of the amino acid/ peptide side chain in the NU:UB compound precursor.

The chemical structure of PL 1 is given in **Figure 18**.

Figure 18: Structure of PL 1



The *in vivo*-active, dual topoisomerase I and II inhibitor NU:UB 31 containing an L-proline residue was used as a starting point. The peptide carrier motif was assembled by sequential coupling of individual amino acids to the extended N-terminus commencing with NU:UB 31. It was recognised that prior assembly of a preformed peptide sequence and subsequent coupling to an aminoalkylanthraquinone offered an alternative route that was adopted in some later experiments.

The rational basis for the design of PL 1 was founded on the published work of McGeehan *et al* (1994) (Glaxo Inc. Research. N. Carolina US) concerning substrate specificities of MMP-1 and MMP-9. The McGeehan study started with a parent substrate: dnp-pro-leu-gly~leu-trp-ala-(D)arg-NH_2, a fluorogenic substrate reported earlier (Stack and Gray 1989) and utilising 88 unique amino acid substitutions at each position; over the 4 subsites (P_2 through P_2') 352 potential substrates were evaluated.

Table 12 shows selected single amino acid substitutions made in the study; the figures in parentheses refer to the relative rate of turnover by MMP-9 for each amino acid substitution compared to the parent substrate dnp-pro-leu-gly~leu-trp-ala-(D)arg-NH_2. Combined results from the peptide mapping afforded an optimised substrate, dnp-pro-cha-abu~smc-his-ala-(D)arg-NH_2 (dnp=2,4-dinitrophenyl, cha=cyclohexylalanine, abu=2-aminobutyric acid, smc=s-methylcysteine). This study, combined with results of earlier work (Berman *et al* 1992), gave an extended profile of the substrate specificities of both MMP-1 and MMP-9 with emphasis on MMP-1.

This optimised peptide showed a 36-fold and 6-fold increase in turnover (k_{cat}/k_m), versus the parent substrate, by MMP-1 and MMP-9 respectively.

It should be noted that the rate enhancements are valid for single amino acid substitutions, additive effects of multiple substitutions cannot be obtained from these values; synergistic or antagonistic effects of greater than single point changes were thus not determined.

Table 12: Relationship between amino acid substitution and rates of MMP-9 proteolytic cleavage of synthetic substrates

P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	
dnp-pro	leu	gly	leu	trp	ala	(D)arg-NH_2	Parent Substrate (1)[#]
	emc* (3.3)	tha* (3)	smc* (4)	met (3)			
	tha* (3)	cha* (3)	nva (2.3)	ile (1.6)			
	pfc* (2.3		nle (1.4)	leu (1.6)			
dnp-pro	cha	abu	smc	his	ala	(D)arg-NH_2	Optimised Substrate

* Where emc = S-mercaptoethylcysteine, tha = thienylalanine, pfc = parachlorophenylalnine.

[#] Numbers in parentheses refer to relative cleavage rates; parent substrate assigned the value 1.

It is significant that inclusion of the D-isomers of amino acids in the P_2-P_2' subsite tetrapeptide sequence, drastically reduced the ability of the enzymes to cleave the substrate yet inclusion of unnatural amino acids of the L-configuration produced substrates with increased cleavage sensitivity. The P_1 specific requirement for glycine was virtually absolute. Largest enhancements at P_2' were seen with sec and his (for MMP-1) and with tyr and met (for MMP-9).

Furthermore, based on McGeehan's findings, the best P_1' substituents may incorporate straight chain residues at this position. Notable selectivity for γ-S substituted cysteines exists. Additionally, P_1' chain length is an important determinant of selectivity.

The cleavage rate increased with increasing length of unbranched α-substituents at P_1'.

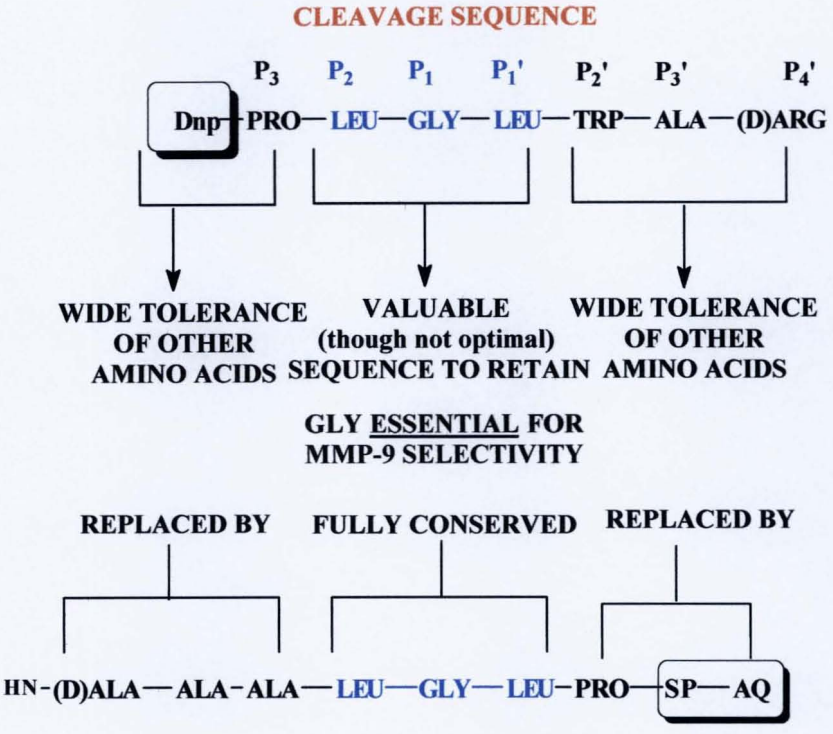
Side chains at the P_2-P_2' positions, in common with P_1' are known to project into deep hydrophobic pockets in MMP-1 and MMP-9. Neither enzyme will tolerate N-α-substitutions or polar functionalities at these positions.

The P_2 position gave noticeable differences between MMP-1 and MMP-9. Specifically, a series of para-substituted phenylalanines were accommodated by MMP-9 but were not hydrolysed readily by MMP-1. Five to ten fold differences in relative k_{cat}/k_m at this position could be exploited in the design of (gelatinase) MMP-9-specific substrates. **Figure 19** outlines the key structural features from the original dnp-pro-leu-gly~leu-trp-ala-(D)arg-NH_2 peptide which have been incorporated into PL1.

Figure 19: **DESIGN OF PL 1**

BASED UPON A PREFERRED CLEAVAGE SUBSTRATE FOR MMP 9

[McGeehan et al, J. Biol. Chem. 1994]



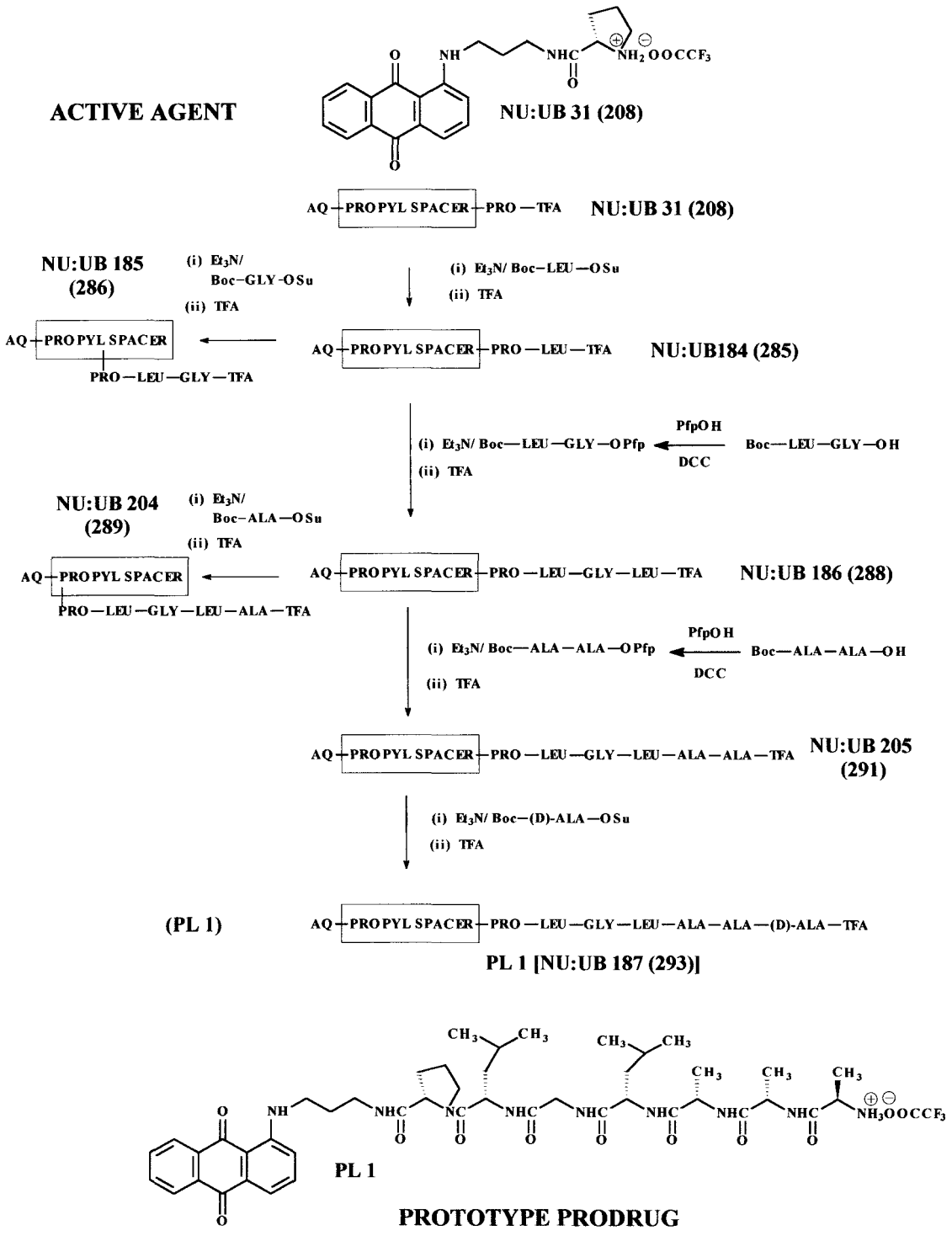
PL 1 preserved the consensus P_1-P_1' gly-leu cleavage site. Given the demonstrated wide tolerance of P_2' substituents and low levels of preference at the P_3' and P_4' positions for cleavage specificity, it was proposed that the *in vivo*-active, experimental warhead NU:UB 31 would span positions P_2' through P_4' (the proline residue occupying the P_2' position). The P5 position was shown to have negligible effect on cleavage specificity although in PL 1 the D-isomer (of ala) was used to help prevent non-specific proteolytic end-degradation of the heptapeptide motif. The reaction chemistry for the synthesis of PL 1 is outlined in **Scheme 26**.

6.2 Synthesis of PL1

The synthesis of the 'warhead' NU:UB 31 was reported in **Section 4.3.1** Briefly, nucleophilic displacement of chlorine from 1-chloroanthraquinone by 1,3-diaminopropane (in excess) in DMSO afforded the anthraquinone-propyl spacer compound. Coupling of N^t-Boc protected, C-activated proline, followed by N-deprotection with TFA afforded, in high yield, the *in vivo*-active, spacer-linked anthracenyl-proline trifluoroacetate salt NU:UB 31.

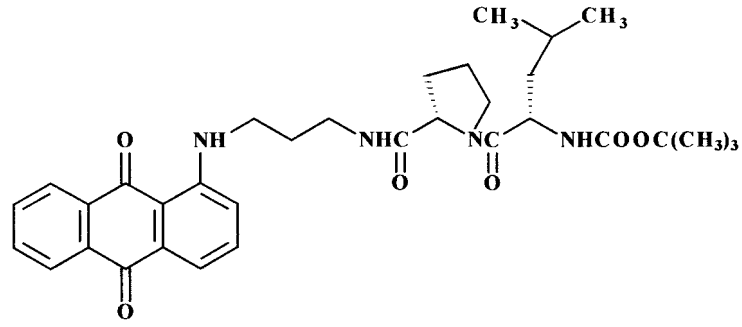
Linear peptide synthesis was carried out on the free amino terminus of NU:UB 31 using N-^{t}Boc-protected α-amino acids, C-activated as either the O-pentafluorophenolate or O-succinimide active esters. Stepwise assembly afforded samples of the potential metabolites of PL1 (as water-soluble N-terminal trifluoroacetate salts), for biological evaluation.

Scheme 26: Outline Synthesis of the Prototype MMP-9 Substrate Prodrug PL 1 (293) [Containing the Experimental Anticancer Agent ('Warhead') NU:UB 31]



6.2.1 1-[3-(N-Tertiarybutoxycarbonyl-L-leucyl-L-prolylamino)propylamino]anthra-quinone (284)

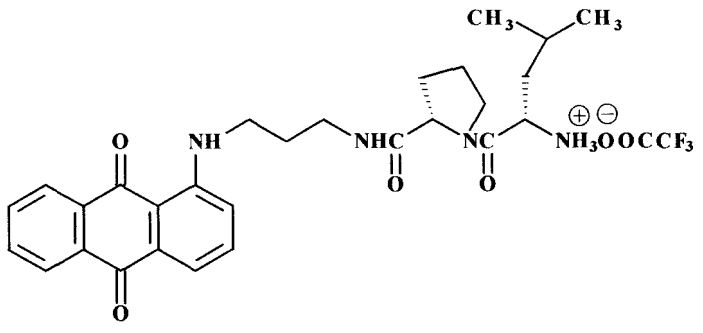
The leucylprolyl motif was introduced by standard N-terminal extension of proline in the experimental antitumour agent, NU:UB 31 (**208**) whose synthesis is described fully in **PART A [Section 4.3.1]**.



(284)

The protected dipeptide conjugate was formed by the reaction of N-^{t}Boc-L-leucine-N-hydroxysuccinimide ester with the free base of the propyl-spaced proline conjugate NU:UB 31 (**208**) (liberated from the trifluoroacetate by triethylamine) in THF. After twelve hours reaction time the crude product was purified by solvent extraction and column chromatography to give the title compound (**284**) in an analytically pure form. The structure was confirmed by its FAB(+) mass spectrum which gave a signal at m/z 613 for the species $(M+Na)^{+}$ and the base peak at m/z 591 $(MH)^{+}$ corresponded to a molecular mass of 590.

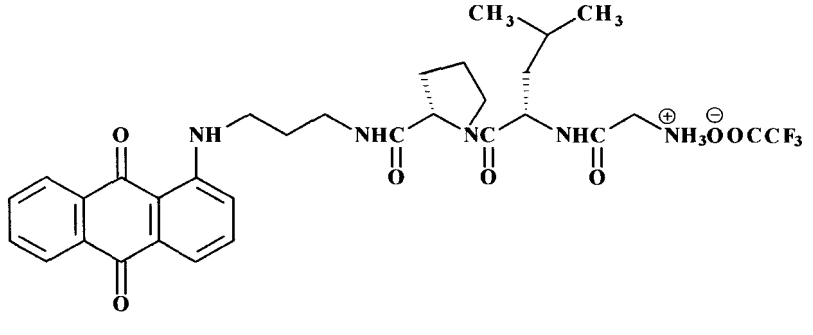
6.2.2 1-[3-(L-Leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (285) (NU:UB 184)



(285)

Deprotection of compound (**284**) was carried out with trifluoroacetic acid. A precipitate of the resultant salt (**285**) was obtained from an ethanol/ ether solution. The electrospray (+) mass spectrum gave signals at m/z 513 for the species $(RNH_3+Na)^+$ and m/z 491 for $(RNH_3)^+$. The presence of the trifluoroacetate anion was confirmed by a peak at m/z 113 in the electrospray (-) mass spectrum.

6.2.3 1-[3-(Glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (286) (NU:UB 185)

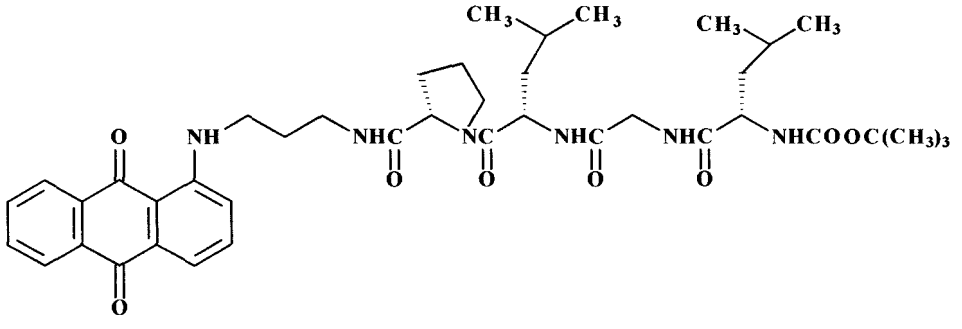


(286)

The N-^{t}Boc protected derivative was prepared by the reaction of N-^{t}Boc-glycine-N-hydroxysuccinimide ester with the dipeptide conjugate (**284**) in THF and triethylamine. The crude product was purified by solvent extraction and column chromatography and was

deprotected using trifluoroacetic acid to give the title compound (**286**). The structure was confirmed by its electrospray (+) mass spectrum which had signals at m/z 570 and 548 corresponding to the species $(RNH_3+Na)^+$ and $(RNH_3)^+$ respectively.

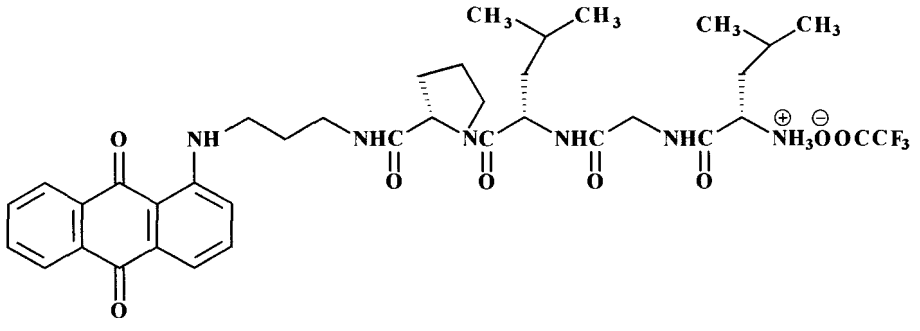
6.2.4 1-[3-(N-Tertiarybutoxycarbonyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)-propylamino]anthraquinone (287)



(287)

The pentafluorophenolate ester of N-^{t}Boc-L-leucylglycine was prepared from the reaction of pentafluorophenol and N-^{t}Boc-L-leucylglycine in dry ethyl acetate using dicyclhexylcarbodiimide (DCC) as the coupling agent. The tetrapeptide compound (**287**) was formed by the addition of N-^{t}Boc-L-leucylglycine pentafluorophenolate to a cooled stirred solution of compound (**284**) (the propyl spaced prolylleucine TFA conjugate) in DMF and triethylamine. Purification by solvent extraction and column chromatography afforded the title compound in an analytically pure form. The FAB(+) mass spectrum had m/z 761 $(MH)^+$, confirming a molecular mass of 760 and a fragmentation peak at m/z 661 corresponding to the loss of the ^{t}Boc protecting group.

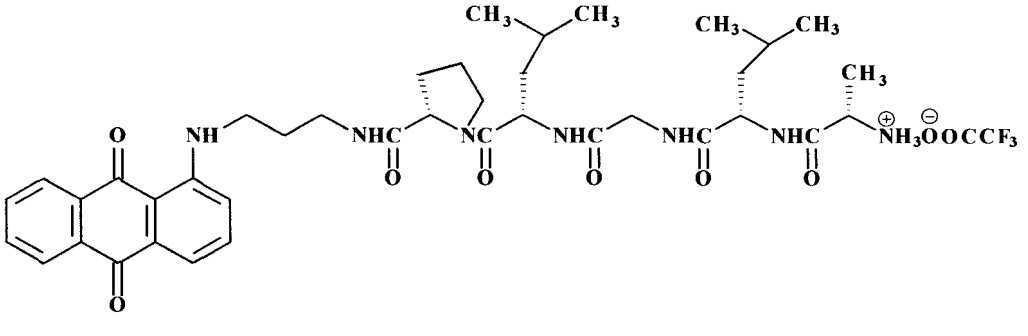
6.2.5 1-[3-(L-Leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate. (288)(NU:UB 186)



(288)

Deprotection of compound (**287**) was carried out using trifluoroacetic acid. The structure of the resultant salt (**288**) was confirmed by its mass spectrum which had a peak at m/z 661 for the species $(RNH_3)^+$. A fragmentation peak at m/z 378, which was also the base peak, corresponded to the species $(AQ\text{-}Propyl\text{-}Sp\text{-}Pro\text{-}NH_2)^+$.

6.2.6 1-[3-(L-Alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]-anthraquinone trifluoroacetate (289) NU:UB 204

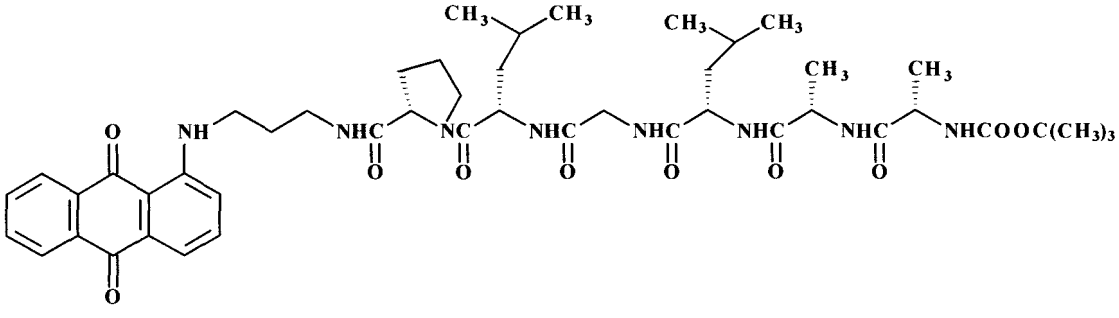


(289)

The N-^{t}Boc protected derivative was prepared by the reaction of N-^{t}Boc-alanine-N-hydroxysuccinimide ester with the tetrapeptide conjugate (**288**) in THF and triethylamine. The crude product was purified using solvent extraction and column chromatography and

was deprotected using trifluoroacetic acid to give the title compound (**289**). The electrospray (+) mass spectrum gave signals at m/z 754 for the species $(RNH_3+Na)^+$ and m/z 732 for $(RNH_3)^+$.

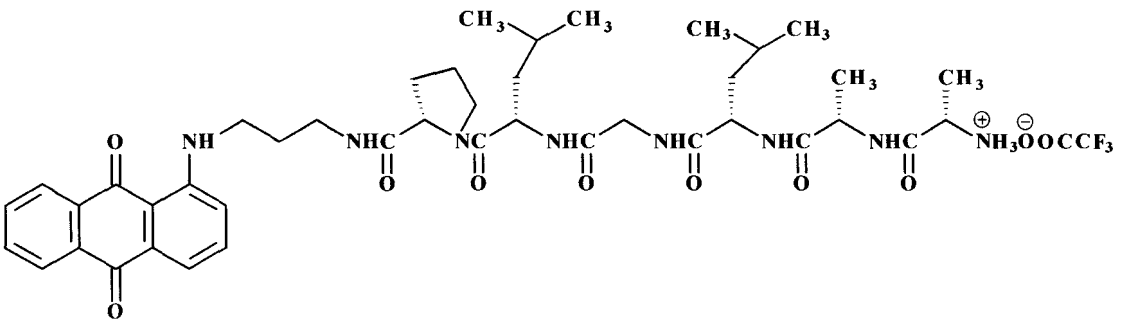
6.2.7 1-[3-(N-Tertiarybutoxycarbonyl-L-alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone (290)



(290)

N-^{t}Boc-L-Alanylalanine pentafluorophenolate ester was prepared from the reaction of pentafluorophenol and N-^{t}Boc-L-alanylalanine in dry ethyl acetate using dicyclhexylcarbodiimide (DCC) as the coupling agent. The title compound was formed by the addition of N-^{t}Boc-L-alanylalanine pentafluorophenolate ester to a cooled stirred solution of the tetrapeptide conjugate (**288**) to which triethylamine had been added to liberate the free amine. After reaction completion, purification by solvent extraction and column chromatography gave the title compound (**290**).

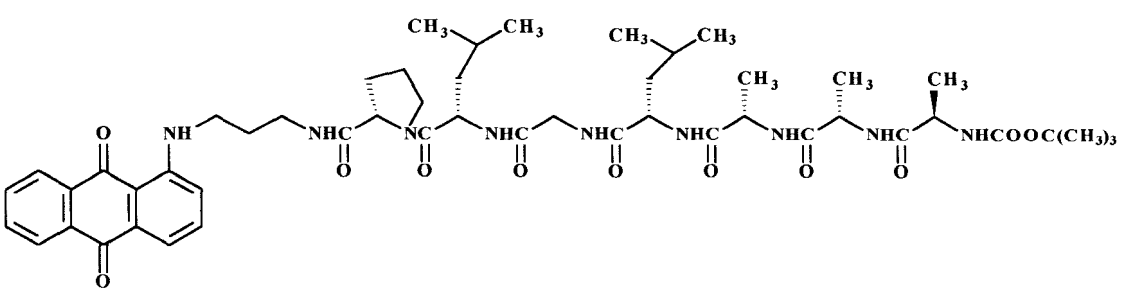
6.2.8 1-[3-(L-Alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propyl-amino]anthraquinone trifluoroacetate (291) NU:UB 205



(291)

Standard deprotection of the intermediate (**290**) was carried out using trifluoroacetic acid. The structure of the resultant salt (**291**) was confirmed by its electrospray (+) mass spectrum which gave signals at m/z 825 for the species $(RNH_3+Na)^+$ and m/z 803 for $(RNH_3)^+$. The presence of the trifluoroacetate anion was confirmed by a signal at m/z 113 in the electrospray (-) mass spectrum.

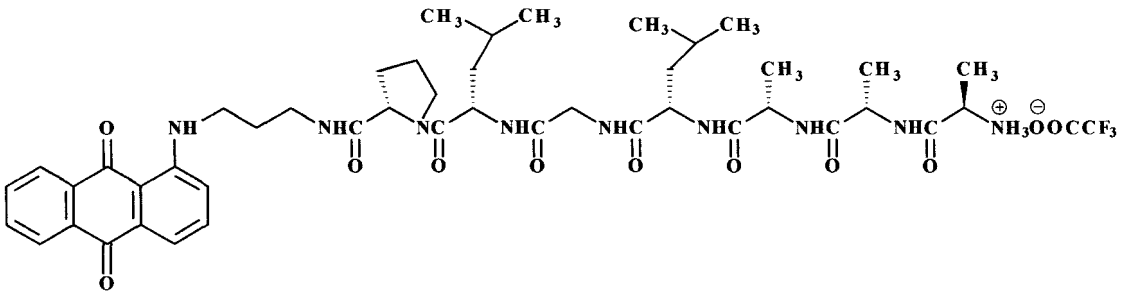
6.2.9 1-[3-(N-Tertiarybutoxycarbonyl-D-alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone (292)



(292)

Compound (**292**) was prepared by the reaction of N-^{t}Boc-D-alanine-N-hydroxysuccinimide ester with the hexapeptide conjugate (**291**) in THF and triethylamine. The crude product was purified by solvent extraction and column chromatography.

6.2.10 1-[3-(D-alanyl-L-Alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)-propylamino]anthraquinone trifluoroacetate (293) (NU:UB 187) PL1



(293)

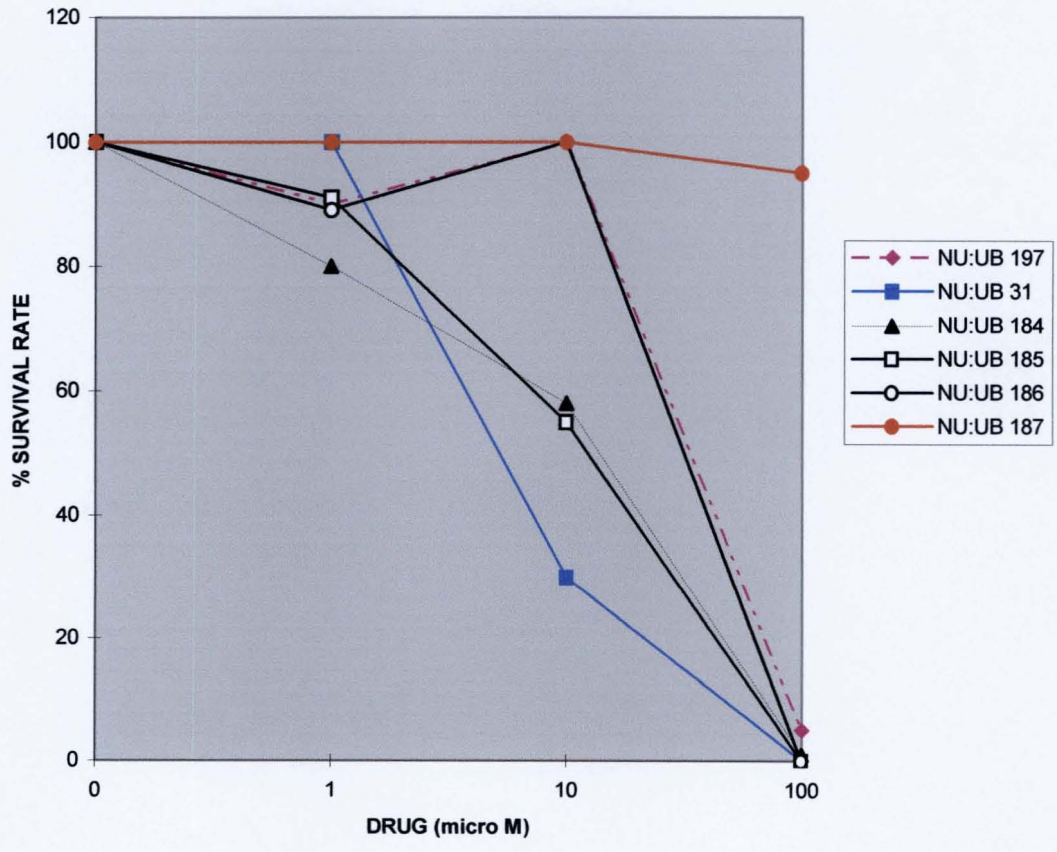
Deprotection of the N-protected intermediate (**292**) was carried out using trifluoroacetic acid. A precipitate of the resultant salt (**293**) was obtained from an ethanol/ ether solution. The electrospray (+) mass spectrum gave signals at m/z 896 for the species $(RNH_3+Na)^+$ and m/z 874 for $(RNH_3)^+$. The base peak at m/z 113 in the electrospray (-) mass spectrum confirmed the presence of the trifluoroacetate anion.

6.3 Biological and Biochemical Evaluation

6.3.1 PL 1 (293) and Intermediates: MAC15A *In Vitro* Chemosensitivity

In vitro cytotoxicity against the MAC15A colon adenocarcinoma cell line was measured after a 1 hour drug exposure by MTT assay. Several intermediate compounds from the synthesis of PL 1 (**293**) were potential metabolites of PL 1 degradation *in vitro* or *in vivo* and as such, were included in this assay with the 'warhead' NU:UB 31 (**208**). The growth curves are depicted in **Figure 20**.

Figure 20: MAC15A Cells Exposed to PL 1 and Intermediates (1 hour exposure)



Over the short exposure time, PL 1 (**293**) was essentially inactive (IC_{50}>>100μM) compared to the 'warhead' NU:UB 31 (**208**) that had an IC_{50} value of 5μM. Notably, the anthraquinone aminopropylamino spacer compound (146) was relatively inactive at 33μM [**Table 13**]. The potential metabolites, dipeptide pro-leu and tripeptide pro-leu-gly intermediates (**285**) and (**286**), respectively were of comparable potency, some 2-3-fold less potent than NU:UB 31. The tetrapeptide conjugate (288) was also relatively inactive (IC_{50} 30μM). It was clear that in this cell line, the extension of NU:UB 31 through addition of a hexapeptide motif, resulted in masking the cytotoxicity of the active agent. Furthermore, over a 96 hour exposure PL1 had an IC_{50} value in excess of 30μM whereas NU:UB 31 had a value of 2.5. The reduction in intrinsic cytotoxic potency of the oligopeptide prodrug was a prerequisite for success of a prodrug strategy. The generality of this feature would require additional experiments using different cell lines and extension to alternative 'warheads'.

Table: 13 Inhibition of topoisomerase I-mediated relaxation of pBR322 DNA and in vitro cytotoxicity against MAC15A colon adenocarcinoma (1h exposure)/ PL 1 and its intermediates

COMPOUND	LEVEL OF TOPOISOMERASE I INHIBITION	MAC15A IC_{50} (μM)
AQ-SP/ NU:UB 197 (**146**)	Complete inhibition of relaxation at 25 μM	33
AQ-SP-P/ NU:UB 31 (**208**)	Complete inhibition of relaxation at 25 μM	5
AQ-SP-P-L/ NU:UB 184 (**285**)	Complete inhibition of relaxation at 5 μM	14
AQ-SP-P-L-G/ NU:UB 185 (**286**)	Partial inhibition of relaxation at 50 μM	13
AQ-SP-P-L-G-L/ NU:UB 186 (**288**)	No inhibition of relaxation between 1-100 μM	30
AQ-SP-P-L-G-L-A/ NU:UB 204 (**289**)	No inhibition of relaxation between 1-100 μM	Not Determined
AQ-SP-P-L-G-L-A-A/ NU:UB 205 (**291**)	No inhibition of relaxation between 1-100 μM	Not Determined
PL 1 (**293**)	No inhibition of relaxation between 1-100 μM	>>100

6.3.2 PL 1 (293) and Intermediates: Topoisomerase Inhibition

A limited number of experiments were conducted to compare the anti-topoisomerase properties of the prototype prodrug and potential metabolites with NU:UB 31; the summary data is presented in **Table 13** for the inhibitory effects upon topoisomerase I-mediated relaxation of supercoiled pBR322 plasmid DNA by gel electrophoresis. Neither the prodrug nor the intermediate tetra-, pent- or hexa-peptide conjugates showed inhibition of enzyme activity at concentrations up to 100μM; the tripeptide conjugate (**286**) showed some weak inhibitory action at 50μM. Most notable was the relatively potent inhibitory action of the dipeptide pro-leu conjugate (**285**) which was 5-fold more potent than NU:UB 31 in this assay. Furthermore, the intact prodrug had no enzyme poisoning activity and did not bind to DNA (data not shown). It was clear that extending the peptide motif nullified topoisomerase enzyme interaction and imparted substantially different biochemical properties to the active agent.

6.3.3 Incubation of PL1 with Human Recombinant MMP-9

Purified prodrug PL 1 (**293**) [the ES(+) mass spectrum (m/z 874, RNH_3^+) of which is shown in **Figure 21**] containing the putative MMP-9 sensitive cleavage sequence was incubated with recombinant enzyme and product extracts were analysed by HPLC/MS methods.

Figure 21: ES(+) Mass Spectrum of PL 1

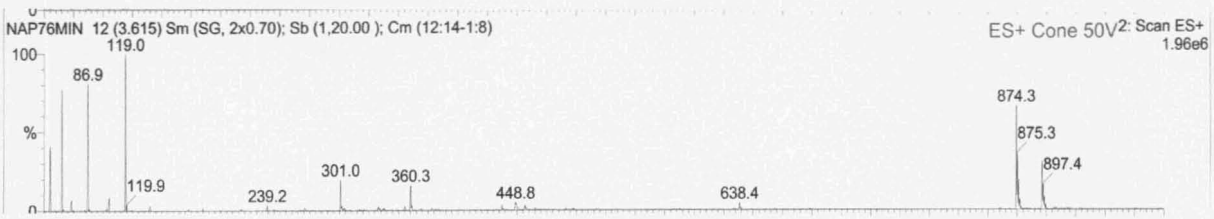
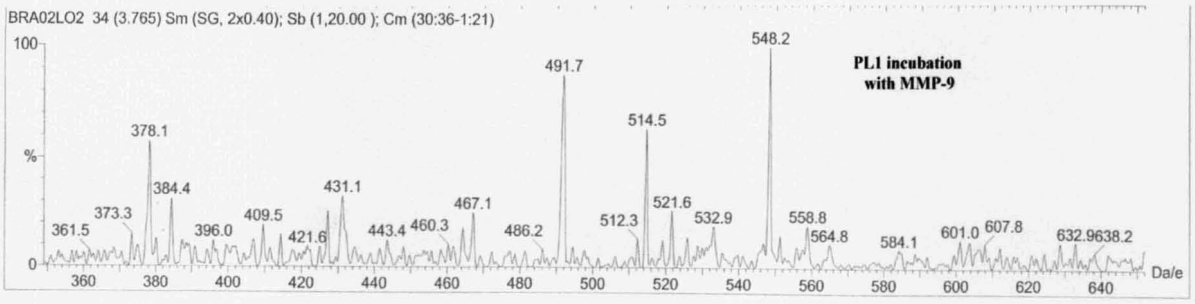
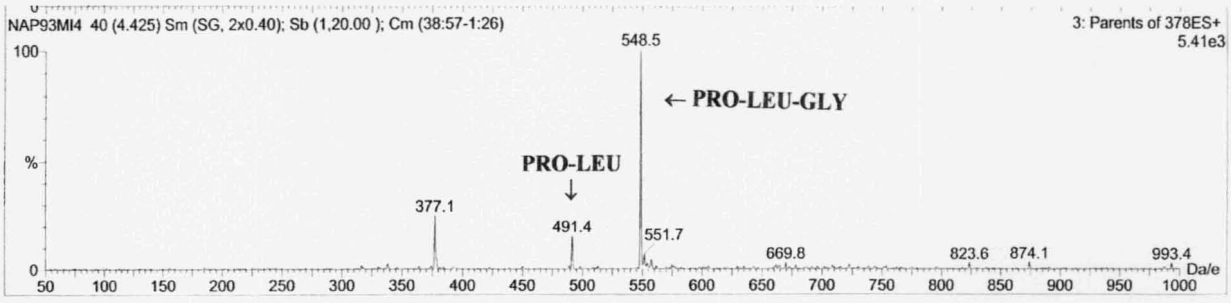


Figure 22: PL1 Incubation with Recombinant MMP-9 [ES(+) Mass Spectrum]



LC-MS and MS-MS analysis of the metabolites resulting from incubation of PL 1 (**293**) with human recombinant MMP-9 [protocol in **Appendix 1**] established that PL 1 was cleaved, principally to the dipeptide pro-leu (**285**) and the tripeptide pro-leu-gly (**286**) [m/z 548 RNH_3^+] intermediates at the predicted cleavage sites [**Figure 22**]. Furthermore, the parent ion spectra of the signal m/z 378 revealed that this breakdown signal was derived from the principal products of *in vitro* metabolism, the parent conjugates, rather than the presence of released active agent NU:UB 31 [**Figure 23**].

Figure 23: PL1 Incubation with Recombinant MMP-9 [ES(+)Parent Ion Mass Spectrum]



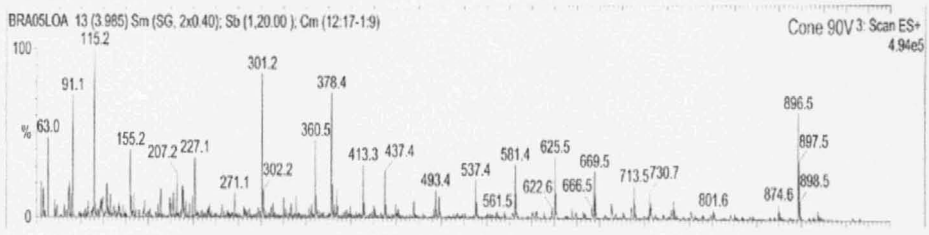
The mass spectral data and hplc traces (not shown) of the *in vitro* metabolites of the incubation of the prototype prodrug PL1 in comparison to pure standards (available by

synthesis of the intermediates) thus, confirmed that the cleavage MMP-9 induced cleavage pattern was consistent with prediction.

6.3.4 Incubation of PL1 with Human Recombinant MMP-2

PL 1 (**293**) was similarly incubated with the closely related human recombinant MMP-2; the ES(+) mass spectrum of the product extract is shown in **Figure 24**.

Figure 24: ES(+) Mass Spectrum of PL1 Incubation with Human Recombinant MMP-2



Despite the very similar substrate specificity of the two proteases and homology in the catalytic site region, surprisingly, PL 1 was shown to be a poor substrate for MMP-2, at least *in vitro*, and was largely unchanged with little or no evidence of prospective, truncated peptide conjugate metabolites (after 24 h) [**Figure 24**]. This marked difference in behaviour towards the closely homologous gelatinases MMP-2 and -9 strongly suggests that it should be feasible to 'design in' molecular features for recognition by specific metalloproteinases.

6.3.5 Preliminary Pharmacokinetic Study of the Stability of PL1

In preliminary PK studies of the stability of prodrug PL1 (**293**) in mice, hplc studies and mass spectra of samples extracted from plasma after an i.v. dose at 40mg/Kg (<MTD), confirmed that the predominant species was intact prodrug at 30 and 60 minute time points [**Figure 25**]. Encouragingly, the significant presence of PL1 indicated that it was relatively long-lived which was a desirable feature in the prodrug design strategy.

Parent ion spectra [**Figure 26**] also confirmed that none of the active agent had been released and that breakdown signals were from the parent prodrug.

Figure 25: ES(+) Mass Spectrum of PL1 [40mg/Kg iv] Extracted from Plasma at 30 minutes

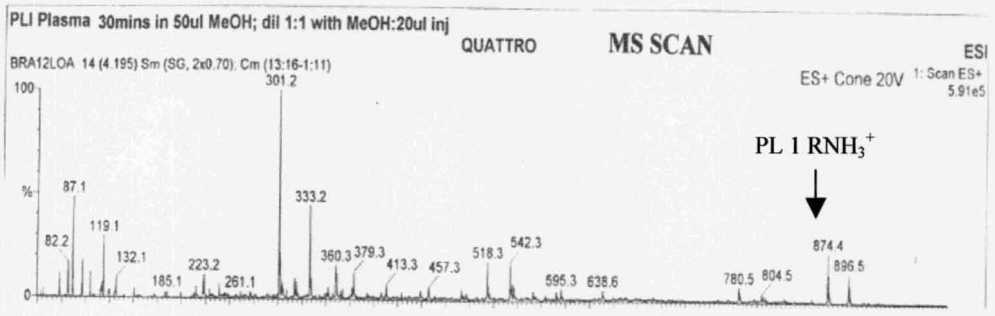
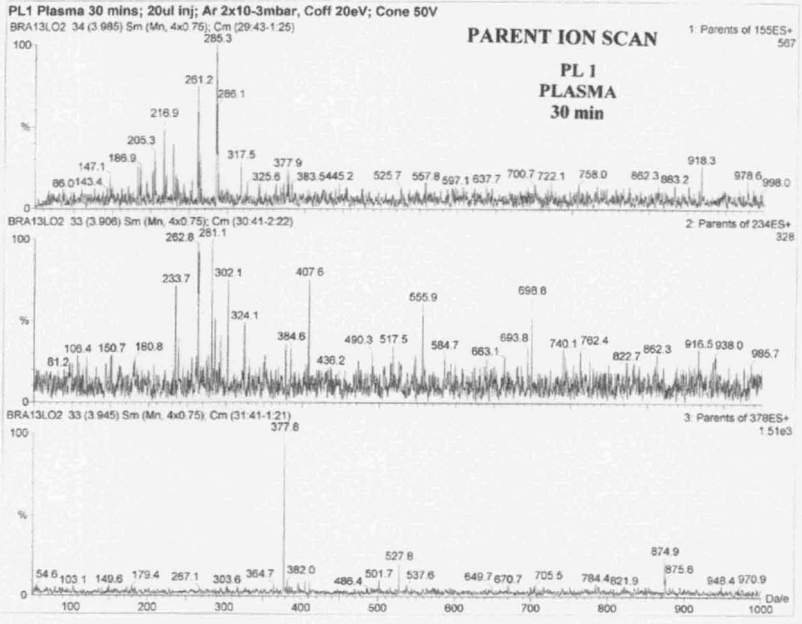
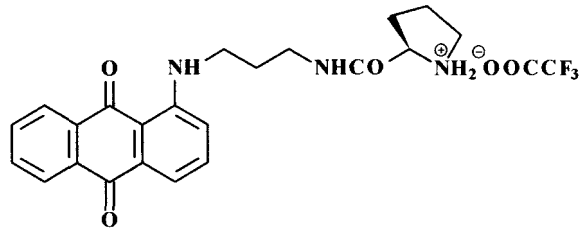


Figure 26: ES(+)Parent Ion Mass Spectrum of PL1 [40mg/Kg iv] Extracted From Plasma at 30 minutes ESI Mass



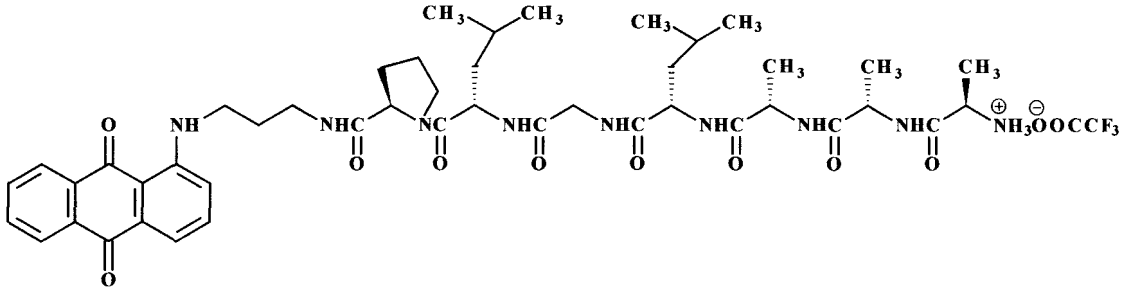
6.3.6 Structural Modification of the Oligopeptide Motif

Clearly, the composition of the oligopeptide carrier can be varied in number of ways that include alternative amino acid substitution patterns to PL 1 and/or changes to the active agent or replacement of peptide bonds. In order to begin to probe the effects of inclusion of amino acids with unnatural, D-isomer configurations, the warhead in PL 1 was replaced by the enantiomeric D-proline conjugate NU:UB 46 (**210**) (Mincher 2002b).



NU:UB 46
(210)

Retention of the hexapeptide sequence in PL 1 thus afforded the isomeric PL 2 (**303**).



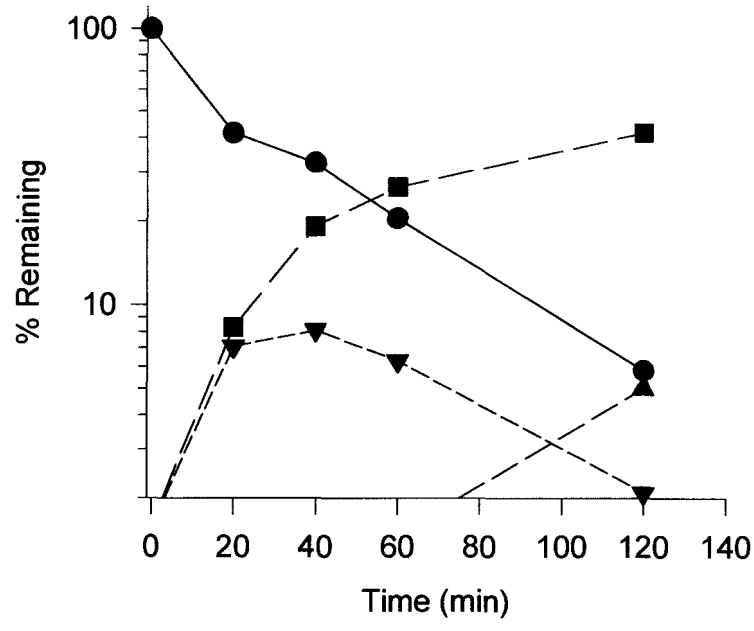
PL2 (303)

The prototype conjugates PL 1 and PL 2 were studied by hplc and hplc/ms for their *in vitro* metabolism [protocol in **Appendix 1**] in (diluted) tissue homogenates of a highly MMP-9 expressing human fibrosarcoma, HT1080.

Both conjugates were rapidly metabolised; furthermore, the cleavage site specificity for PL 1 mirrored that observed with human recombinant protein affording the residual

anthraquinone di- and tri- peptide conjugates. Initial metabolism of PL 2 (**303**) was similar giving principally the D-pro-gly-leu tripeptide conjugate metabolite (**301**) as shown in **Figure 27**. In contrast to PL 1, over extended exposure times (24 hours) proteolytic degradation to release the active agent did not extend to release of anthraquinone-spacer (**145**), thus the presence of the D-isomer considerably stabilised the spacer-(pro) amino acid junction whilst preserving the primary cleavage site.

Figure 27: PL 2 (303) in HT1080 homogenate (1/500)



- PL2 (**303**)
- ▼ AQ(D-Pro)-Leu-Gly-Leu (**302**)
- ■ AQ(D-Pro)-Leu-Gly (**301**)
- ▲ AQ(D-Pro)-Leu (**300**)

6.3.7 Oligopeptide Prodrug Summary

For reasons of commercialisation restrictions and the sake of brevity, the discussion of prototype prodrugs has been limited to preliminary work to establish that prototypes can function as MMP substrates. It is clear that the inclusion of a gly-leu-gly sequence in the peptide carrier facilitates cleavage by MMP-9. That MMP-2 does not, at least *in vitro*, metabolise PL1 (**293**) in a directly analogous manner is as intriguing as it is surprising and indicates that tailoring substrates to individual MMPs may be feasible; this may not, however, be necessary or desirable since MMPs occur in 'gangs' and a broad spectrum substrate may therefore be more effective. Encouragingly, recent work has shown that the prototypes have shown differential tissue metabolism between tumour and liver tissue (used as an indicator of normal tissue metabolism); this differential applies not only to cleavage rates but also to differential selectivity for cleavage sites in the oligopeptide carrier. Work is ongoing in parallel projects and in collaborating laboratories to broaden the differential metabolism studies to a panel of tissues, including heart, lung, and kidney. Pharmacokinetic and antitumour studies have been instigated. Exploiting non-specific protease action has been used in the past to effect release of active agents from short peptide conjugates, for example, doxorubicin (**1**) from an N-succinyl-(β-alanyl-L-leucyl-L-alanyl-L-leucyl) derivative (Fernandez *et al* 2001) and similar doxorubicin (and vinblastine)-peptide substrates of the protease, prostate specific antigen (PSA) have been reported (DeFeo-Jones *et al* 2000 and 2002). Since patent applications were filed (earliest priority date 09-03-2001) based on work arising from this research programme (Mincher *et al* 2002b), an MMP cleavage sequence-containing peptide has been used to link doxorubicin to a human serum albumin conjugate (Kratz *et al* 2001), illustrating the growing interest in tumour-activated prodrug therapy.

CHAPTER SEVEN

CONCLUSIONS

7 Conclusions and Suggestions for Further Work

PART A

In this research programme, it was demonstrated that novel spacer-linked anthraquinone-amino acid and peptide conjugates could be successfully synthesised by the coupling of suitably N-protected amino acids (or peptides) as amides or esters (to the free amino or hydroxy terminus, respectively) of precursor anthraquinone-spacer molecules, followed by deprotection to give water-soluble amine salts.

A large library of conjugates [NU:UB], spacer compounds and intermediates was prepared and characterised by spectroscopic methods, principally mass (EI, CI, electrospray and FAB) spectrometry and nmr spectroscopy; several conjugates were shown to be cytotoxic in the low micromolar range *in vitro* against panels of human and animal tumour cell lines. Key conjugates have progressed to preliminary *in vivo* antitumour studies and have potent activity in tumour models that are refractory to clinical agents, including the related anthraquinone topo II inhibitors doxorubicin and mitoxantrone.

Selected conjugates were further studied for their interaction with topoisomerase enzymes *in vitro* and concomitantly for their pattern of cell kill in a panel of Chinese hamster ovarian (CHO) cell lines with altered topoisomerase expresion. For the lead compounds, the pattern of cell kill was, at least in part, consistent with their inhibitory and poisoning effects against topoisomerase enzymes over-expressed in these cell lines.

DNA binding studies showed that the amide-linked, propyl-spaced proline conjugate NU:UB 31 (**208**) binds to DNA, via a mixed-modal, part-intercalative, part groove-binding mechanism that may correlate with its dual action on type I and type II topoisomerase enzymes; a more detailed study of the relationship between mode of DNA binding and topo inhibitory effects for this and related conjugates in the library

would be necessary to devolve structure-activity relationships. The anti-topoisomerase investigations here (and in parallel projects) established that NU:UB 31 and several related conjugates interacted with DNA topoisomerase enzymes in *in vitro* assays using plasmid DNA substrates as well as in intact (HL60) cancer cells. For NU:UB 31 both inhibition of topo I-mediated DNA relaxation (catalytic inhibition) and cleavage complex formation (poisoning) were observed. Additionally, anti-topo II (α and β) activities (catalytic inhibition and poisoning) were also revealed. Thus, there is significant evidence to suggest that NU:UB 31 and other NU:UB compounds, merit classification as dual topo I- and II-targeting agents. These agents may be considered non-classical inhibitors or poisons given their propensity to antagonise the cleavage reaction at high drug concentrations. The ester-linked, butyl-spaced alanine conjugate NU:UB 73 (**194**) is unique in its ability to inhibit topo I and the beta-isoform only of topo II. Cytotoxic potency across the NU:UB series was determined by the α-substituent of the amino acid residue; optimum residues (and preferred dual topo inhibitors) were small, hydrophobic residues which in proximity to the cationic charge probably facilitates cellular uptake of the drug. Changes to absolute configuration at the α-carbon did not significantly influence potency, whereas the structure of the spacer group had a greater effect; in general, steric hindrance at the anthraquinone terminus or branching typically diminshed activity 10-fold for a common amino acid residue. The data generated is valuable in defining the structural requirements for dual enzyme inhibition and for the design of new topo-targeting antitumour agents that are capable of circumventing multi-drug resistance phenomena. It is recognised that many other factors, beyond the scope of the present study, influence drug cytotoxicity; this project has spawned others in which cell cycle dependence, p53 status and induction of apoptotic mechanisms are now being studied for lead compounds from this project.

PART B

Part B comprised a novel approach to the design of anticancer prodrugs to selectively deliver cytotoxic compounds to the site of a tumour.

The chemistry of spacer-linked anthraquinone-amino acid and peptide conjugates was extended to include prototype oligopeptide prodrug substrates for the tumour-associated protease matrix metalloproteinase (MMP-9), in which the *in vivo*-active lead compound, NU:UB 31, was linked to a hexapeptide motif containing an MMP-9 cleavage 'hotspot'. Incubation with human recombinant MMP-9 protein and MMP-9 over-expressing cell lines confirmed that cleavage of the peptide carrier occurred at the intended gly-leu cleavage site to release the active agent, after non-specific cleavage of residual peptide carrier fragments, providing evidence that the approach to subvert the proteolytic capacity of MMPs is a viable one to improve tumour targeting.

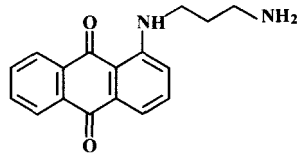
The chemistry requires further extension to optimise the peptide carrier sequence and to tailor the prodrugs to the proteolytic specificity of individual MMPs. Specifically, efforts should be concentrated on the P_1-P_1' cleavage site, exploiting the deep hydrophobic pocket in the S_1' subsite in MMPs by introduction of long straight-chain hydrophobic residues such as norvaline, norleucine etc to enhance cleavage rates and site-specific cleavage; the latter may also be achievable by selective peptide bond replacement (peptide mimics). Attachment of the peptide carrier served to inactivate the intrinsic cytotoxic potency of the active agent ('warhead') NU:UB 31; in future work a principal objective would be to investigate the generality of this outcome by substituting the active agent with a more potent drug. If effective, the technology could be applied to improve the therapeutic index of existing agents in the clinical setting.

CHAPTER EIGHT

STRUCTURE LIBRARY

[OF COMPOUNDS SYNTHESISED IN THIS STUDY]

ANTHRAQUINONE SPACER COMPOUNDS
[SYNTHESIS REPORTED IN SECTIONS 9.2.3 TO 9.2.18]



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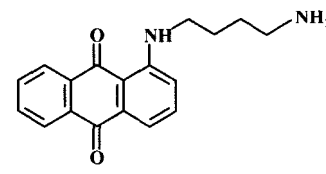
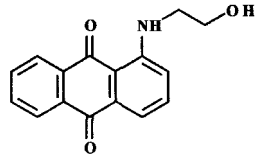


(147)

(b) .$HOOCCF_3$
(146) NU:UB 197



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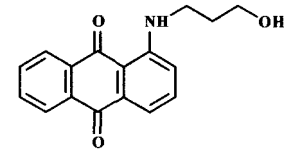

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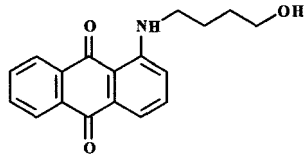

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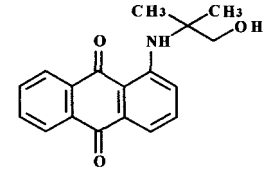

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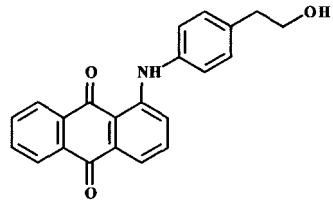

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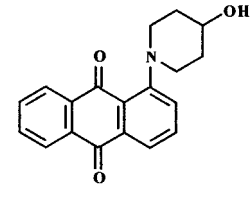

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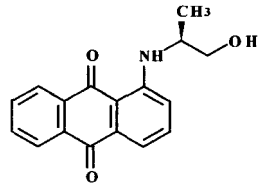

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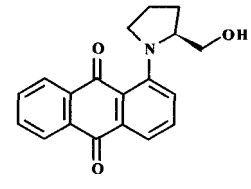

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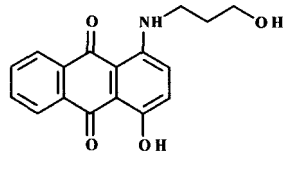

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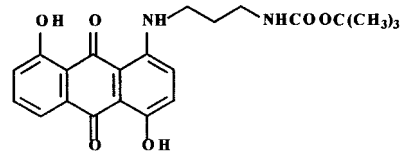

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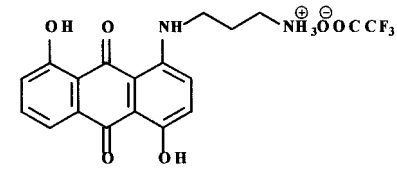

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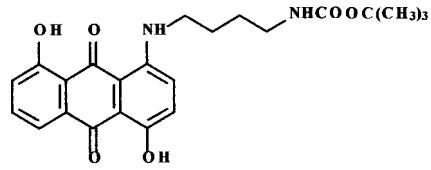

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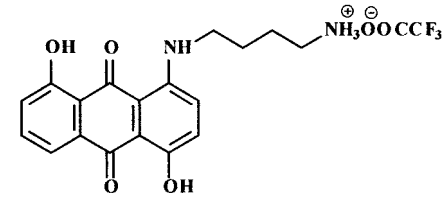

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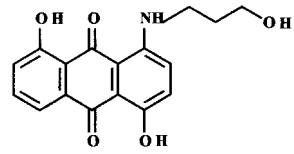

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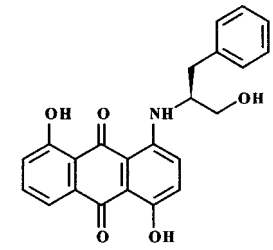

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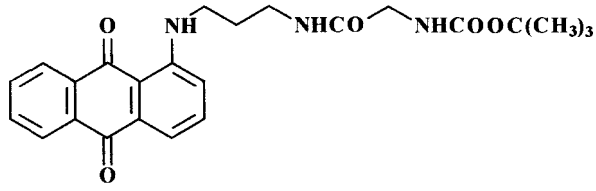
GLYCINE CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

[SYNTHESIS REPORTED IN SECTIONS 3.4.1 TO 3.4.18]



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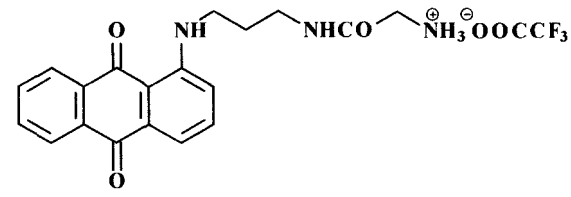

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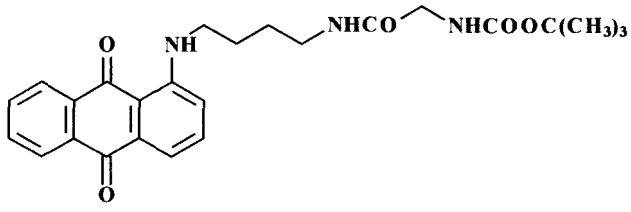

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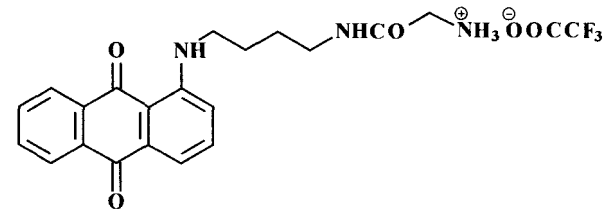

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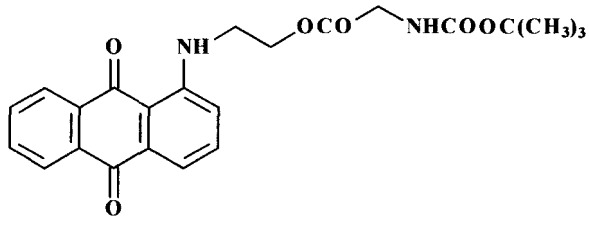

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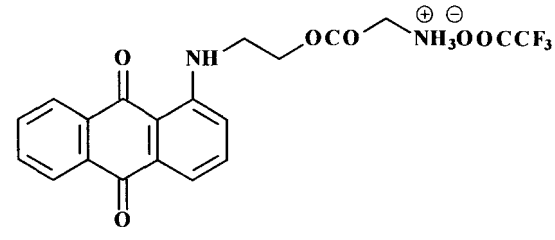

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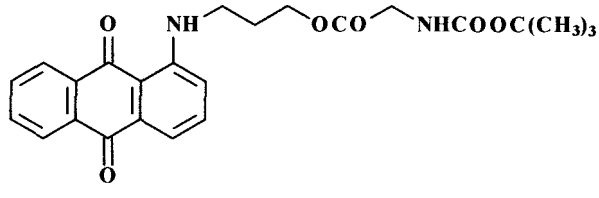

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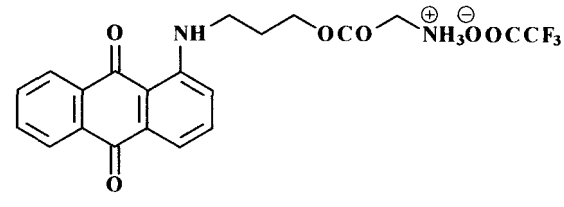

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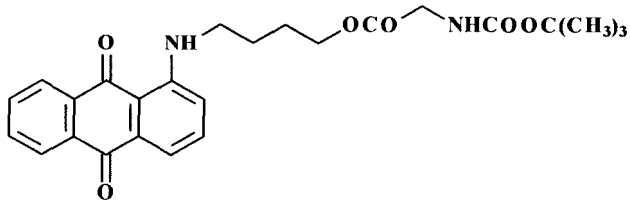

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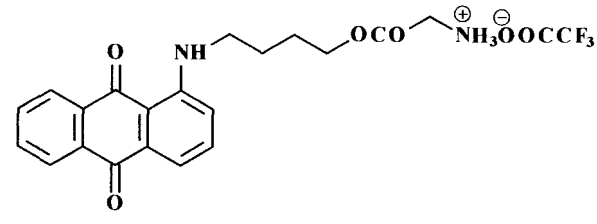

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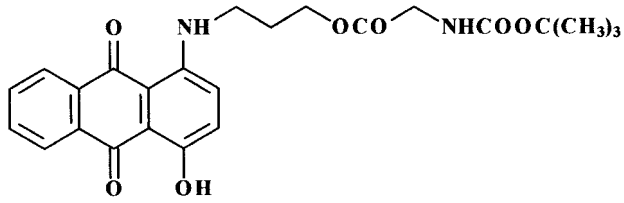

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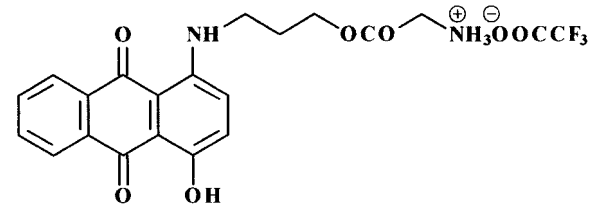

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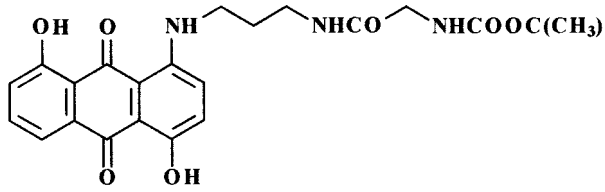

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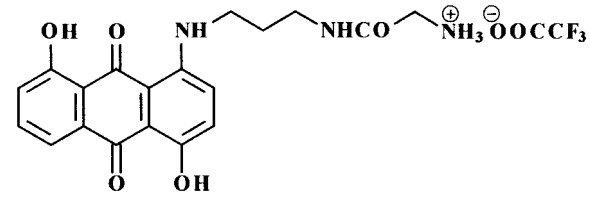

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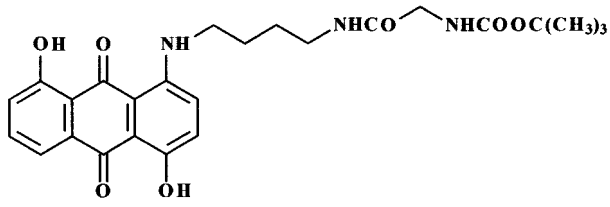

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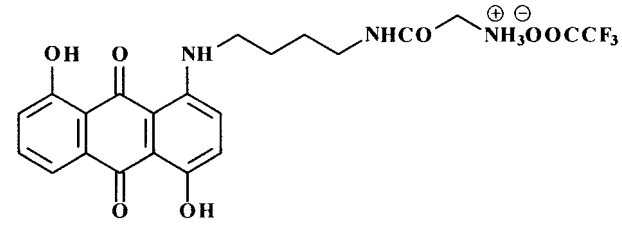

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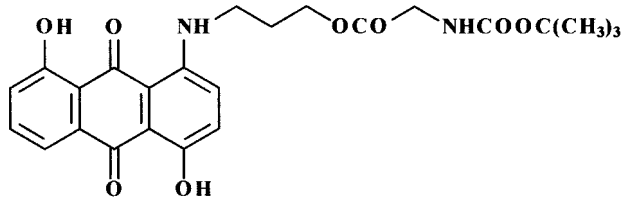

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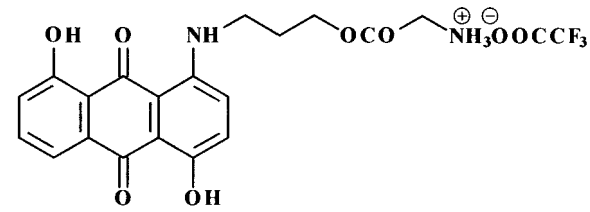

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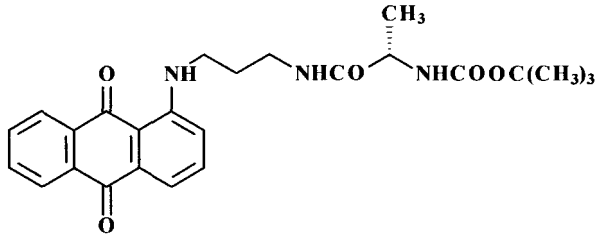
ALANINE CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

[SYNTHESIS REPORTED IN SECTIONS 3.5.1 TO 3.5.26]



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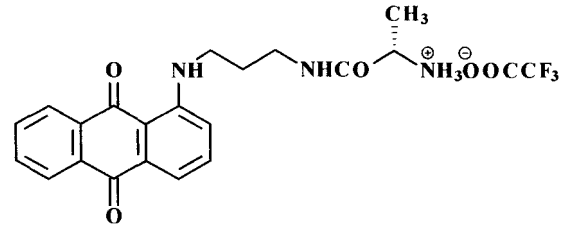

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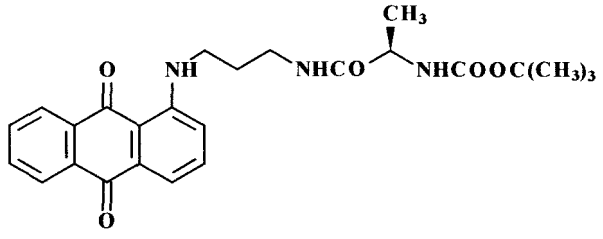

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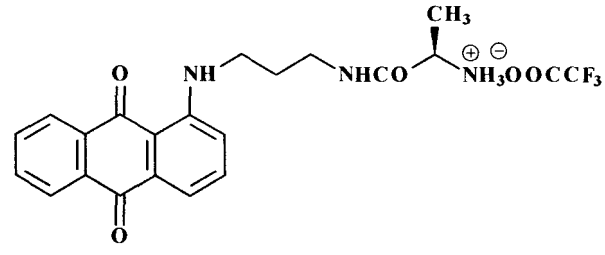

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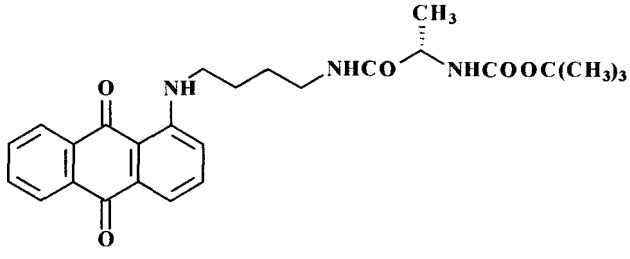

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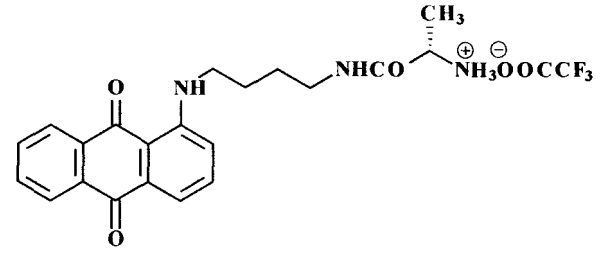

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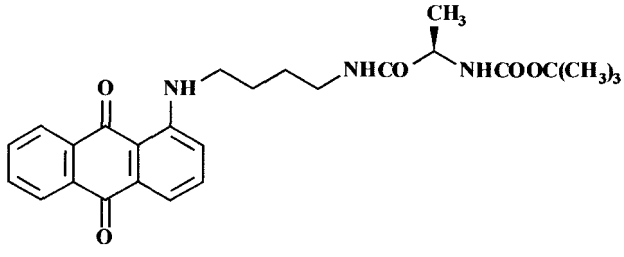

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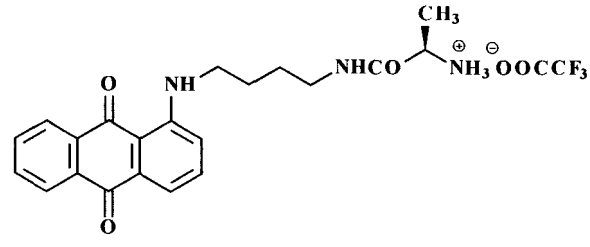

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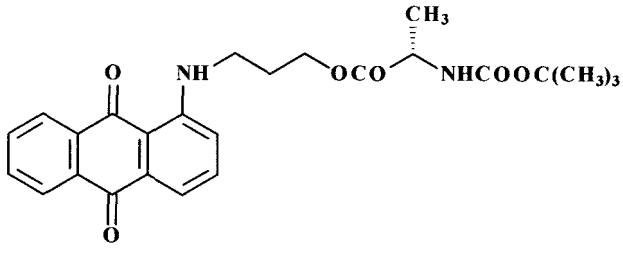

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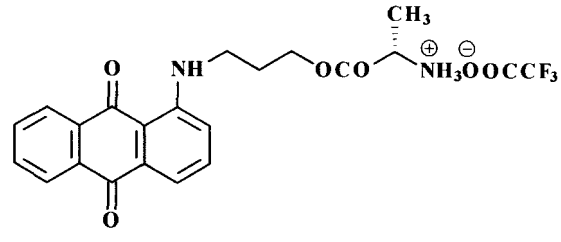

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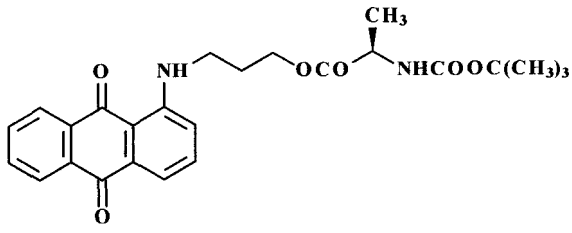

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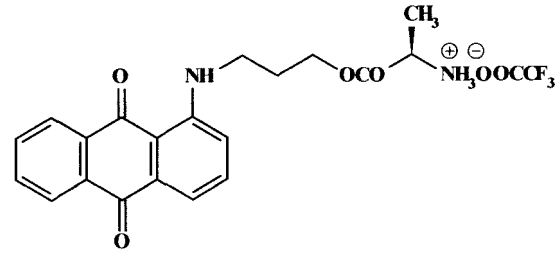

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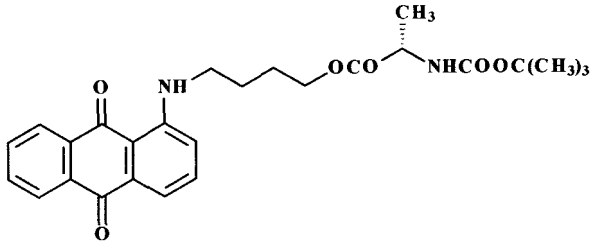

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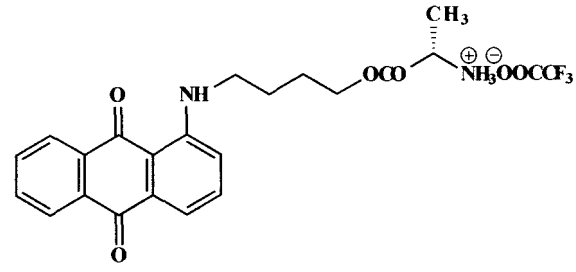

NU:UB 73
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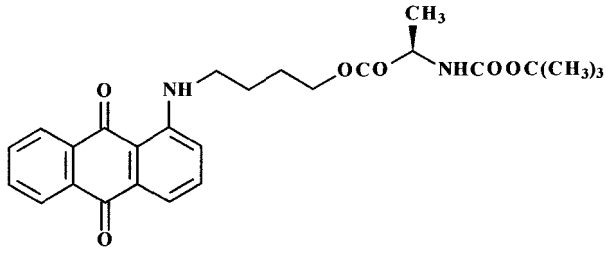

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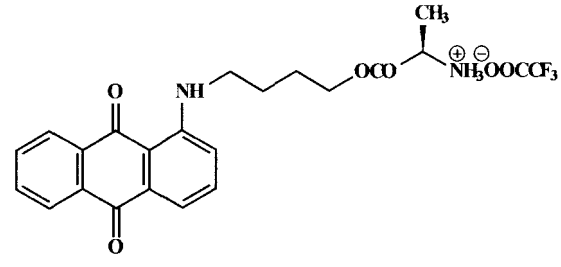

NU:UB 76
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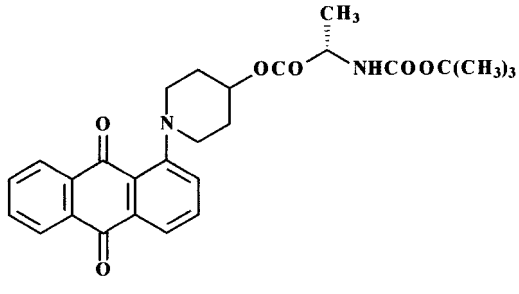

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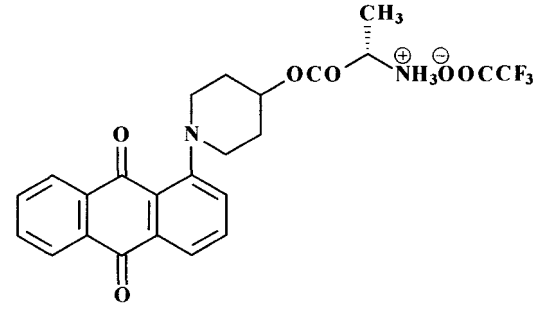

NU:UB 158
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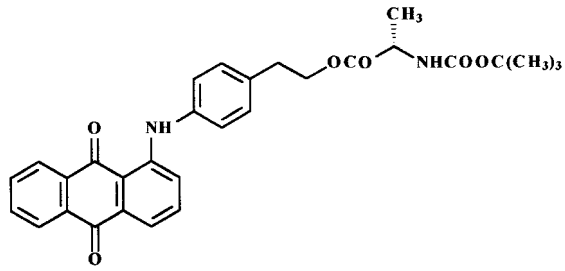

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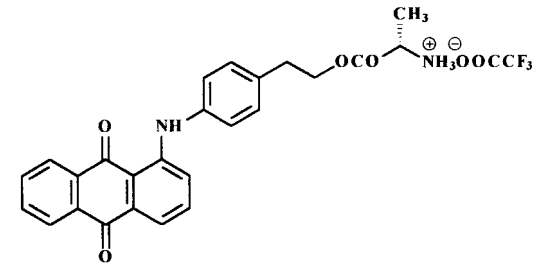

NU:UB 156
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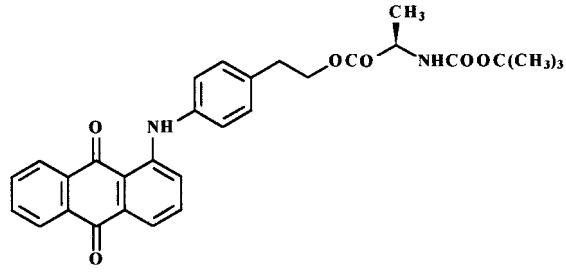

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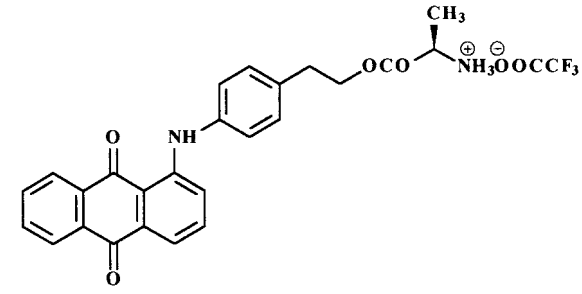
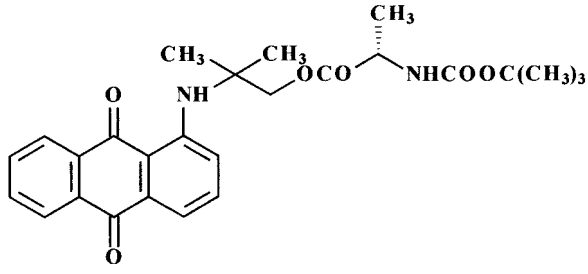


NU:UB 157
(202)



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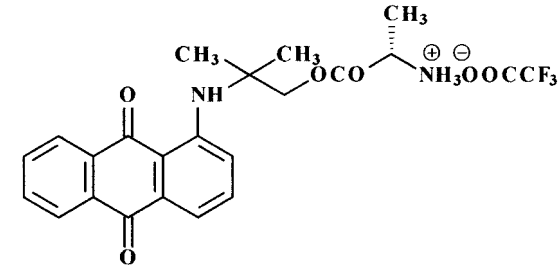

NU:UB 128
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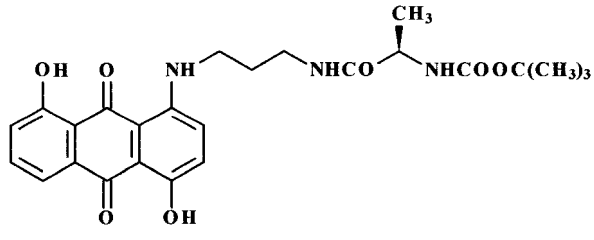

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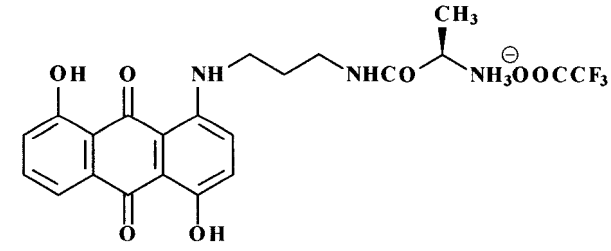
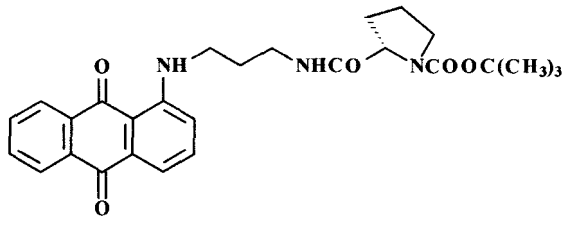


NU:UB 118
(206)

PROLINE CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

[SYNTHESIS REPORTED IN SECTIONS 3.6.1 TO 3.6.19]



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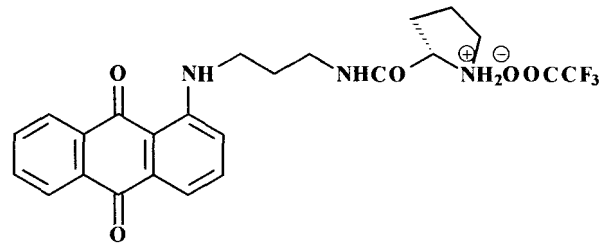

NU:UB 31
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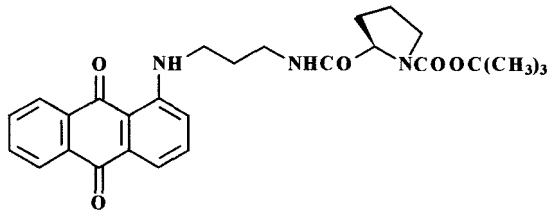

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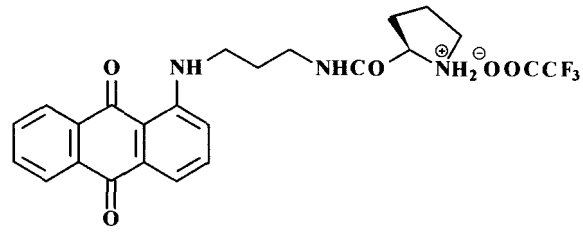

NU:UB 46
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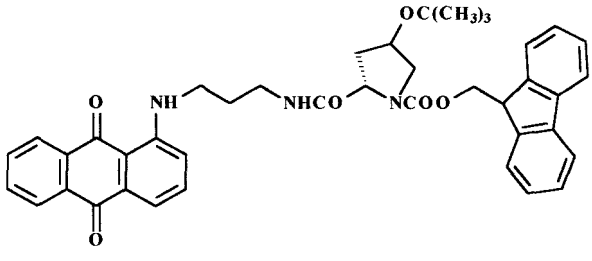

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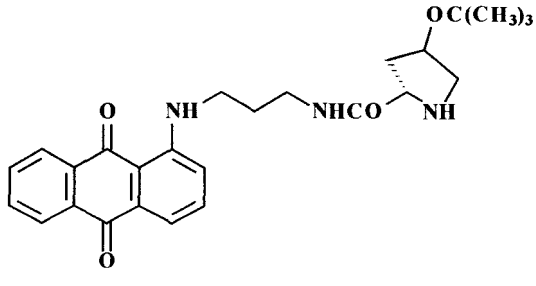

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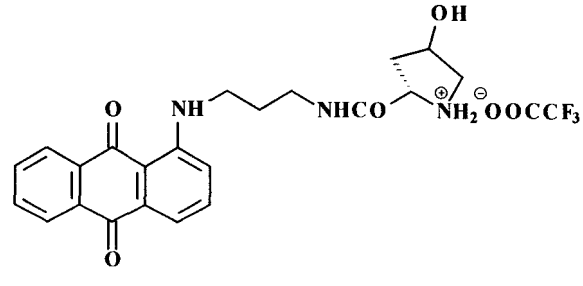

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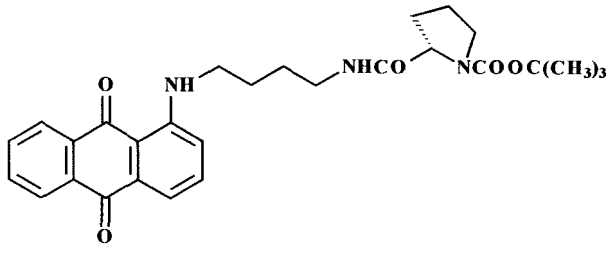

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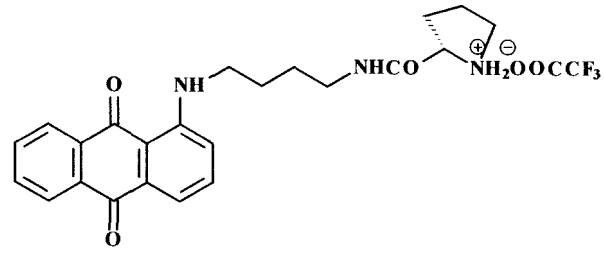

NU:UB 43
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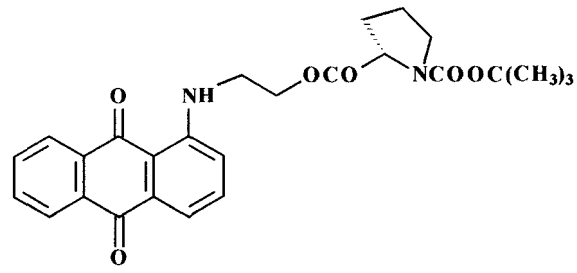

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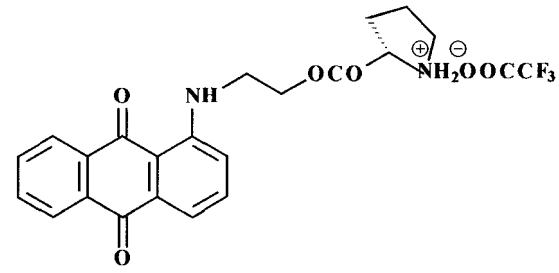

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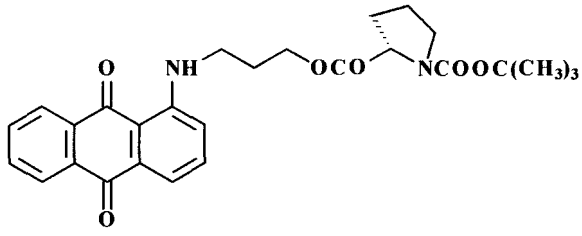

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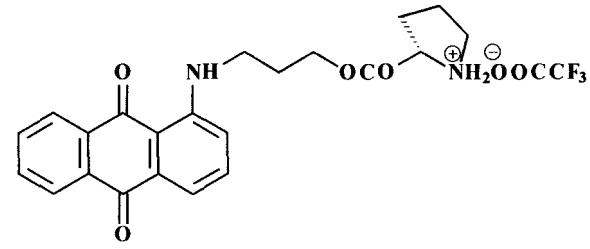

NU:UB 111
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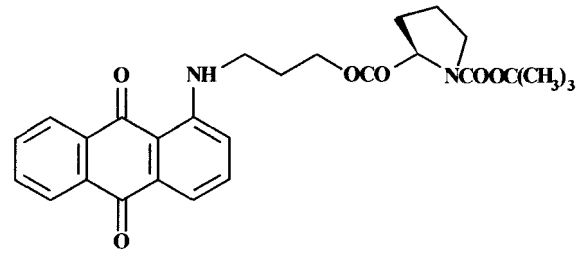

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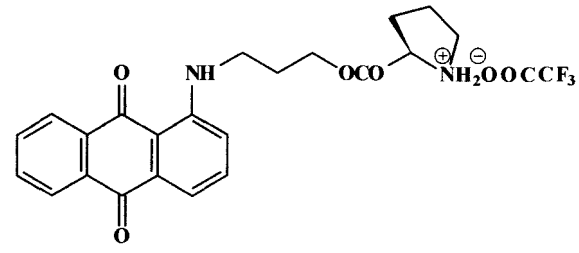

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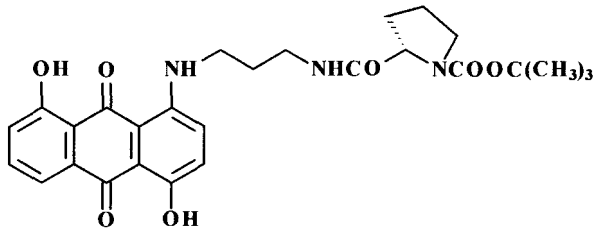

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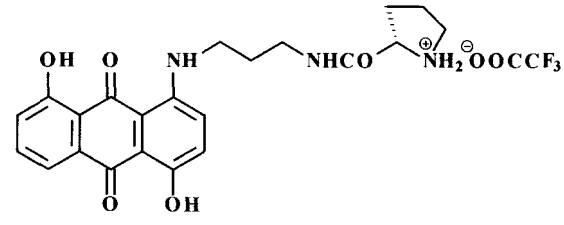

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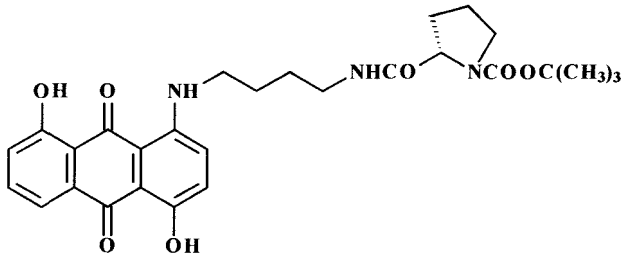

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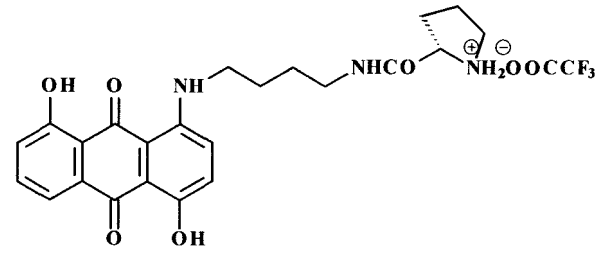
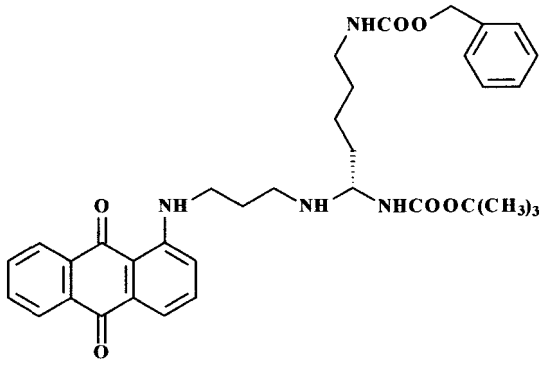


NU:UB 85
(225)

LYSINE AND ORNITHINE CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

[SYNTHESIS REPORTED IN SECTIONS 3.7.1 TO 3.7.23]



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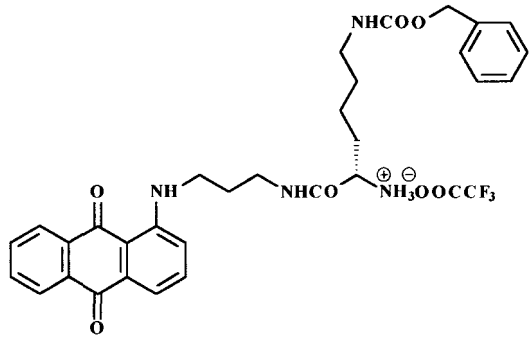

NU:UB 8
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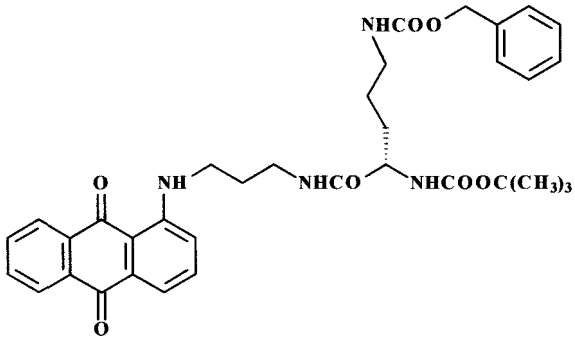

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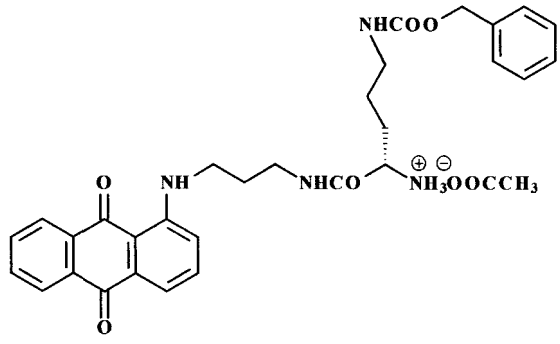
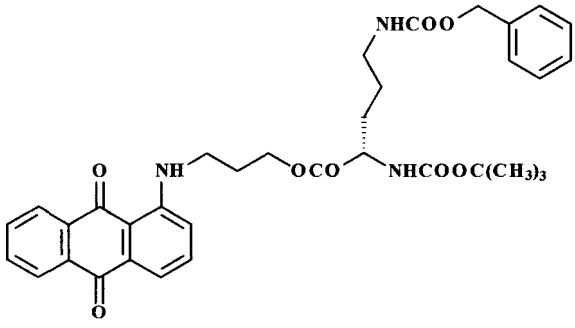


NU:UB 47
(229)



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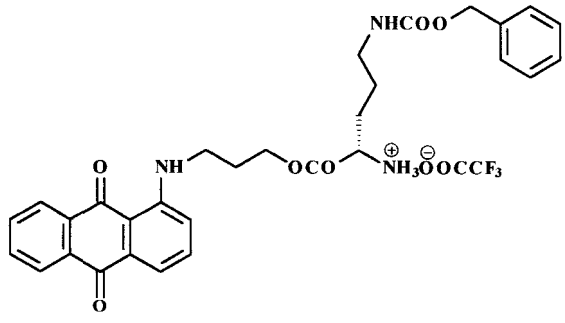

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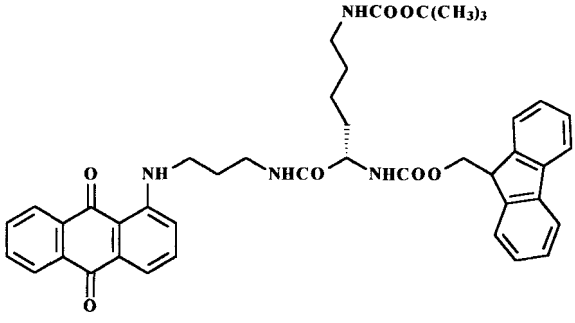

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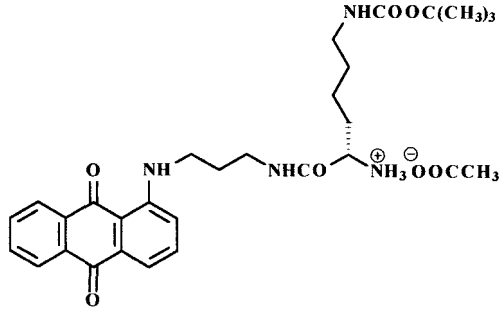

NU:UB 45
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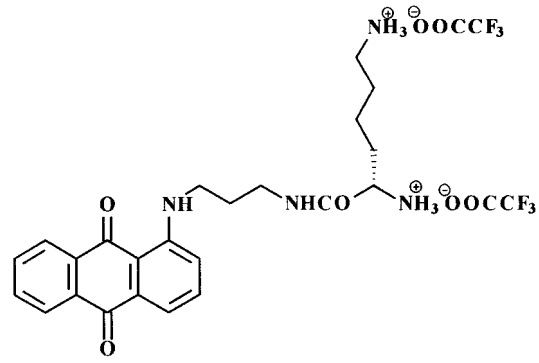

NU:UB 16
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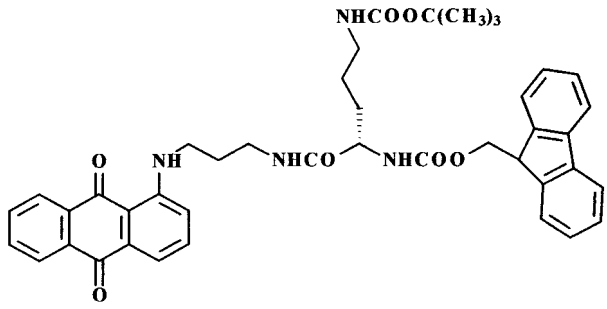

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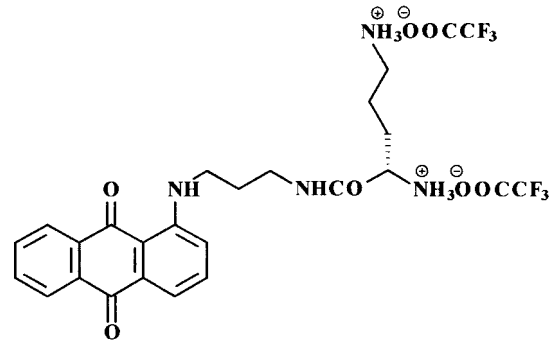
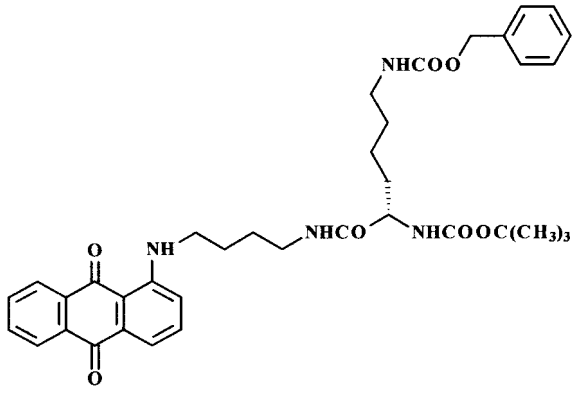
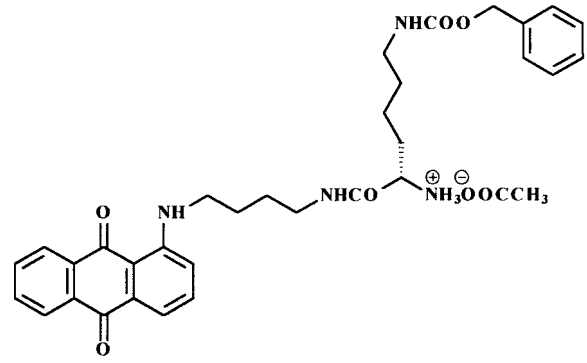


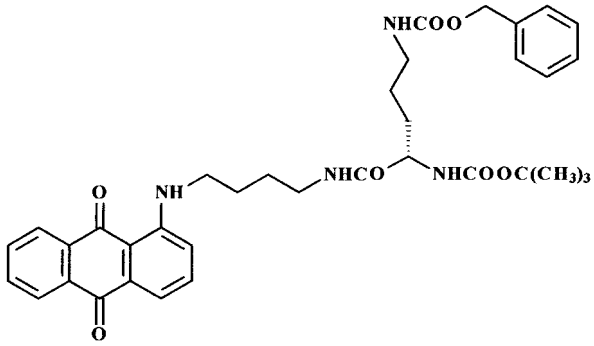
NU:UB 24
(236)



(237)



NU:UB 19
(238)



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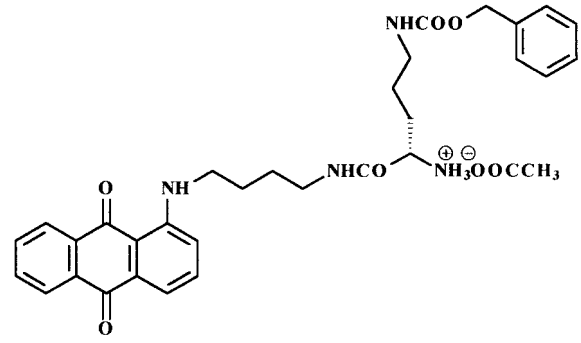
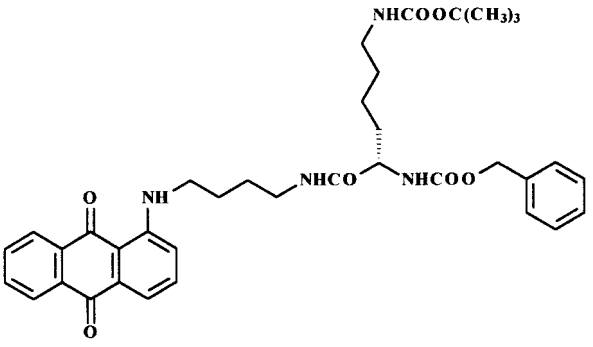


NU:UB 48
(240)



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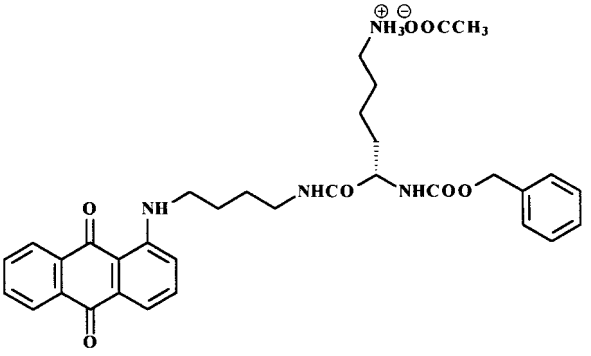
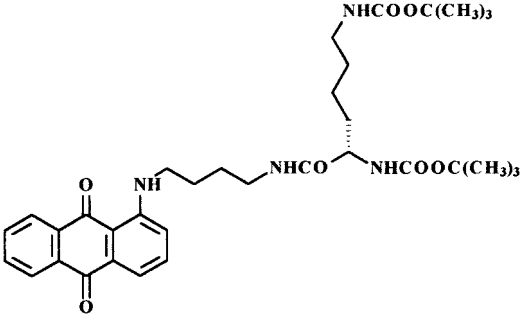


NU:UB 42
(242)



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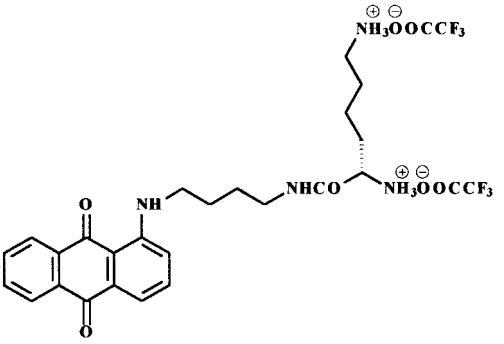
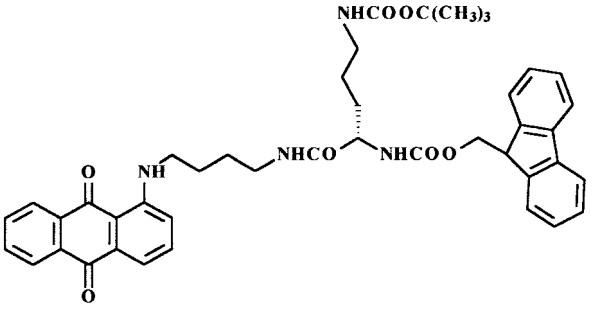
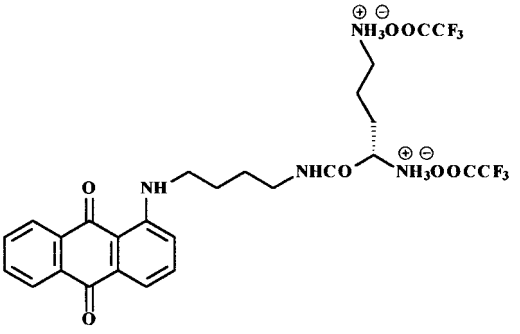


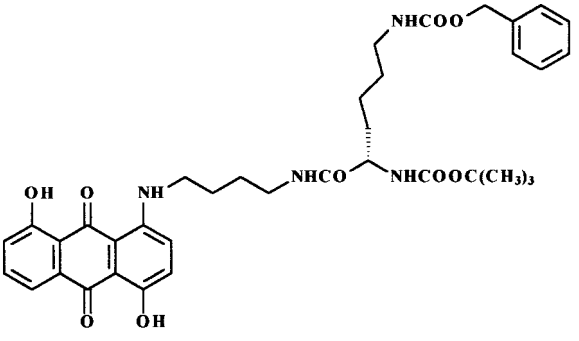
NU:UB 20
(244)



(245)



NU:UB 99
(246)



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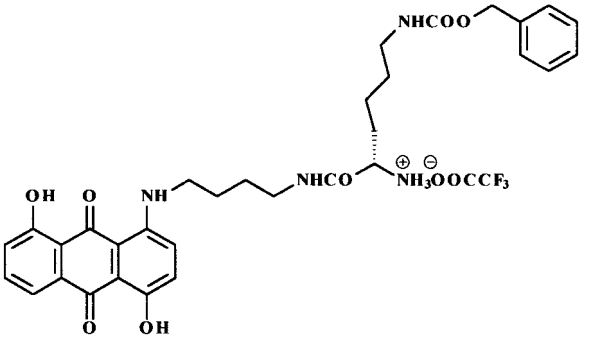
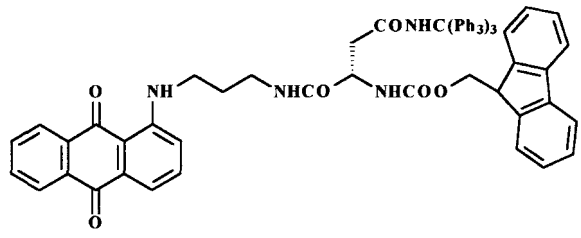
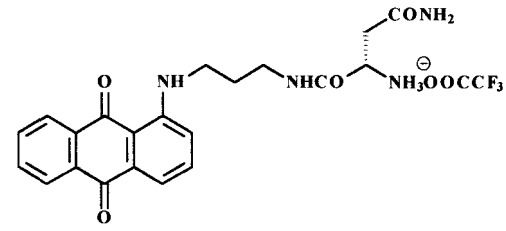


(248)

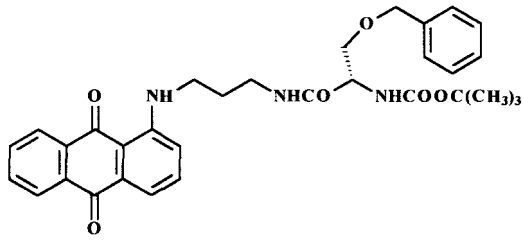
OTHER SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES
[SYNTHESIS REPORTED IN SECTIONS 3.8.1 TO 3.8.19]



(249)



NU:UB 49
(250)



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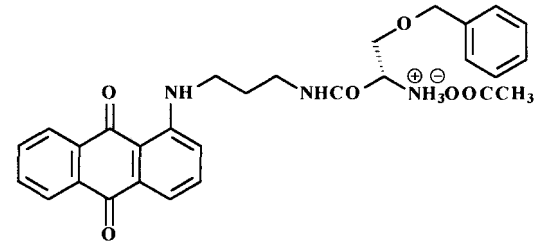
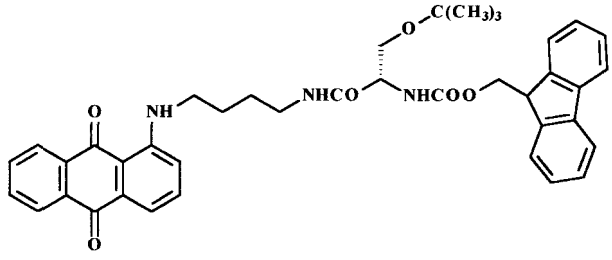


NU:UB 22
(252)



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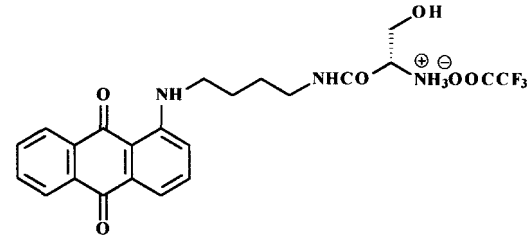
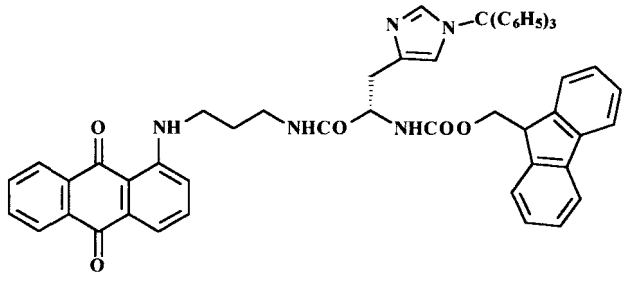
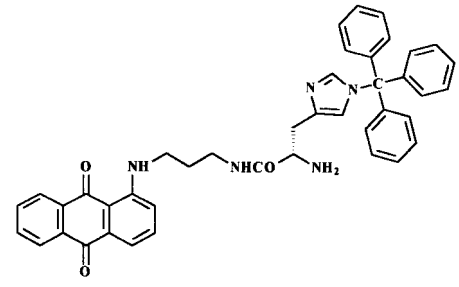


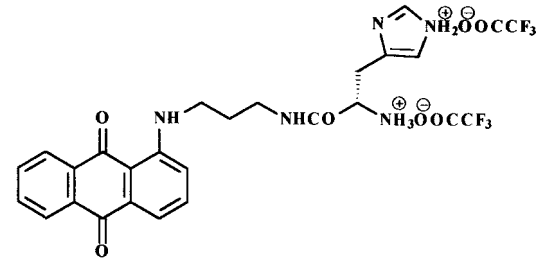
NU:UB 44
(254)



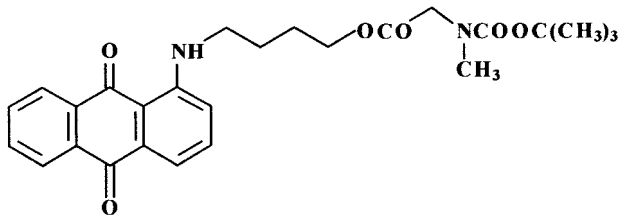
(255)



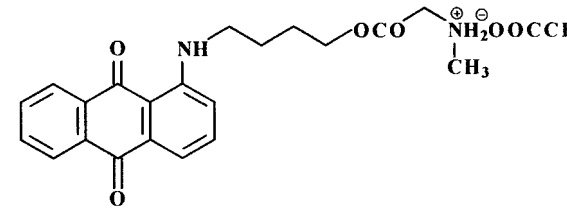
(256)



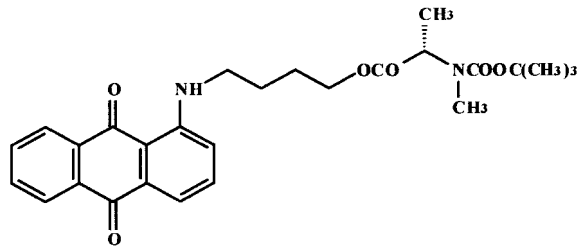
NU:UB 30
(257)



(258)



NU:UB 163
(259)



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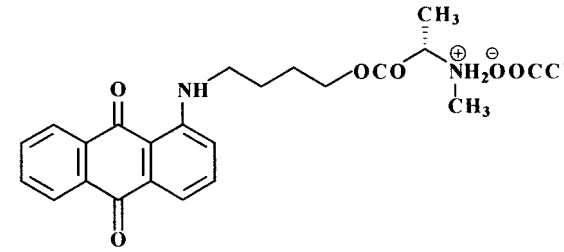
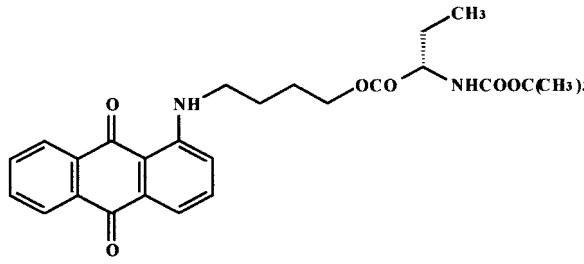
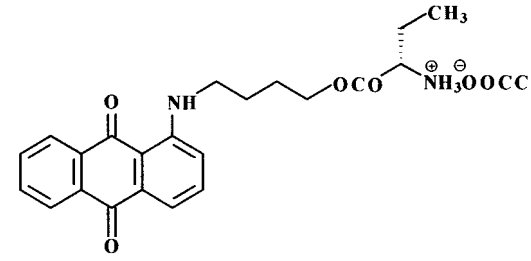


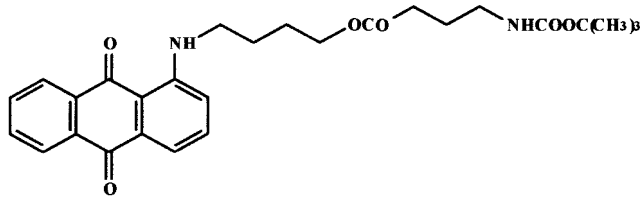
NU:UB 166
(261)



(262)



NU:UB 167
(263)



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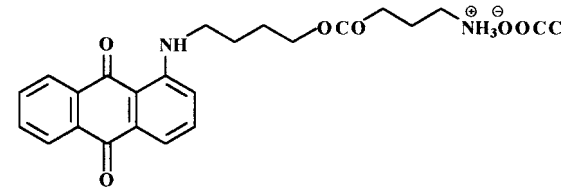
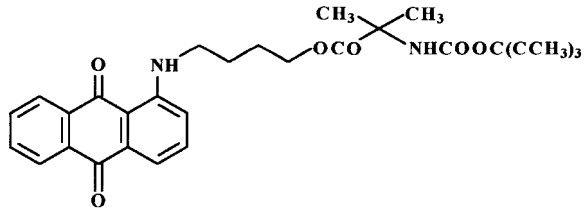
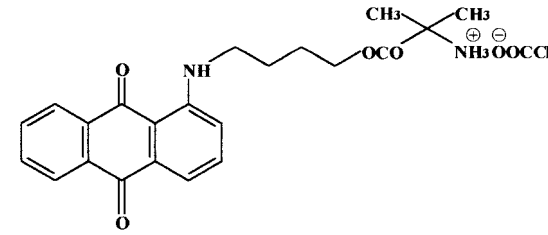


NU:UB 168
(265)



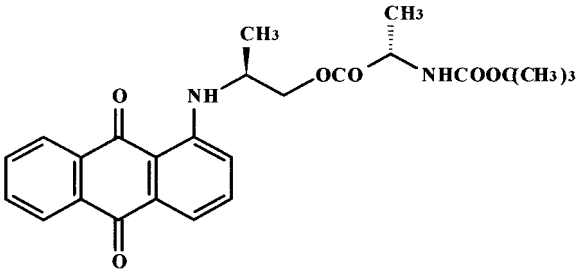
(266)



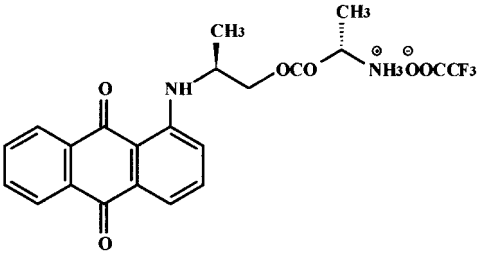
NU:UB 169
(267)

CHIRAL SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

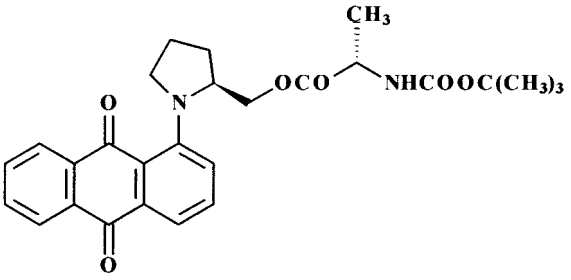
[SYNTHESIS REPORTED IN SECTIONS 3.9.1 TO 3.9.6]



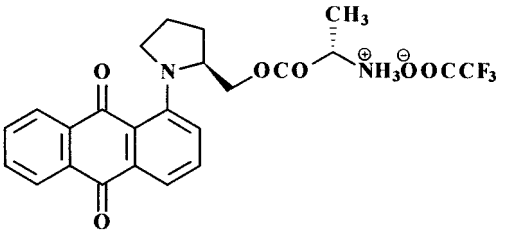
(268)



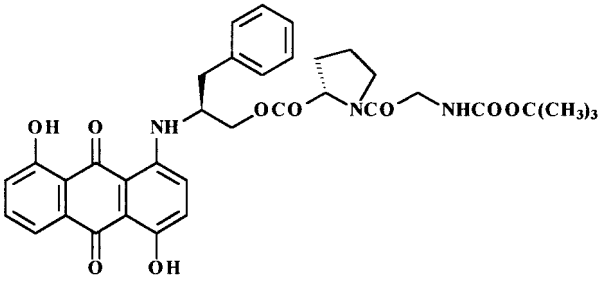
NU:UB 170
(269)



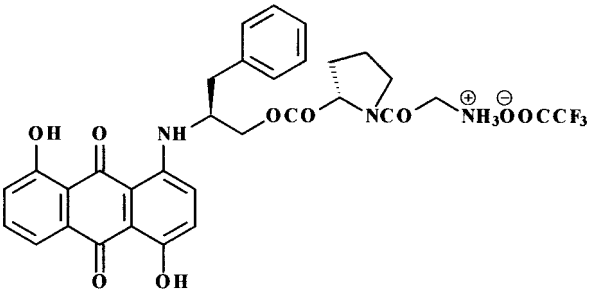
(270)



NU:UB 171
(271)

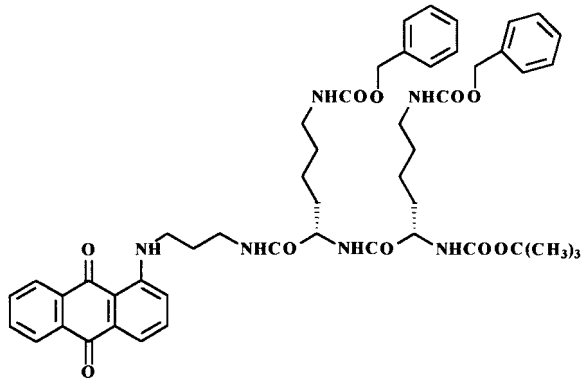


(272)

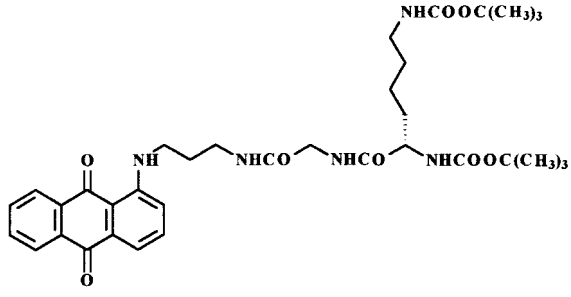


NU:UB159
(273)

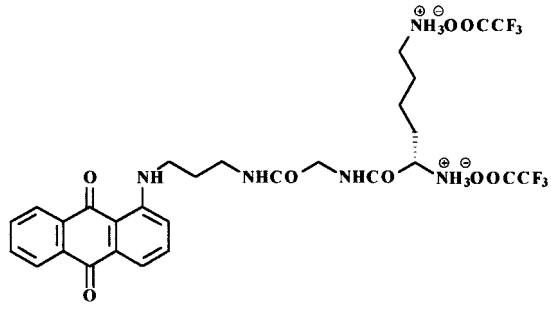
SPACER-LINKED ANTHRAQUINONE DIPEPTIDES
[SYNTHESIS REPORTED IN SECTIONS 3.10.1 TO 3.10.10]



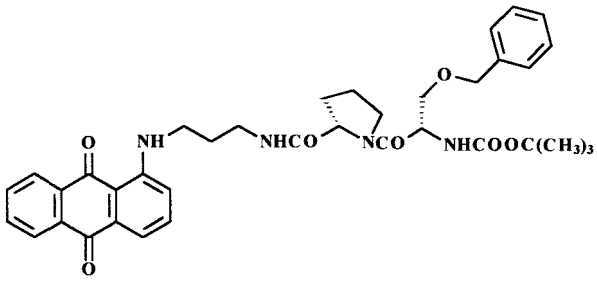
(274)



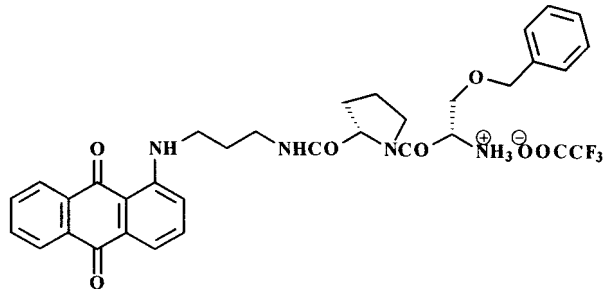
(275)



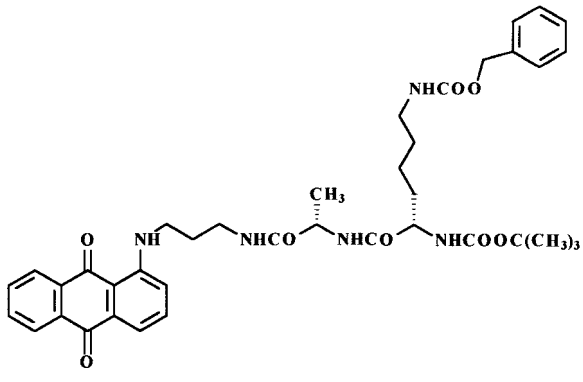
NU:UB 23
(276)



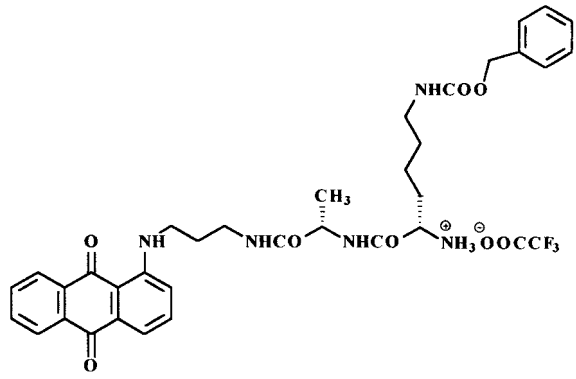
(277)



NU:UB 41
(278)



(279)



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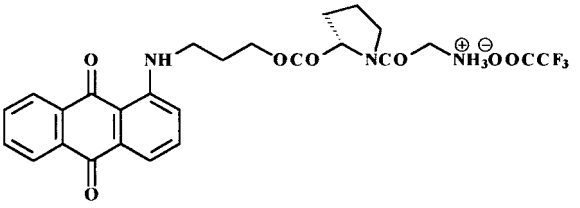
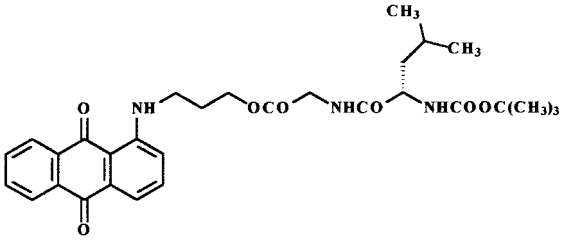


NU:UB 116
(281)



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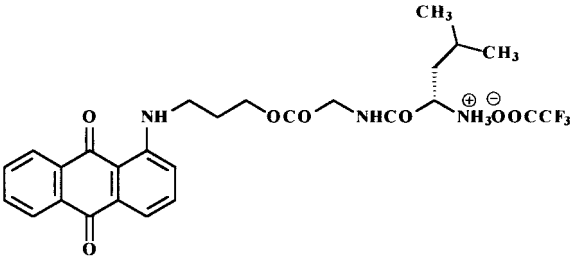
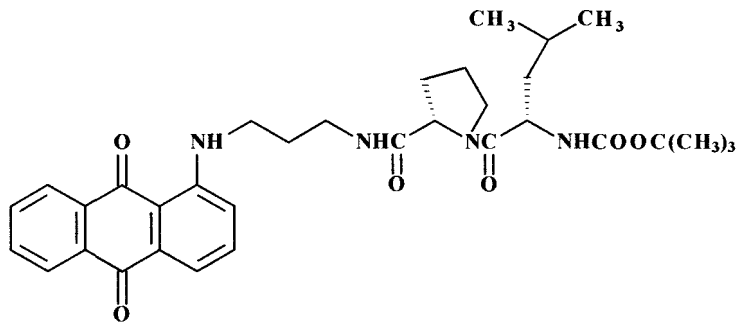
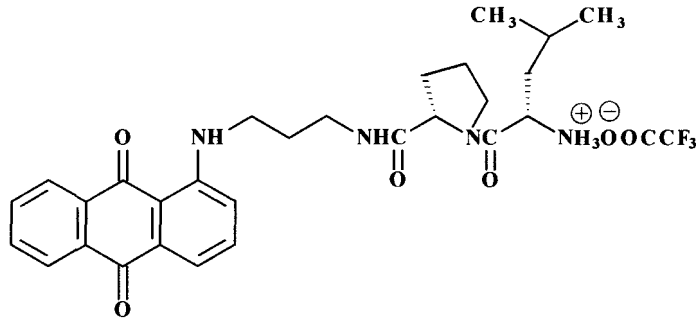


NU:UB 127
(283)

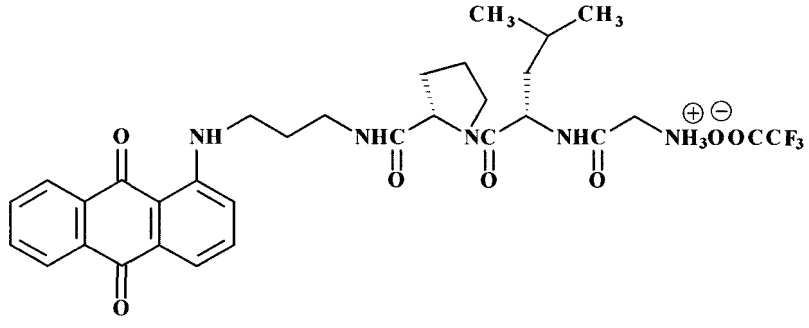
SPACER-LINKED ANTHRAQUINONE OLIGOPEPTIDES
[SYNTHESIS REPORTED IN SECTIONS 9.11.1 TO 9.11.10]



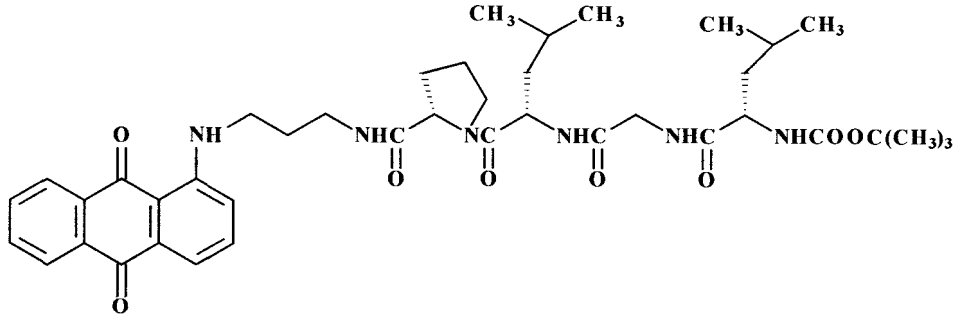
(284)



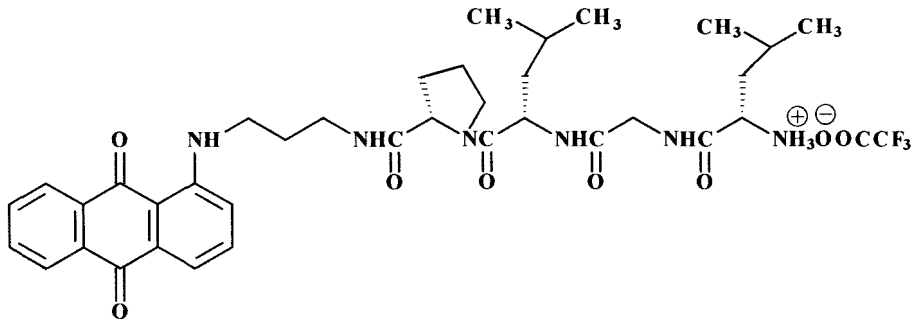
NU:UB 184
(285)



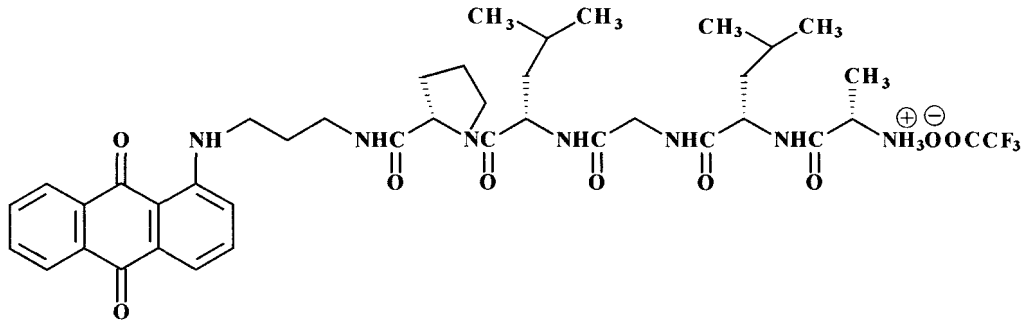
NU:UB 185
(286)



(287)



NU:UB 186
(288)



NU:UB 204
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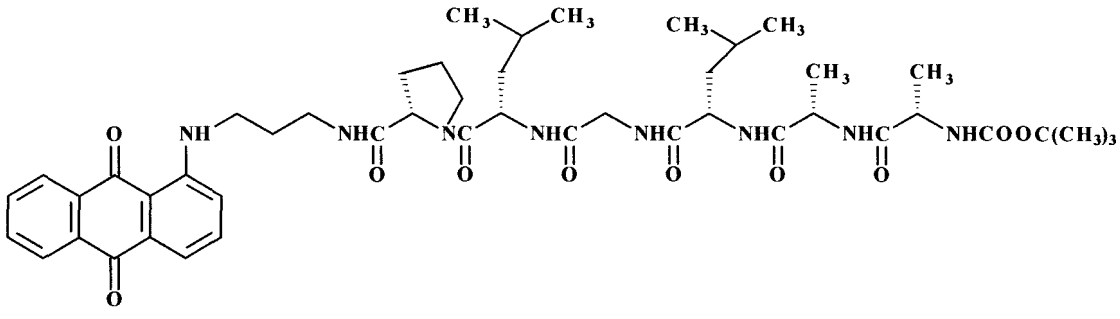

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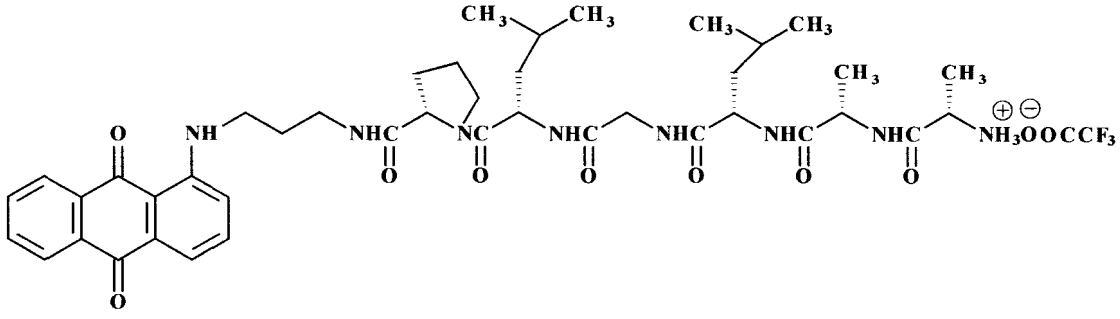
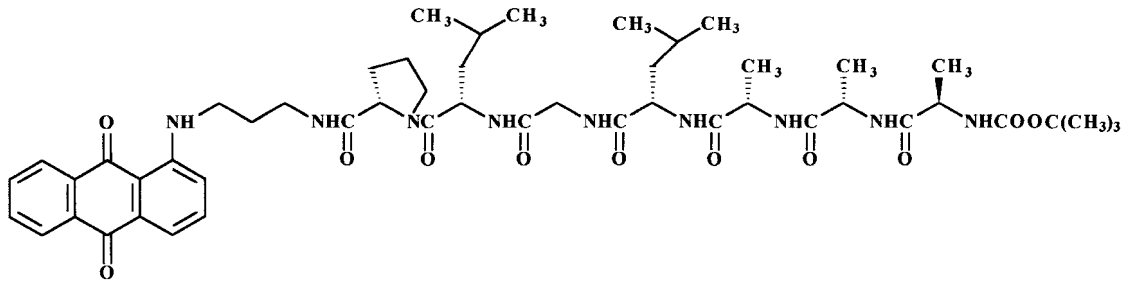
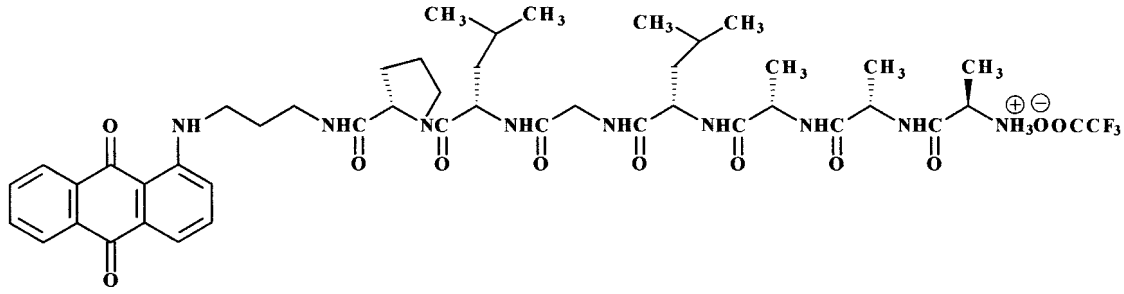


NU:UB 205
(291)



(292)



PL1
NU:UB 187
(293)

CHAPTER NINE

EXPERIMENTAL

[PART A + PART B]

9.1 Experimental techniques

Elemental analysis

An N-Carlo-Erba 1160 elemental analyser was used for microanalysis of carbon, hydrogen and nitrogen.

NMR

^{1}H nmr spectra were obtained on either a Brucker WP250 (200 MHz), a Brucker DPX FT multinuclear (400 MHz) or a Jeol JNM GMX (300 MHz) nmr spectrophotometer.

^{13}C nmr spectra were obtained on a Brucker DPX 400 nmr spectrophotometer.

NOTE: In ^{13}C nmr spectral data the +ve and –ve signals correspond to a DEPT spectrum and 'ab' corresponds to quaternary C signals which are absent in DEPT but appear in normal ^{13}C spectra.

Mass spectrometry

FAB(+) mass spectra were recorded on either a VG/MS9 or a Micromass Autospec using a FAB gun source. The sample was dissolved in 3-nitrobenzyl alcohol (NOBA) and its spectrum subtracted from the sample spectrum.

Low resolution EI, CI and Electrospray mass spectra were recorded on a Micromass Quattro II triple quadrapole instrument.

Chromatography

Thin layer chromatography was carried out on Kieselgel 60 F_{254} pre-coated aluminium plates. Most compounds synthesised absorb in the visible region, additional visualisation where required, was by short-wave U.V. light.

Flash chromatography was carried out in columns packed with silica gel, particle size 40-63μm, with a positive pressure applied via an air pump.

Solvent systems used for thin layer chromatography

1. chloroform : methanol, 9:1
2. toluene : ethyl acetate, 4:1
3. butanol : glacial acetic acid : water, 4:5:1

9.2 SYNTHESIS OF ANTHRAQUINONE SPACER COMPOUNDS

9.2.1 Method A

General method for the preparation of 'anthraquinone-spacer arm' compounds.

Applicable to hydroxyalkylamino- or aminoalkylamino-anthraquinones.

1-Chloroanthraquinone (40 mmol) was suspended in DMSO (15 cm^3); an α,ω-diaminoalkane or ω-aminoalkanol (200 mmol) was added and the mixture was heated for 30 min over a boiling water bath (or heated at reflux as appropriate). The solution was cooled and added to a large excess of water (500 cm^3). The red precipitated solid was filtered off, dried and used for subsequent reactions without further purification. Analytically pure samples were prepared by column chromatography [chloroform : methanol (9:1)] or recrystallisation from ethanol.

9.2.2 Method B

General method for the preparation of '4,8-dihydroxylated-anthraquinone-spacer arm' compounds.

Applicable to hydroxyalkylamino-anthraquinones [Method B(i)] or aminoalkylamino-anthraquinones [2 stages Method B(i) and (ii)].

(i) Leuco-1,4,5-trihydroxyanthraquinone (3.90 mmol) was suspended in dichloromethane (200 cm^3). An N-^{t}Boc–α,ω-diaminoalkane or an ω-aminoalkanol (3.90 mmol) was added and the mixture was stirred at room temperature for 6h followed by the addition of triethylamine (2 cm^3) and aeration for 2h. The solution was concentrated to half volume (rotary evaporator) before applying to a silica gel chromatography column (4×40 cm) prepared with dichloromethane. The column

was eluted firstly with dichloromethane to remove high running impurities before the addition of ethyl acetate to give the spacer compound as a purple solid after recrystallisation from ethanol.

The foregoing procedure afforded hydroxyalkylamino-anthraquinones directly; isolation of aminoalkylamino-anthraquinones required the following deprotection step.

(ii) N-^{t}Boc-aminoalkylamino-anthraquinone was dissolved in trifluoroacetic acid (7 cm^3) at room temperature. After 0.5h the solvent was evaporated and the residue was re-evaporated from ethanol (3×10 cm^3) before dissolving in a minimum volume of ethanol (3 cm^3). Addition of ether (100 cm^3) gave a precipitate of the deprotected anthraquinone-spacer compound as the trifluoroacetate salt which was filtered off and dried.

9.2.3 (a) 1-[(3-Aminopropyl)amino]anthraquinone (145)

Compound (**145**) was prepared by the reaction of 1-chloroanthraquinone (10g, 36 mmol) with 1,3-diaminopropane (50 cm^3, 590 mmol) [following method A]. T.l.c.(solvent system 1): R_f 0.00 (red) product, 0.90 (red), 0.95 (yellow) 1-chloroanthraquinone. T.l.c.(solvent system 3): R_f 0.40 (red) product. Yield (9.5g)(82%).

Found: mp 106-108 °C.

(b) 1-[(3-Aminopropyl)amino]anthraquinone trifluoroacetate (146)(NU:UB 210)

Compound (**145**) was purified by column chromatography, eluting with butanol : glacial acetic acid : water (4:5:1). 1-[(3-aminopropyl)amino]anthraquinone acetate was

partitioned between chloroform and water and neutralised with triethylamine to give 1-[(3-aminopropyl)amino]anthraquinone. The chloroform extract was dried ($NaSO_4$), filtered, evaporated to dryness and dissolved in a small volume of trifluoroacetic acid (2 cm^3). Addition of ether (100 cm^3) afforded the title compound as a red solid. T.l.c.(solvent system 3): R_f 0.35 (red) product.

Found: mp 196 °C.

ESMS(+)(Cone 20V) m/z: 281 (100%)$(RNH_3)^+$, 263 (20%), 102 (15%).

9.2.4 1-[(4-Aminobutyl)amino]anthraquinone (147)

1,4-Diaminobutane (30g, 340 mmol) was reacted with 1-chloroanthraquinone (10g, 41 mmol) to give the title compound (**147**) [following method A]. T.l.c.(solvent system 1): R_f 0.00 (red) product, 0.90 (red), 0.95 (yellow) 1-chloroanthraquinone. T.l.c.(solvent system 3): R_f 0.65 (red) product. Yield (10.2g)(84%).

Found: mp 108-118 °C.

FABMS(+) m/z: 295 (30%)$(MH)^+$, 277 (18%), 225 (37%), 89 (40%), 72 (100%). M, 294.

9.2.5 1-[(2-Hydroxyethyl)amino]anthraquinone (148)

Compound (**148**) was prepared using ethanolamine (70 cm^3, 1130 mmol) and 1-chloroanthraquinone (8.0g, 33 mmol) [following method A]. T.l.c.(solvent system 1); R_f 0.20 (red) product. Yield (6.94g)(79%).

Found: mp 124-130 °C.

EIMS m/z: 267 (5%)$(M)^+$, 249 (5%)$[M - (H_2O)]^+$, 236 (100%). M, 267.

9.2.6 1-[(3-Hydroxypropyl)amino]anthraquinone (149)

Compound (**149**) was prepared by the reaction of 3-amino-1-propanol (40 cm^3, 530 mmol) with 1-chloroanthraquinone (7.5g, 31 mmol) [following method A]. T.l.c. [chloroform : methanol (2:1)]: R_f 0.75 (red) product, 0.95 (yellow) 1-chloroanthraquinone.Yield (6.9g)(79%).

Found: mp 166 °C.

9.2.7 1-[(4-Hydroxybutyl)amino]anthraquinone (150)

Compound (**150**) was prepared by the reaction of 4-amino-1-butanol (5.0g, 56 mmol) with 1-chloroanthraquinone (3.0g, 12 mmol) [following method A]. T.l.c.(solvent system 1): R_f 0.10 (red) product, 0.95 (yellow) 1-chloroanthraquinone. The crude product was partially purified by dissolving in chloroform (200 cm^3) and passing through a silical gel pad to remove high running impurities. Yield (2.7g)(73%).

Found: mp 114-124 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.65-1.95 (5H, m, unresolved, CH_2-CH_2-CH_2-CH_2-OH); 3.35 (2H, t, Aq-NH-CH_2); 3.75 (2H, t, CH_2-OH, J_{HCCH} 6Hz); 7.05 (1H, dd, H-2, $J_{2,3}$ 8Hz, $J_{2,4}$ 1.5 Hz); 7.45-7.55 (2H, m, H-3 and H-4); 7.60-7.75 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.60-9.90 (1H, br. s, Aq-NH).

EIMS m/z: 295 (12%)$(M)^+$, 277 (5%)$[M-(H_2O)]^+$, 236 (100%), 165 (20%). M, 295.

9.2.8 1-[(2-Hydroxytertiarybutyl)amino]anthraquinone (151)

2-Amino-2-methyl-1-propanol (50 g, 560 mmol) and 1-chloroanthraquinone (10 g, 36 mmol) were heated under relux for 0.5h to give the title compound [following method A]. T.l.c.(solvent system 1): R_f 0.30 (red) product. Yield (8.5 g)(80%).

Found: mp 106-110 °C.

Accurate mass measurement EI peak match [M+H] (reference compound: perfluorotributylamine): Calculated mass m/z: 295.1215. Measured mass m/z: 295.1208.

9.2.9 1-{[4-(2-Hydroxyethyl)phenyl]amino}anthraquinone (152)

2-(4-Aminophenyl)ethanol (10g, 73 mmol) and 1-chloroanthraquinone (2.0g, 8.5 mmol) were heated under reflux for 0.5h to give the title compound (**152**) [following method A]. T.l.c.(solvent system 2): R_f 0.10 (red) product. Yield (2.3g)(85%).

Found: mp 148 °C.

CIMS(+) m/z: 344 (8%)$(MH)^+$, 330 (8%), 72 (100%). M, 343.

9.2.10 1-(4-Hydroxypiperidyl)anthraquinone (153)

Compound (**153**) was prepared using 4-hydroxypiperidine (5.0g, 50 mmol) and 1-chloroanthraquinone (2.0g, 8 mmol) [following method A]. T.l.c.(solvent system 1): R_f 0.25 (red) product, 0.95 (yellow) 1-chloroanthraquinone. Yield (2.0g)(80%).

Found: mp 130-134 °C.

CIMS(+) m/z: 308 (8%)$(MH)^+$, 260 (100%). M, 307.

9.2.11 (1S)-1-[(2-Hydroxyisopropyl)amino]anthraquinone (154)

Compound (**154**) was prepared using (S)-2-amino-1-propanol (5.0 cm^3, 67 mmol) and 1-chloroanthraquinone (3.0g, 12 mmol). [following method A]. The crude product was purified by column chromatography eluting with chloroform : methanol (20:1). Yield (0.3g)(10%). T.l.c.(solvent system 1): R_f 0.55 (red) product.

Found: mp 202 °C.

FABMS(+) m/z: 304 (10%)$(M+Na)^+$, 282 (100%)$(MH)^+$. M, 281.

9.2.12 (2S)-1-[2-(Hydroxymethyl)pyrrolidinyl]anthraquinone (155)

Compound (**155**) was prepared using L-prolinol (2.0g, 19 mmol), 1-chloroanthraquinone (1.0g, 4.0 mmol) and pyridine (1 cm^3) [following method A]. T.l.c.(solvent system 2): R_f 0.18 (red) product, 0.95 (yellow) 1-chloroanthraquinone.Yield (1.0g, 78%).

Found: mp 134 °C.

FABMS(+) m/z: 330 (15%)$(M+Na)^+$, 308 (100%)$(MH)^+$, 276 (40%). M, 307.

9.2.13 4-Hydroxy-1-[(3-hydroxypropyl)amino]anthraquinone (156)

1,4-Dihydroxyanthraquinone (2g, 8 mmol) was suspended in ethanol (50 cm^3) and THF (50 cm^3) containing 3-amino-1-propanol (15g, 200 mmol) and heated over a water bath (at 95°C) for 1.75h. T.l.c. of the crude product (solvent system 2): R_f 0.00 (brown), 0.15 (blue) 1,4-bis substituted compound, 0.40 (purple) product, 0.90 (orange) 1,4-dihydroxyanthraquinone. The solution was cooled and immediately applied to a silica gel chromatography column using toluene : ethyl acetate (4:1) as the eluting solvent to give compound (**156**) as a purple solid after recrystallisation of the residue from the major fraction from ethanol. Yield (0.51g)(21%).

Found: mp 152 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.50 (2H, qn, CH_2-CH_2-CH_2); 3.45 (2H, q, Aq-NH-CH_2); 3.55 (2H, q, CH_2OH); 4.70 (1H, t, OH); 7.30 (1H, d, H-2); 7.45 (1H, d, H-3); 7.80-7.90 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 10.70 (1H, t, Aq-NH); 13.65 (1H, s, 4-OH).

FABMS(+) m/z: 320 (10%)$(M+Na)^+$, 298 (100%)$(MH)^+$, 252 (20%). M, 297.

9.2.14 Attempted synthesis of 4,8-dihydroxy-1-[(3-aminopropyl)amino]-anthraquinone

Leuco-1,4,5-trihydroxyanthraquinone (1.0g, 3.9 mmol) and 1,3-diaminopropane (0.29g, 3.9 mmol) were suspended in dichloromethane (250 cm^3) and stirred at room temperature for 6h followed by the addition of triethylamine (2 cm^3) and aeration for 2h. During the reaction time the mixture turned from orange to very dark purple and contained a large amount of preciptated dark green sticky solid. T.l.c. examination (solvent system 3) showed the presence of the (presumed) target compound [R_f 0.30 (purple)] along with 1,4,5-trihydroxyanthraquinone [R_f 0.95 (orange)] and a large amount of dark green baseline material. This was thought to be the unoxidised intermediate which, due to poor solubility, was being precipitated from solution before oxidation could take place. The reaction was abandoned at this point.

9.2.15 4,8-Dihydroxy-1-[(3-aminopropyl)amino]anthraquinone trifluoroacetate (158) /(NU:UB 59)

(i) 4,8-Dihydroxy-1-[3-(N-tertiarybutoxycarbonylamino)propylamino]anthraquinone /(157)

Compound (**157**) was prepared using N-^{t}Boc-1,3-diaminopropane (1.0g, 5.7 mmol) and leuco-1,4,5-trihydroxyanthraquinone (1.45g, 5.60 mmol) [following method B(i)]. T.l.c. of the crude product (solvent system 1): R_f 0.00 (brown), 0.15 (pink), 0.50 (purple) product, 0.75 (pink), 0.90 (yellow) 1,4,5-trihydroxyanthraquinone. Yield (0.73g)(46%).

Found: mp 173 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40 (9H, s, ^{t}Boc); 1.75 (2H, qn, CH_2-CH_2-CH_2); 3.10 (2H, q, CH_2NHCO); 3.45 (2H, q, Aq-NH-CH_2); 6.95 (1H, t, NHCO); 7.30-7.40 (2H, m, H-2 and H-3); 7.55 (1H, d, H-7); 7.65-7.75 (2H, m, H-5 and H-6); 10.00 (1H, t, Aq-NH); 13.40 (1H, s, 4-OH); 13.95 (1H, s, 8-OH).

FABMS(+) m/z: 413 (2%)$(MH)^+$, 149 (95%), 57 (100%). M, 412.

(ii) 4,8-Dihydroxy-1-[(3-aminopropyl)amino]anthraquinone trifluoroacetate (158) /(NU:UB 59)

The ^{t}Boc protected derivative (**157**)(0.63g) was deprotected using trifluoroacetic acid [following method B(ii)]. The product was recrystallised from an ethyl acetate/ethanol (4:1) solution. T.l.c.(solvent system 3): R_f 0.65 (purple) product. Yield (0.44g)(68%).

Found: mp 128 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.90 (2H, qn, CH_2-CH_2-CH_2); 2.95 (2H, t, $CH_2NH_3^+$, J_{HCCH} 8Hz); 3.50 (2H, q, Aq-NH-CH_2); 7.20-7.35 (2H, m, H-2 and H-3); 7.45 (1H, d, H-7, $J_{6,7}$ 10Hz); 7.60-7.70 (2H, m, H-5 and H-6); 9.80 (1H, t, Aq-NH).

FABMS(+) m/z: 313 (15%)$(RNH_3)^+$, 279 (14%), 176 (20%), 149 (100%).

9.2.16 4,8-Dihydroxy-1-[(4-aminobutyl)amino]anthraquinone trifluoroacetate (160) /(NU:UB 60)

(i) 4,8-Dihydroxy-1-[4-(N-tertiarybutoxycarbonylamino)butylamino]anthraquinone /(159)

Compound (**159**) was prepared using N-^{t}Boc-1,4-diaminobutane (1.0g, 5.7 mmol) and leuco-1,4,5-trihydroxyanthraquinone (1.5g, 5.8 mmol) [following method B(i)]. T.l.c. of the crude product [chloroform : methanol (5:1)]: R_f 0.00 (brown), 0.15 (orange), 0.50 (blue), 0.80 (purple) product, 0.95 (yellow) 1,4,5-trihydroxyanthraquinone. Yield (0.55g)(22%).

Found: mp 172 °C.

CIMS(+) m/z: 427 (20%)$(MH)^+$, 189 (60%), 115 (100%). M, 426.

(ii) 4,8-Dihydroxy-1-[(4-aminobutyl)amino]anthraquinone trifluoroacetate (160) /(NU:UB 60)

Compound (**159**)(0.50g) was deprotected using trifluoroacetic acid [following method B(ii)]. T.l.c. of the crude product (solvent system 1): R_f 0.10 (purple) product, 0.82 (purple)(AT74). An analytically pure sample of compound (**160**) was prepared by recrystallisation from an ethyl acetate/ethanol (4:1) solution. Yield (0.36g)(69%).

Found: mp 186 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.55-1.75 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 2.85 (2H, t, $CH_2NH_3^+$); 3.45 (2H, q, Aq-NH-CH_2); 7.20-7.35 (2H, m, H-2 and H-3);

7.50 (1H, d, H-7, $J_{6,7}$ 10Hz); 7.65-7.70 (2H, m, H-5 and H-6); 9.85 (1H, t, Aq-NH, J_{HNCH} 4Hz).

FABMS(+) m/z: 327 (23%)$(RNH_3)^+$, 257 (14%), 69 (100%). M, 440.

9.2.17 4,8-Dihydroxy-1-[(3-hydroxypropyl)amino]anthraquinone (161)

Compound (**161**) was prepared using 3-amino-1-propanol (0.59g, 7.9 mmol) and leuco-1,4,5-trihydroxyanthraquinone (2.0g, 7.8 mmol) [following method B(i)]. T.l.c. of the crude product (solvent system 1): R_f 0.05 (yellow), 0.10 (green), 0.15 (yellow), 0.35 (blue), 0.45 (purple) product, 0.90 (orange) 1,4,5-trihydroxyanthraquinone. Yield (0.51g)(21%).

Found: mp 168 °C.

$C_{17}H_{15}NO_5$ requires: C 65.17, H 4.83, N 4.47 %. Found; C 65.07, H 4.72, N 4.45 %.

FABMS(+) m/z: 336 (5%)$(M+Na)^+$, 314 (55%)$(MH)^+$, 259 (15%), 107 (100%). M, 313.

9.2.18 4,8-Dihydroxy-1-{[(S)-2-hydroxy-1-benzylethyl]amino}anthraquinone (162)

Compound (**162**) was prepared using (S)-2-amino-3-phenyl-1-propanol (L-phenylalaninol) (1.0g, 6.6 mmol) and leuco-1,4,5-trihydroxyanthraquinone (1.7g, 6.6 mmol) [following method B(i)]. T.l.c. of the crude product (solvent system 2): R_f 0.00 (green), 0.15 (purple) product, 0.85 (orange) 1,4,5-trihydroxyanthraquinone. Yield (1.22g)(47%).

Found: mp 140 °C

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 2.35 (1H, t, OH); 3.05 (2H, m, CH_2-phe); 3.85 (2H, m, CH_2OH); 4.05 (1H, m, Aq-NH-CH); 7.05 (1H, d, H-2); 7.15-7.35 (7H, m,

unresolved, $C_6\underline{H}_5$, H-3 and H-7); 7.55 (1H, d, H-6, $J_{5,6}$ 8Hz); 7.75 (1H, d, H-5); 10.20 (1H, d, Aq-N<u>H</u>, J_{HNCH} 8Hz); 13.20 (1H, s, 4-O<u>H</u>); 13.80 (1H, s, 8-O<u>H</u>).

<u>FABMS(+)</u> m/z: 801 (1%)$(2M+Na)^+$, 779 (2%)$(2M+H)^+$, 412 (2%)$(M+Na)^+$, 390 (100%)$(MH)^+$, 358 (10%)$[M-(CH_2OH)]^+$, 298 (80%). M, 389.

9.3 <u>SYNTHESIS OF SPACER-LINKED ANTHRAQUINONE AMINO ACID (PEPTIDE) CONJUGATES</u>

AMIDE-LINKED Anthraquinone-Amino Acid (Peptide) Conjugates

9.3.1 <u>Method C</u>

General method for coupling of an N-α-protected-C-activated amino acid to a pre-formed anthraquinone-aminoalkylamino spacer compound.

The (aminoalkylamino)anthraquinone spacer compound (3.0 mmol) was suspended in DMF (70cm^3) and stirred at 0°C. An N-α-protected amino acid-O-pentafluorophenolate ester (3.3 mmol) in DMF (30 cm^3) [or an N-α-protected amino acid-N-hydroxysuccinimide ester (3.3 mmol) in THF (30 cm^3)] was added drop-wise and the reaction mixture was allowed to reach room temperature. Stirring was continued for a further 12h.

The mixture was partitioned between chloroform and water. The chloroform extracts were washed with saturated sodium bicarbonate$_{(aq)}$, then water, dried ($MgSO_4$), filtered and evaporated to a low volume (10 cm^3). The foregoing concentrated solution was then applied to a silica gel chromatography column (4×30 cm) prepared with chloroform : ethyl acetate (4:1) and eluted initially in the same solvent mixture to remove a little coloured highly mobile impurity. The major product was eluted using the same solvent containing

increasing gradients of methanol (2–5% v/v). Fractions containing the major product were combined, filtered and evaporated to give a red solid. Recrystallisation (or precipitation) from a suitable solvent [typical solvents included: ethanol, methanol/ hexane (1:50), ethyl acetate/ hexane (1:50), ethyl acetate/ petroleum ether (1:100)] afforded the spacer-linked anthraquinone (N-protected) amino acid conjugate in an analytically pure form.

9.3.2 Method D

General method for activation of an N-α-protected amino acid by conversion to a pentafluorophenolate ester.

Pentafluorophenol (3.3 mmol) was added to a stirred solution of an N-protected amino acid (3.0 mmol) in dry ethyl acetate (70 cm^3) at 0°C. A solution of dicyclohexylcarbodiimide (3.6 mmol) in dry ethyl acetate (30 cm^3) was added dropwise and stirring was continued for 12h as the mixture was allowed to reach room temperature. The precipitated dicyclohexylurea was filtered off and the solution evaporated to yield a crystalline precipitate of the N-protected amino acid-O-pentafluorophenolate ester which was used for subsequent reaction without further purification. (Analytically pure samples could be obtained by recrystallisation according to published procedures; Bodanszky and Bodanszky 1994).

ESTER-LINKED Anthraquinone-Amino Acid (Peptide) Conjugates

9.3.3 Method E

General method for coupling of an N-α-protected amino acid to a pre-formed anthraquinone-hydroxyalkylamino spacer compound.

Dicyclohexylcarbodiimide (DCC) (3.3 mmol) and 4-dimethylaminopyridine (DMAP) (0.15 mmol) in dichloromethane (35 cm^3) were added to a cooled stirred solution of a hydroxyalkylaminoanthraquinone (3 mmol) and an N-α-^{t}Boc-protected amino acid (3.3 mmol) in dichloromethane (35 cm^3). Stirring was continued for 12h as the mixture was allowed to reach room temperature. The precipitated dicyclohexylurea (DCU) was filtered off and the solution partitioned between chloroform and water (1:1, 100 cm^3), washed with saturated sodium bicarbonate solution (2×50 cm^3) and water (2×50 cm^3), dried ($MgSO_4$), filtered and evaporated to dryness. The residual solid was dissolved in toluene, applied to a silica gel column and eluted with toluene/ethyl acetate (4:1) with increasing gradients of ethyl acetate. Fractions containing the major product were combined, evaporated, dissolved in ethyl acetate, cooled (0 °C for 12h) and filtered to remove any remaining DCU. Recrystallisation (or precipitation) from a suitable solvent [typical solvents included: ethanol, methanol/ hexane (1:50), ethyl acetate/ hexane (1:50), ethyl acetate/ petroleum ether (1:100)] afforded the spacer-linked anthraquinone (N-protected) amino acid conjugate in an analytically pure form.

Methods for deprotection of N-protected compounds

9.3.4 Method F

General method for the deprotection of N-tertiarybutoxycarbonyl (^{t}Boc) protected anthraquinone spacer (ester or amide) linked amino acid conjugates.

The ^{t}Boc protected compound (3 mmol) was dissolved in trifluoroacetic acid (7 cm^3) at room temperature. After 0.5h the solvent was evaporated and the residual solid re-evaporated with ethanol (3×10 cm^3) before dissolving in a minimum volume of ethanol (3 cm^3). Addition of ether (100 cm^3) gave a precipitate of the deprotected anthraquinone spacer-linked amino acid conjugate as the N-terminal trifluoroacetate salt which was filtered off and dried *in vacuo*.

9.3.5 Method G

General method for the deprotection of N-α-fluorenylmethoxycarbonyl (Fmoc) protected anthraquinone spacer-linked amino acid conjugates.

The Fmoc protected compound (1 mmol) was dissolved in 20% (v/v) piperidine in DMF (20 cm^3) and stirred at room temperature for 5 min. The solution was partitioned between chloroform and water (1:1, 100 cm^3), washed with water (3×50 cm^3), dried (Na_2SO_4), filtered and evaporated to a low volume before application to a silica gel chromatography column [chloroform : methanol (19:1)] eluting with chloroform : methanol, (increasing gradient,19:1→5:1). The fractions containing the product were combined and evaporated to dryness to yield the free N-terminal amino compound.

9.4 GLYCINE-CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

9.4.1 1-[3-(N-Tertiarybutoxycarbonylglycylamino)propylamino]anthraquinone. (163) (NU:UB 1)

N-^{t}Boc-glycine was converted to its N-^{t}Boc-glycine-O-pentafluorophenolate ester (0.60g, 1.80 mmol) and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(0.45g, 1.60 mmol) [following methods C and D]. T.l.c.(solvent system 1): R_f 0.00 (red) spacer, 0.40 (red) product. The title compound was obtained as fine red crystals after recrystallisation from ethanol (0.31g)(44%).

Found: mp 168 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 2.00 (2H, qn, CH_2-CH_2-CH_2); 3.40 (2H, q, CH_2NHCO); 3.50 (2H, q, Aq-NH-CH_2); 3.85 (2H, d, CH_2-gly, J_{HCCH} 6Hz); 5.45 (1H, br. s, NH-^{t}Boc); 6.60 (1H, t, NH-gly); 7.00 (1H, dd, H-2, $J_{2,3}$ 8Hz, $J_{2,4}$ 1.5Hz); 7.45-7.60 (2H, m, H-3 and H-4); 7.70-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH, exchangable).

$C_{24}H_{27}N_3O_5$ requires: C 65.89, H 6.22, N 9.61 %. Found C 65.04, H 6.01, N 9.40 %.

FABMS(+) m/z: 438 (25%)$(MH)^+$, 236 (40%), 115 (50%), 57 (100%). M, 437.

9.4.2 1-[3-(Glycylamino)propylamino]anthraquinone trifluoroacetate (164) /(NU:UB 2)

The ^{t}Boc-glycine conjugate (**163**)(0.25g) was deprotected using trifluoroacetic acid [following method F] giving the title compound (**164**) as a brownish-red powder (0.23g)(88%). T.l.c. [chloroform : methanol (3:1)]: R_f 0.20 (red) product.

Found: mp 177 °C.

^{1}H nmr spectrum (d_6-DMSO, 400MHz) δ: 1.80 (2H, qn, CH_2-C$\underline{H}_2$-CH_2); 3.30 (2H,q, C$\underline{H}_2$); 3.40 (2H, q, C$\underline{H}_2$); 3.60 (2H, s, C$\underline{H}_2$-gly); 7.25 (1H, dd, H-2); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.80 (2H, m, H-6 and H-7); 7.95-8.20 (5H, unresolved, H-5, H-8 and N$\underline{H}_3^+$); 8.50 (1H, t, N$\underline{H}$CO); 9.65 (1H, t, Aq-N$\underline{H}$).

^{13}C nmr spectrum (d_6-DMSO, 100MHz) δ: 29.33 (–ve, Aq-NH-CH_2-$\underline{C}H_2$); [37.33 (–ve), 37.33 (–ve), 39.72 (–ve), 41.03 (–ve), Aq-NH-$\underline{C}H_2$-CH_2-$\underline{C}H_2$-NHCO-C$\underline{H}_2$-]; 112.90 (ab, aromatic b); 115.90 (+ve, aromatic $\underline{C}$H); 119.35 (+ve, aromatic $\underline{C}$H); 127.09 (+ve, aromatic $\underline{C}$H); 127.23 (+ve, aromatic $\underline{C}$H); 133.17 (ab, aromatic c); 134.30 (+ve, aromatic $\underline{C}$H); [134.78 (ab), 135.15 (ab), aromatic e and f]; [135.32 (+ve, aromatic $\underline{C}$H), 136.46 (+ve, aromatic $\underline{C}$H) 5 and 8]; 152.10 (ab, aromatic $\underline{C}$-1); 166.84 (ab, NH$\underline{C}$O); 183.67 (ab, $\underline{C}$=O); 184.83 (ab, $\underline{C}$=O).

FABMS(+) m/z: 676 (1%)$(2RNH_3)^+$, 360 (11%) $(RNH_2+Na)^+$, 338 (100%)$(RNH_3)^+$, 236 (18%)$(AqNHCH_2)^+$. M, 451.

9.4.3 1-[4-(N-Tertiarybutoxycarbonylglycylamino)butylamino]anthraquinone (165)

N-^{t}Boc-glycine was converted to its N-^{t}Boc-gly-O-pentafluorophenolate ester (1.3g, 3.8 mmol) which was subsequently reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(1.0g, 3.4 mmol) [following methods C and D] to give the title compound (**165**). Yield [precipitation from ethyl acetate/ hexane (1:50)] (0.60g)(39%). T.l.c.(solvent system 1): R_f 0.40 (red) product.

Found: mp 119 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.60-1.85 (4H, m, unresolved, Aq-NH-CH_2-C$\underline{H}_2$-C$\underline{H}_2$); 3.35 (4H, m, unresolved, Aq-NH-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$); 3.80 (2H, d, C$\underline{H}_2$-gly, J_{HCNH} 6Hz); 5.30 (1H, br.s, N$\underline{H}$-^{t}Boc); 6.40 (1H, t, N$\underline{H}$-Gly); 7.00 (1H, dd, H-

2, $J_{2,3}$ 8Hz, $J_{2,4}$ 1.5Hz); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.75 (2H, m, H-6 and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 9.65 (1H, t, Aq-NH).

FABMS(+) m/z: 474 (4%)$(M+Na)^+$, 452 (10%)$(MH)^+$+, 57 (100%)$[(CH_3)_3C]^+$. M, 451.

9.4.4 1-[4-(Glycylamino)butylamino]anthraquinone trifluoroacetate (166) /(NU:UB 18)

Treatment of the N-^{t}Boc protected glycine conjugate (**165**)(0.50g) with TFA [following method F] gave the title compound (**166**)(0.38g)(73%). T.l.c.(solvent system 1): R_f 0.00 (red) product, 0.40 (red)(AT8). T.l.c.(solvent system 3): R_f 0.50 (red) product.

Found: mp 119 °C.

^{1}H nmr spectrum (d_6-DMSO, 300MHz) δ: 1.50-1.75 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.20 (2H, q, CH_2-NHCO); 3.40 (2H, q, Aq-NH-CH_2); 3.55 (2H, s, CH_2-gly); 7.25 (1H, d, H-2); 7.45 (1H, d, H-4); 7.65 (1H, t, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.05 (3H, s, RNH_3^+); 8.05-8.20 (2H, m, H-5 and H-8); 8.40 (1H, t, NHCO); 9.75 (1H, t, Aq-NH).

$C_{22}H_{22}N_3O_5F_3$ requires: C 56.77, H 4.76, N 9.03 %. Found C 56.45, H 4.60, N 8.89 %.

FABMS(+) m/z: 352 (56%)$(RNH_3)^+$, 225 (15%), 77 (45%), 31 (100%). M, 465.

9.4.5 1-[2-(N-Tertiarybutoxycarbonylglycyloxy)ethylamino]anthraquinone (167)

Compound (**167**) was prepared from the reaction of 1-[(2-hydroxyethyl)amino]anthraquinone (**148**)(0.75g, 2.81 mmol) with N-^{t}Boc-glycine (0.50g, 2.85 mmol) [following method E]. T.l.c. [chloroform : methanol (7:1)]: R_f 0.90 (red) product. Yield (from methanol) (0.57g)(48%).

Found: mp 122 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 3.65 (2H, q, Aq-NH-CH_2); 3.95 (2H, d, CH_2-gly); 4.42 (2H, t, CH_2-OCO); 5.05 (1H, br.s, NH-^{t}Boc); 7.08 (1H, dd, H-2); 7.55-7.65 (2H, m, H-3 and H-4); 7.70-7.80 (2H, m, H-6 and H-7); 8.15-8.30 (2H, m, H-5 and H-8); 9.85 (1H, t, Aq-NH).

$C_{23}H_{24}N_2O_6$ requires: C 65.08, H 5.70, N 6.60 %. Found C 64.47, H 5.37, N 6.47 %.

CIMS(+) m/z: 425 (37%)$(MH)^+$, 236 (88%), 210 (100%). M, 424

Accurate mass measurement CI peak match [M+H] (reference compound: perfluorotributylamine): Calculated mass m/z: 425.1712. Measured mass m/z: 425.1705.

9.4.6 1-[2-(Glycyloxy)ethylamino]anthraquinone trifluoroacetate (168) (NU:UB 117)

Deprotection of the ^{t}Boc-glycine conjugate (**167**)(0.45g) using TFA [following method F] afforded the title compound after recrystallisation from ethanol. T.l.c.(solvent system 1): R_f 0.35 (red) product. Yield (0.36g)(78%).

Found: mp 180 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 3.70 (2H, q, Aq-NH-CH_2); 3.80 (2H, s, CH_2-gly, J_{HCCH} 5Hz); 4.40 (2H, t, CH_2-OCO, J_{HCCH} 6Hz); 7.30 (1H, dd, H-2); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.05-8.35 (5H, m, H-5, H-8 and RNH_3^+); 9.75 (1H, t, Aq-NH).

ESMS(+)(Cone 50V) m/z: 325 (75%)$(RNH_3)^+$, 307 (15%), 250 (100%)(Aq-NH-CH_2-$CH_2)^+$.

ESMS(-)(Cone -20V) m/z: 113(100%). M, 438.

9.4.7 1-[3-(N-Tertiarybutoxycarbonylglycyloxy)propylamino]anthraquinone (169)

Compound (**169**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**)(0.75g, 2.63 mmol) with N-^{t}Boc-glycine (0.47g, 2.68 mmol) [following method E]. T.l.c. of the crude product (solvent system 1): R_f 0.75 (red) product, 0.95 (yellow) 1-chloroanthraquinone. Yield [from methanol/ ether (1:5)](0.58g)(50%)

Found: mp 84 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 2.15 (2H, qn, CH_2-CH_2-CH_2); 3.45 (2H, q, Aq-NH-CH_2); 4.00 2H, d, CH_2-gly); 4.35 (2H, t, CH_2-OCO, J_{HCCH} 5Hz); 5.20 (1H, br.s, NH-^{t}Boc); 7.05 (1H, dd, H-2, $J_{2,3}$ 8 Hz, $J_{2,4}$ 1.5 Hz); 7.50-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-NH).

$C_{24}H_{26}N_2O_6$ requires: C 65.74, H 5.98, N 6.39 %. Found C 65.70, H 6.04, N 6.35%.

FABMS(+) m/z: 461 (1%)$(M+Na)^+$, 439 (4%)$(MH)^+$, 383 (14%), 57 (100%). M, 438.

9.4.8 1-[3-(Glycyloxy)propylamino]anthraquinone trifluoroacetate (170) /(NU:UB 109)

The N-^{t}Boc protected derivative (**169**) (0.47g) was deprotected using TFA [following method F] to give an analytically pure sample of the title compound (**170**). T.l.c.(solvent system 3): R_f 0.60 (red) product. Yield (0.38g)(81%).

Found: mp 198 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 2.05 (2H, qn, CH_2-CH_2-CH_2); 3.50 (2H, q, Aq-NH-CH_2); 3.90 (2H, s, CH_2-gly); 4.30 (2H, t, CH_2-OCO); 7.25 (1H, dd, H-2); 7.40 (1H,

dd, H-4); 7.65 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.00-8.45 (5H, m, H-5, H-8 and $RN\underline{H}_3^+$); 9.75 (1H, t, Aq-N<u>H</u>).

$\underline{C_{21}H_{19}N_2O_6F_3}$ requires: C 55.76, H 4.23, N 6.19 %. Found C 56.12, H 4.07, N 6.18 %.

FABMS(+) m/z: 379 (4%), 339 (100%)$(RNH_3)^+$, 263 (20%), 236 (25%), 69 (40%). M, 452.

9.4.9 1-[4-(N-Tertiarybutoxycarbonylglycyloxy)butylamino]anthraquinone (171)

Compound (**171**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**)(0.75g, 2.54 mmol) with N-^{t}Boc-glycine (0.45g, 2.57 mmol) [following method E]. T.l.c. of the crude product (solvent system 1): R_f 0.75 (red) product, 0.95 (yellow) 1-chloroanthraquinone. Yield (from methanol)(0.49g)(43%) Found: mp 66 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.85 (4H, m, unresolved, CH_2-$C\underline{H}_2$-$C\underline{H}_2$-CH_2); 3.35 (2H, q, Aq-NH-$C\underline{H}_2$); 3.90 (2H, d, $C\underline{H}_2$-gly); 4.20 (2H, t, $C\underline{H}_2$-OCO); 5.00 (1H, br.s, N<u>H</u>-^{t}Boc); 7.00 (1H, dd, H-2); 7.50-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-N<u>H</u>).

$\underline{C_{25}H_{28}N_2O_6}$ requires: C 66.36, H 6.24, N 6.19 %. Found C 66.24, H 6.17, N 6.04 %.

CIMS(+) m/z: 453 (17%)$(MH)^+$, 439 (5%), 70 (100%). M, 452.

9.4.10 1-[4-(Glycyloxy)butylamino]anthraquinone trifluoroacetate (172) /(NU:UB 110)

Treatment of the N-^{t}Boc protected glycine conjugate (**171**)(0.44g) with TFA [following method F] gave the title compound (**172**)(0.33g)(73%). T.l.c. (solvent system 3): R_f 0.65 (red) product.

Found: mp 154 °C.

^{1}H nmr spectrum (d_6-DMSO)(200MHz) δ: 1.65-1.85 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 3.40 (2H, q, Aq-NH-CH_2); 3.80 (2H, s, CH_2-gly); 4.20 (2H, t, CH_2-OCO); 7.20 (1H, dd, H-2); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.00-8.40 (5H, m, H-5, H-8 and RNH_3^+); 9.75 (1H, t, Aq-NH).

$C_{22}H_{21}N_2O_6F_3$ requires: C 56.65, H 4.54, N 6.01 %. Found C 56.24, H 4.16, N 6.07 %.

ESMS(+)(Cone 20V) m/z: 727 (1%)$(2RNH_2+Na)^+$, 705 (2%)$[(RNH_2)RNH_3)]^+$, 353 (100%)$(RNH_3)^+$, 74 (15%).

ESMS(–)(Cone 20V) m/z: 113 (75%)$(OOC.CF_3)^-$, 69 (100%)$(CF_3)^-$. M, 466.

9.4.11 4-Hydroxy-1[-3-(N-tertiarybutoxycarbonylglycyloxy)propylamino]-anthraquinone (173)

Compound (**173**) was prepared from the reaction of 4-hydroxy-1-[(3-hydroxypropyl)amino]anthraquinone (**156**) (0.30g, 1.71 mmol) with N-^{t}Boc-glycine (0.50g, 1.68 mmol) [following method E]. T.l.c. of the crude product (solvent system 2): R_f 0.00 (brown), 0.08 (purple) spacer, 0.25 (purple) product, 0.80 (yellow) 1,4,5-trihydroxyanthraquinone. Yield [from ethyl acetate/ pentane (1:50)](0.77g)(88%)

Found: mp 124 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 2.10 (2H, qn, CH_2-CH_2-CH_2); 3.50 (2H, q, Aq-NH-CH_2); 4.00 (2H, d, CH_2-gly, J_{HCNH} 5Hz); 4.35 (2H, t, CH_2-OCO, J_{HCCH} 6Hz); 5.20 (1H, br.s, NH-^{t}Boc); 7.25-7.30 (2H, m, H-2 and H-3); 7.65-7.80 (2H, m, H-6 and H-7); 8.25-8.35 (2H, m, H-5 and H-8); 10.30 (1H, t, Ar-NH); 13.60 (1H, s, 4-OH).

$C_{24}H_{26}N_2O_7$ requires: C 63.42, H 5.77, N 6.16 %. Found C 62.52, H 5.57, N 6.00 %.

FABMS(+) m/z: 477 (22%)$(M+Na)^+$, 455 (100%)$(MH)^+$, 399 (77%), 355 (10%), 278 (44%), 252 (80%). M, 454.

9.4.12 4-Hydroxy-1-[3-(glycyloxy)propylamino]anthraquinone trifluoroacetate (174) /(NU:UB 165)

The title compound (**174**) was obtained by deprotection of the ^{t}Boc-glycine conjugate (**173**) using TFA [following method F]. Yield (0.62g)(89%). T.l.c. (solvent system 1): R_f 0.2 (purple) product.

Found: mp 188 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 2.00 (2H, qn, CH_2-CH_2-CH_2); 3.55 (2H, q, Aq-NH-CH_2); 3.90 (2H, s, CH_2-gly); 4.30 (2H, t, CH_2-OCO); 7.35 (1H, d, H-2); 7.50 (1H, d, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 10.30 (1H, t, Aq-NH).

$C_{21}H_{19}N_2O_7F_3$ requires C 53.85, H 4.09, N 5.98 %. Found C 53.45, H 3.96, N 5.93 %.

ESMS(+)(Cone 20V) m/z: 377 (2%)$(RNH_2+Na)^+$, 355 (45%)$(RNH_3)^+$, 129 (50%), 97 (100%).

ESMS(−)(Cone 20V) m/z: 113 (100%)$(OOC.CF_3)^-$. M, 468.

9.4.13 4,8-Dihydroxy-1[-3-(N-tertiarybutoxycarbonylglycylamino)propylamino]-anthraquinone (175)

N-^{t}Boc-glycine-N-hydroxysuccinimide ester (0.15g, 0.55 mmol) was added to a cooled, stirred solution of 4,8-dihydroxy-1-[(3-aminopropyl)amino]anthraquinone trifluoroacetate (**158**)(0.20g, 0.47 mmol) and triethylamine (1 mmol) in THF [following method C]. T.l.c. of the crude product (solvent system 1): R_f 0.04 (purple) spacer, 0.40 (blue), 0.50 (purple)

product, 0.95 (yellow) 1,4,5-trihydroxyanthraquinone. Recrystallisation from ethanol gave (**175**) as purple solid (0.12g)(55%).

9.4.14 4,8-Dihydroxy-1-[3-(glycylamino)propylamino]anthraquinone trifluoroacetate (176) (NU:UB 51)

The N- ^{t}Boc protected compound (**175**) was deprotected with trifluoroacetic acid [following method F]. The resultant salt was purified by column chromatography using chloroform : methanol (9:1) as the eluting solvent. Recrystallisation from ethyl acetate afforded the title compound (**176**) (0.056g)(46%). T.l.c. (solvent system 3): R_f 0.55 (purple) product.

Found: mp 155 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.80 (2H, qn, CH_2-CH_2-CH_2); 3.25 (2H, q, CH_2-NHCO); 3.35 (2H, m, Aq-NH-CH_2); 3.55 (2H, s, CH_2-gly); 7.15-7.25 (2H, m, H-2 and H-3); 7.35 (1H, d, H-7, $J_{6,7}$ 8Hz); 7.55-7.70 (2H, m, H-5 and H-6); 8.55 (1H, t, NHCO); 9.85 (1H, t, Aq-NH).

FABMS(+) m/z: 370 (10%)$(RNH_3)^+$, 329 (15%), 176 (45%), 151 (20%), 63 (100%). M, 483.

9.4.15 4,8-Dihydroxy-1-[4-(N-tertiarybutoxycarbonylglycylamino)butylamino]-anthraquinone (177)

N-^{t}Boc-glycine-N-hydroxysuccinimide ester (0.20g, 0.74 mmol) was reacted with 4,8-dihydroxy-1-[(4-aminobutyl)amino]anthraquinone trifluoroacetate (**160**)(0.30g, 0.68 mmol) in THF (50 cm^3) and triethylamine (1.5 cm^3) [following method C].

Recrystallisation from ethanol/ethyl acetate (1:1) afforded the title compound (**177**) as a dark purple solid (0.09g)(27%). T.l.c. (solvent system 1): R_f 0.60 (purple) product.

Found: mp 205 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.65-1.90 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 3.35-3.50 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2); 3.80 (2H, d, CH_2-gly, J_{HCNH} 6Hz); 5.10 (1H, br.s, NHCO-^{t}Boc); 6.20 (1H, br.s, spacer-NHCO-gly); 7.20-7.30 (3H, m, H-2, H-3 and H-7); 7.60 (1H, m, H-6); 7,85 (1H, dd, H-5, $J_{5,6}$ 8Hz, $J_{5,7}$ 1Hz); 9.95 (1H, br. s, Ar-NH); 13.35 (1H, s, 4-OH); 13.90 (1H, s, 8-OH).

CIMS(+) m/z: 484 (42%)$(MH)^+$, 428 (12%), 246 (28%), 70 (100%). M, 483.

9.4.16 4,8-Dihydroxy-1-[4-(glycylamino)butylamino]anthraquinone trifluoroacetate (178) (NU:UB 61)

Deprotection of the ^{t}Boc-glycine conjugate (**177**)(0.60g) using TFA [following method F] gave the title compound (**178**)(0.045g)(73%). T.l.c. (solvent system 3): R_f 0.55 (purple) product.

Found: mp 192 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.50-1.80 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 3.20 (2H, q, CH_2-NHCO); 3.40 (2H, m, Aq-NH-CH_2); 3.60 (2H, s, CH_2-gly); 7.20-7.30 (2H, m, H-2 and H-3); 7.40 (1H, d, H-7); 7.60-7.75 (2H, m, H-5 and H-6); 8.40 (1H, t, NHCO); 9.80 (1H, t, Aq-NH).

FABMS(+) m/z: 384 (9%)$(RNH_3)^+$, 176 (11%), 149 (27%), 69 (65%), 40 (100%). M, 497.

9.4.17 4,8-Dihydroxy-1[-3-(N-tertiarybutoxycarbonylglycyloxy)propylamino]-anthraquinone (179)

Compound (**179**) was prepared from the reaction of 4,8-dihydroxy-1-[(3-hydroxypropyl)amino]anthraquinone (**161**) (0.35g, 1.12 mmol) with N-^{t}Boc-glycine (0.20g, 1.14 mmol) [following method E]. T.l.c. of the crude product (solvent system 1): R_f 0.05 (brown), 0.40 (purple) spacer, 0.60 (blue), 0.75 (purple) product, 0.95 (yellow) 1,4,5-trihydroxyanthraquinone. Yield [from ethanol/ toluene (1:20)](0.47g)(89%).

Found: mp 152 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 2.10 (2H, qn, CH_2-CH_2-CH_2); 3.50 (2H, q, Aq-NH-CH_2); 3.95 (2H, d, CH_2-gly); 4.35 (2H, t, CH_2-OCO); 5.05 (1H, br. s, NH-^{t}Boc); 7.10-7.25 (3H, unresolved, H-2, H-3 and H-7); 7.55 (1H, m, H-6); 7.75 (1H, dd, H-5); 9.85 (1H, t, Aq-NH); 13.12 (1H, s, OH-4); 13.72 (1H, s, OH-8).

$C_{24}H_{26}N_2O_8$ requires: C 61.27, H 5.57, N 5.95 %. Found C 61.57, H 5.49, N 5.89 %.

FABMS(+) m/z: 493 (8%)$(M+Na)^+$, 470 (45%)$(MH)^+$, 415 (28%), 294 (46%), 268 (100%). M, 470.

9.4.18 4,8-Dihydroxy-1-[3-(glycyloxy)propylamino]anthraquinone trifluoroacetate (180) (NU:UB 129)

Treatment of the ^{t}Boc-glycine conjugate (**179**) with TFA [following method F] gave the title compound (**180**)(0.38g)(86%). T.l.c. [chloroform : methanol (4:1)]: R_f 0.40 (purple) product.

Found: mp 196 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 2.00 (2H, qn, CH_2-CH_2-CH_2); 3.50 (2H, q, Aq-NH-CH_2); 3.82 (2H, s, CH_2-gly); 4.25 (2H, t, CH_2-OCO); 7.25-7.35 (2H, m, H-2 and H-3); 7.45 (1H, d, H-7); 7.60-7.70 (2H, m, H-5 and H-6); 9.85 (1H, t, Aq-NH).

$C_{21}H_{19}N_2O_8F_3$ requires: C 52.07, H 3.95, N 5.78 %. Found C 51.83, H 3.73, N 5.70%.

ESMS(+)(Cone 80V) m/z: 741 (28%)$[(RNH_2)RNH_3]^+$, 371 (100%)$(RNH_3)^+$. M, 484.

9.5 ALANINE-CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

9.5.1 1-[3-(N-Tertiarybutoxycarbonyl-L-alanylamino)propylamino]anthraquinone /(181)

N-^{t}Boc-L-alanine was converted to its N-^{t}Boc-L-alanine-O-pentafluorophenolate ester (0.94g, 2.65 mmol) [t.l.c. (solvent system 1): R_f 0.30 (u.v. active) pentafluorophenol, 0.70 (u.v. active) ^{t}Boc-ala-Opfp] and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(0.67g, 2.4 mmol) [following methods C and D]. T.l.c. of the crude product (solvent system 1): R_f 0.40 (purple), 0.60 (red) product, 0.95 (red). The title compound (**181**) was obtained as fine red crystals from ethanol and deprotected in full (0.47g)(44%).

9.5.2 1-[3-(L-Alanylamino)propylamino]anthraquinone trifluoroacetate /(182) (NU:UB 4)

Deprotection of the ^{t}Boc-alanine conjugate (**181**)(0.47g) using TFA [following method F] gave the title compound (**182**)(0.17g)(34%). T.l.c. (solvent system 1): R_f 0.10 (red).

Found: mp 140 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.40 (3H, d, CH_3-ala); 1.85 (2H, qn, Aq-CH_2-CH_2); 3.25-3.50 (4H, m, unresolved, NH-CH_2-CH_2-CH_2); 3.85 (1H, q, α-CH, J_{HNCH} 6Hz);

7.25 (1H, dd, H-2, $J_{2,3}$ 8Hz, $J_{2,4}$ 1Hz); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.00-8.25 (5H, m, unresolved, H-5, H-8 and $N\underline{H}_3^+$); 8.50 (1H, t, N<u>H</u>CO); 9.75 (1H, t, Aq-N<u>H</u>, J_{HNCH} 4Hz).

<u>ESMS(+)</u> m/z: 703 (8%)$[(RNH_2)RNH_3]^+$, 374 (5%), 352 (100%)$(RNH_3)^+$.

<u>ESMS(−)</u> m/z: 113 (100%). M, 465.

9.5.3 1-[3-(N-Tertiarybutoxycarbonyl-D-alanylamino)propylamino]anthraquinone /(183)

N-^{t}Boc-D-alanine was converted to its N-^{t}Boc-D-alanine-O-pentafluorophenolate ester (2.80g, 7.89 mmol) and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(2.00g, 7.14 mmol) [following methods C and D]. The title compound was obtained as fine red crystals from ethanol (1.38g)(43%). T.l.c. (solvent system 1): R_f 0.60 (red) product.

Found: <u>mp</u> 176 °C.

<u>^{1}H nmr spectrum ($CDCl_3$, 200MHz)</u> δ: 1.35-1.45 (12H, m, unresolved, ^{t}Boc and C<u>H</u>$_3$-ala); 2.00 (2H, qn, CH_2-C<u>H</u>$_2$-CH_2); 3.30-3.60 (4H, m, unresolved, Aq-NH-C<u>H</u>$_2$-CH_2-CH_2-C<u>H</u>$_2$); 4.20 (1H, qn, α-C<u>H</u>-ala); 5.20 (1H, br.d, N<u>H</u>-^{t}Boc); 6.55 (1H, t, CH_2-N<u>H</u>CO); 7.05 (1H, dd, H-2); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-N<u>H</u>).

<u>$C_{25}H_{29}N_3O_5$ requires:</u> C 66.50, H 6.47, N 9.31 %. Found; C 66.06, H 6.28, N 9.10 %.

<u>CIMS(+)</u> m/z: 452 (30%)$(MH)^+$, 338 (10%), 210 (95%), 58 (100%). M, 451.

9.5.4 1-[3-(D-Alanylamino)propylamino]anthraquinone trifluoroacetate (184) /(NU:UB 21)

The ^{t}Boc-D-alanine conjugate (**184**)(1.05g) was deprotected using TFA [following method F] to give a red solid of the title compound (0.70g)(65%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 137 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.35 (3H, d, CH_3-ala, J_{HCCH} 8Hz); 1.85 (2H, qn, Aq-CH_2-CH_2); 3.20-3.50 (4H, m, unresolved, NH-CH_2-CH_2-CH_2); 3.85 (1H, q, α-CH); 7.20 (1H, d, H-2); 7.40 (1H, d, H-4); 7.60 (1H, m, H-3); 7.80-7.90 (2H, m, H-6 and H-7); 7.95-8.20 (5H, m, unresolved, H-5, H-8 and NH_3^+); 8.50 (1H, t, NHCO); 9.70 (1H, t, Aq-NH, J_{HNCH} 5Hz).

FABMS(+) m/z: 704 (2%)$(2RNH_3)^+$, 374 (7%)$(RNH_2+Na)^+$, 352 (77%)$(RNH_3)^+$, 129 (55%), 31 (100%). M, 465.

9.5.5 1-[4-(N-Tertiarybutoxycarbonyl-L-alanylamino)butylamino]anthraquinone /(185)

N-^{t}Boc-L-alanine was converted to its N-^{t}Boc-L-alanine-O-pentafluorophenolate ester (2.70g, 7.61 mmol) {t.l.c. [dichloromethane : methanol (8:1)]: R_f 0.35 (u.v. active) PfpOH, 0.80 (u.v. active) ^{t}Boc-ala-Opfp} and reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(2.00g, 6.80 mmol) [following methods C and D]. T.l.c. (solvent system 1): R_f 0.40 (red) product. The title compound was obtained as fine red crystals after recrystallisation from ethanol (0.81g)(81%).

Found: mp 116 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.35 (3H, d, CH_3-ala) 1.40 (9H, s, ^{t}Boc); 1.60-1.85 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 3.25-3.40 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2); 4.15 (1H, qn, α-CH-ala); 5.10 (1H, d, NH-^{t}Boc); 6.45 (1H, t, CH_2-NHCO-ala) 7.00 (1H, dd, H-2); 7.45-7.55 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

$C_{26}H_{31}N_3O_5$ requires: C 67.08, H 6.71, N 9.03 %. Found C 66.57, H 6.80, N 8.76%.

CIMS(+) m/z: 466 (5%)$(MH)^+$, 179 (8%), 144 (8%), 91 (10%), 70 (100%). M, 465.

9.5.6 1-[4-(L-Alanylamino)butylamino]anthraquinone trifluoroacetate (186) /(NU:UB 33)

The title compound was obtained by deprotection of the ^{t}Boc-alanine conjugate (**185**)(0.50g) using TFA [following method F]. Yield (0.25g)(48%). T.l.c. (solvent system 3): R_f 0.40 (red) product.

Found: mp 126 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.35 (3H, d, CH_3-ala); 1.45-1.80 (4H, m, unresolved, Aq-CH_2-CH_2-CH_2); 3.25 (2H, q, CH_2-NHCO); 3.40 (2H, q, Aq-NH-CH_2); 3.75 (1H, q, α-CH); 7.25 (1H, d, H-2); 7.40 (1H, d, H-4); 7.65 (1H, t, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.00-8.30 (5H, m, unresolved, H-5, H-8 and NH_3^+); 8.45 (1H, t, NHCO); 9.70 (1H, t, Aq-NH).

ESMS(+)(Cone 50V) m/z: 388 (30%)$(RNH_2+Na)^+$, 366 (10%)$(RNH_3)^+$, 95 (30%), 74 (100%). M, 479.

9.5.7 1-[4-(N-Tertiarybutoxycarbonyl-D-alanylamino)butylamino]anthraquinone /(187)

N-^{t}Boc-D-alanine was converted to its N-^{t}Boc-D-alanine-O-pentafluorophenolate ester (1.50g, 4.23 mmol) and reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(1.13g, 3.84 mmol) [following methods C and D]. T.l.c. of the crude product [chloroform : ethyl acetate (4:1)]: R_f 0.00 (red) spacer, 0.20 (red) product. The title compound was obtained as fine red crystals from ethyl acetate (0.71g)(40%).

Found: mp 112 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.35 (3H, d, CH_3-ala) 1.45 (9H, s, ^{t}Boc); 1.60-1.85 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 3.25-3.40 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2); 4.15 (1H, qn, α-CH-ala); 5.05 (1H, br.d, NH-^{t}Boc); 6.40 (1H, t, spacer-NHCO-ala) 7.05 (1H, dd, H-2); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.10-8.20 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

$C_{26}H_{31}N_3O_5$ requires: C 67.08, H 6.71, N 9.03 %. Found C 66.37, H 6.36, N 8.73 %.

CIMS(+) m/z: 466 (5%)$(MH)^+$, 452 (7%), 210 (23%), 70 (77%), 44 (100%). M, 465.

9.5.8 1-[4-(D-Alanylamino)butylamino]anthraquinone trifluoroacetate (188) /(NU:UB 72)

Deprotection of the above ^{t}Boc compound (**187**)(0.54g) using TFA [following method F] afforded a red solid of the title compound (**188**)(0.25g)(45%). T.l.c. (solvent system 3): R_f 0.40 (red) product.

Found: mp 122 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.35 (3H, d, CH_3-ala); 1.50-1.85 (4H, m, unresolved, Aq-CH_2-CH_2-CH_2); 3.25 (2H, q, CH_2-NHCO); 3.40 (2H, q, Aq-NH-CH_2);

3.85 (1H, q, α-CH); 7.25 (1H, d, H-2); 7.40 (1H, d, H-4); 7.65 (1H, t, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.00-8.30 (5H, m, unresolved, H-5, H-8 and NH_3^+); 8.45 (1H, t, NHCO); 9.70 (1H, t, Aq-NH).

ESMS(+) m/z: 366 (100%)$(RNH_3)^+$, 143 (25%), 72 (10%).

ESMS(–) m/z: 113(100%). M, 479.

9.5.9 1-[3-(N-Tertiarybutoxycarbonyl-L-alanyloxy)propylamino]anthraquinone /(189)

Compound (**189**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (0.75g, 2.67mmol) with N-^{t}Boc-L-alanine (0.49g, 2.59 mmol) [following method E]. T.l.c. of the crude product (solvent system 1): R_f 0.60 (red) spacer, 0.75 (red) product, 0.95 (yellow) 1-chloroanthraquinone. Yield (from methanol)(0.45g)(37%).

Found: mp 60 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.35-1.45 (12H, m, unresolved, CH_3-ala and ^{t}Boc); 2.15 (2H, qn, CH_2-CH_2-CH_2); 3.50 (2H, q, Aq-NH-CH_2); 4.25-4.45 (3H, unresolved, α-CH and CH_2-OCO); 5.25 (1H, d, NH-^{t}Boc), 7.05 (1H, dd, H-2); 7.55-7.65 (2H, m, H-3 and H-4); 7.70-7.80 (2H, m, H-6 and H-7); 8.20-8.35 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-NH).

$C_{25}H_{28}N_2O_6$ requires; C 66.36, H 6.24, N 6.19 %. Found C 66.04, H 6.35, N 6.07 %.

FABMS(+) m/z: 475 (1%)$(M+Na)^+$, 453 (7%)$(MH)^+$, 397 (12%), 236 (15%), 57 (100%). M, 452

9.5.10 1-[3-(L-Alanyloxy)propylamino]anthraquinone trifluoroacetate (190) /(NU:UB 108)

The ^{t}Boc-L-alanine conjugate (**189**)(0.40g) was deprotected using TFA [following method F] to give a red solid of the title compound (0.31g)(76%). T.l.c. (solvent system 3): R_f 0.85 (red) product.

Found: mp 128 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.20 (3H, d, CH_3-ala); 1.80 (2H, qn, CH_2-CH_2-CH_2); 3.30 (2H, q, Aq-NH-CH_2); 3.85 (1H, q, α-CH); 4.05 (2H, t, CH_2-OCO); 7.05 (1H, d, H-2); 7.20 (1H, d, H-4); 7.45 (1H, t, H-3); 7.60-7.80 (2H, m, H-6 and H-7); 7.90-8.05 (2H, m, H-5 and H-8); 9.50 (1H, t, Aq-NH).

FABMS(+) m/z: 353 (100%)$(RNH_3)^+$, 282 (28%), 236 (30%), 69 (20%), 44 (79%). M, 466.

9.5.11 1-[3-(N-Tertiarybutoxycarbonyl-D-alanyloxy)propylamino]anthraquinone /(191)

Compound (**191**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (0.75g, 2.67mmol) with N-^{t}Boc-D-alanine (0.49g, 2.59 mmol) [following method E]. Yield (from methanol) (0.51g)(42%). T.l.c. (solvent system 1): R_f 0.75 (red) product.

Found: mp 58 °C.

^{1}H nmr spectrum ($CDCl_3$)(200MHz) δ: 1.35-1.45 (12H, m, unresolved, CH_3-ala and ^{t}Boc); 2.10 (2H, qn, CH_2-CH_2-CH_2); 3.45 (2H, q, Aq-NH-CH_2); 4.20-4.45 (3H, m, unresolved, α-CH and CH_2-OCO); 5.20 (1H, d, NH-^{t}Boc), 7.05 (1H, dd, H-2); 7.50-7.60 (2H, m, H-3

and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

$C_{25}H_{28}N_2O_6$ requires: C 66.36, H 6.24, N 6.19 %. Found C 65.71, H 6.12, N 5.96 %.

FABMS(+) m/z: 475 (2%)$(M+Na)^+$, 453 (10%)$(MH)^+$, 353 (2%), 397 (17%), 57 (100%). M, 452.

9.5.12 1-[3-(D-Alanyloxy)propylamino]anthraquinone trifluoroacetate (192) /(NU:UB 107)

Deprotection of the ^{t}Boc compound (**191**)(0.45g) using TFA [following method F] afforded an analytically pure sample of the title compound (**192**)(0.28g)(61%). T.l.c. (solvent system 3): R_f 0.85 (red).

Found: mp 132 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.40 (3H, d, CH_3-ala); 2.02 (2H, qn, CH_2-CH_2-CH_2); 3.50 (2H, q, Aq-NH-CH_2); 4.15 (1H, q, α-CH); 4.30 (2H, t, CH_2-OCO); 7.20 (1H, d, H-2); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.00-8.50 (5H, m, H-5, H-8 and RNH_3^+); 9.70 (1H, t, Aq-NH).

$C_{22}H_{21}N_2O_6F_3$ requires: C 56.65, H 4.54, N 6.01 %. Found C 56.26, H 4.39, N 5.98 %.

FABMS(+) m/z: 706 (1%)$(2RNH_3)^+$, 375 (1%)$(RNH_2+Na)^+$, 353 (82%)$(RNH_3)^+$, 282 (28%), 236 (36%), 44 (100%). M, 466.

9.5.13 1-[4-(N-Tertiarybutoxycarbonyl-L-alanyloxy)butylamino]anthraquinone (193)

Compound (**193**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (0.45g, 1.53mmol) with N-^{t}Boc-L-alanine (0.29g, 1.53 mmol) [following method E]. T.l.c. of the crude product (solvent system 1):

R_f 0.40 (red) spacer, 0.85 (red) product, 0.95 (yellow). Yield (from methanol)(0.21g)(30%).

Found: mp 102 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.35-1.45 (12H, m, unresolved, CH_3-ala and ^{t}Boc); 1.80-1.95 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-); 3.40 (2H, m, Aq-NH-CH_2); 4.40 (2H, t, CH_2-OCO); 4.35 (1H, m, α-CH); 5.05 (1H, br. s, NH-^{t} Boc), 7.05 (1H, dd, H-2); 7.45 (2H, m, H-3); 7.60-7.80 (3H, m, H-4, H-6 and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

FABMS(+) m/z: 467 (30%)$(MH)^+$, 57 (100%). M, 466

9.5.14 1-[4-(L-Alanyloxy)butylamino]anthraquinone trifluoroacetate (194) /(NU:UB 73)

The ^{t}Boc-L-alanine conjugate (**193**)(0.19g) was deprotected using TFA [following method F] to give a red solid of the title compound (0.15g)(75%). T.l.c.(solvent system 3): R_f 0.75 (red) product.

Found: mp 64 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz): δ: 1.35 (3H, d, CH_3-ala); 1.60-1.80 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.40 (2H, m, Aq-NH-CH_2); 4.10 (2H, t, CH_2-OCO); 4.20 (1H, m, α-CH); 7.25 (1H, dd, H-2); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.75-7.85 (2H, m, H-6 and H-7); 8.10 (3H, br. s, NH_3^+); 8.25-8.35 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

$C_{23}H_{23}N_2O_6F_3$ requires: C 57.50, H 4.83, N 5.83 %. Found C 57.18, H 4.70, N 5.66 %.

FABMS(+) m/z: 367 (100%)$(RNH_3)^+$.

9.5.15 1-[4-(N-Tertiarybutoxycarbonyl-D-alanyloxy)butylamino]anthraquinone /(195)

Compound (**195**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (0.70g, 2.37 mmol) with N-^{t}Boc-D-alanine (0.45g, 2.38 mmol) [following method E]. Yield (from methanol) (0.35g)(32%). T.l.c. (solvent system 1): R_f 0.85 (red) product.

Found: mp 100 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40-1.45 (12H, m, unresolved, CH_3-ala and ^{t}Boc); 1.80-1.95 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-); 3.40 (2H, q, Aq-NH-CH_2); 4.20-4.40 (3H, m, unresolved, CH_2-OCO and α-CH); 5.05 (1H, d, NH-^{t}Boc); 7.05 (1H, dd, H-2, $J_{2,3}$ 8Hz, $J_{2,4}$ 1.5Hz); 7.40-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 489 (20%)$(M+Na)^+$, 467 (100%)$(MH)^+$, 410 (75%), 278 (87%), 149 (95%). M, 466.

9.5.16 1-[4-(D-Alanyloxy)butylamino]anthraquinone trifluoroacetate (196)/ (NU:UB 76)

Treatment of the N-^{t}Boc protected D-alanine conjugate (**195**)(0.28g) with TFA [following method F] gave a red solid of the title compound (0.17g)(59%). T.l.c. (solvent system 3): R_f 0.75 (red), product.

Found: mp 62 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.35 (3H, d, CH_3-ala, J_{HCCH} 7Hz); 1.65-1.80 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.30 (2H, m, Aq-NH-CH_2); 4.10 (1H, m, α-CH, J_{HCCH} 7Hz); 4.25 (2H, t, CH_2-OCO); 7.20 (1H, dd, H-2); 7.35 (1H, dd, H-4); 7.60

(1H, m, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.05-8.20 (2H, m, H-5 and H-8); 8.25-8.50 (3H, br. s, $N\underline{H}_3^+$); 9.70 (1H, t, Aq-N<u>H</u>).

<u>ESMS(+)(Cone 20V)</u> m/z: 755 (30%), 733 (20%)$[(RNH_2)RNH_3]^+$, 389 (55%)$(RNH_2+Na)^+$, 367 (100%)$(RNH_3)^+$.

<u>ESMS(–)(Cone -20V)</u> m/z: 113 (50%)$(CF_3.COO)^-$, 69 (100%)$(CF_3)^-$. M, 480.

9.5.17 1-(9,10-Dioxoanthryl)-4-piperidyl-(2S)-2-[(tertiarybutoxy)carbonylamino]-propanoate (197)

Compound (**197**) was prepared from the reaction of 1-(4-hydroxypiperidyl)anthraquinone (**153**) (0.50g, 1.63 mmol) with N-^{t}Boc-L-alanine (0.31g, 1.64 mmol) [following method E]. Yield [from ethyl acetate/ hexane (1:100)] (0.13g)(17%). T.l.c. (solvent system 1): R_f 0.75 (red) product.

Found: <u>mp</u> 98 °C.

<u>^{1}H nmr spectrum ($CDCl_3$, 200MHz)</u> δ: 1.40-1.50 (12H, m, unresolved, $C\underline{H}_3$-ala and ^{t}Boc); 2.10 (2H, m, H-3′,3″); 2.20 (2H, m, H-5′,5″); 3.15 (2H, m, H-2′,2″); 3.35 (2H, m, H-6′,6′); 4.30 (1H, qn, α-C<u>H</u>); 5.00-5.15 (2H, m, unresolved, N<u>H</u>-^{t} Boc and C<u>H</u>OCO); 7.40 (1H, dd, H-4-Aq); 7.60 (1H, m, H-3-Aq); 7.70-7.85 [2H, m, (H-6 and H-7)-Aq]; 7.95 (1H, dd, H-2-Aq); 8.20-8.30 [2H, m, (H-5 and H-8)-Aq].

<u>FABMS(+)</u> m/z: 501 (16%)$(M+Na)^+$, 479 (100%)$(MH)^+$, 423 (33%), 288 (63%), 133 (45%). M, 478.

9.5.18 1-(9,10-Dioxoanthryl)-4-piperidyl-(2S)-2-aminopropanoate trifluoroacetate (198) (NU:UB 158)

Treatment of the N-^{t}Boc protected alanine conjugate (**197**)(0.095g) with TFA [following method F] gave the title compound (**198**)(0.065g)(68%). T.l.c. [chloroform : methanol (2:1)]: R_f 0.75 (red) product.

Found: mp 102 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.50 (3H, d, CH_3-ala); 1.95 (2H, m, H-3′,3″); 2.10 (2H, m, H-5′,5″); 3.15 (2H, m, H-2′,2″); 3.35 (2H, m, H-6′,6′); 4.15 (1H, q, α-CH); 5.05 (1H, qn, CHOCO); 7.55 (1H, dd, H-2-Aq); 7.65-7.80 [2H, m, (H-3 and H-4)-Aq]; 7.80-7.95 [2H, m, (H-6 and H-7)-Aq]; 8.10-8.20 [2H, m, (H-5 and H-8)-Aq]; 8.40 (3H, br. s, NH_3^+).

ESMS(+)(Cone 50V) m/z: 411 (1%)$(RNH_2+Na)^+$, 379 (100%)$(RNH_3)^+$, 290 (50%) 182 (10%).

ESMS(–)(Cone 20V) m/z: 113 (100%). M, 492.

9.5.19 2-{4-[(9,10-Dioxoanthryl)amino]phenyl}ethyl (2S)-2-[(tertiarybutoxy)-carbonylamino]propanoate (199)

Compound (**199**) was prepared from the reaction of 1-{[4-(2-hydroxyethyl)phenyl]amino}anthraquinone (**152**) (0.50g, 1.46 mmol) with N-^{t}Boc-L-alanine (0.28g, 1.48 mmol) [following method E]. Yield [from ethyl acetate/ hexane (1:100)] (0.05g)(7%). T.l.c. (solvent system 2): R_f 0.35 (red) product.

Found: mp 124 °C.

FABMS(+) m/z: 537 (30%)$(M+Na)^+$, 514 (64%)$(MH)^+$, 459 (30%), 326 (100%). M, 513.

9.5.20 2-{4-[(9,10-Dioxoanthryl)amino]phenyl}ethyl (2S)-2-propanoate trifluoro-acetate (200) (NU:UB 156)

The N-^{t}Boc-L-alanine conjugate (**199**) (0.045g) was deprotected using TFA [following method F] to give the title compound (**200**)(0.028g)(61%). T.l.c. (solvent system 3): R_f 0.75 (red) product.

Found: mp 168 °C.

ESMS(+)(Cone 20V) m/z: 437 (15%)$(RNH_2+Na)^+$, 415 (100%)$(RNH_3)^+$.

ESMS(–)(Cone 50V) m/z: 113 (28%), 69 (100%). M, 528.

9.5.21 2-{4-[(9,10-Dioxoanthryl)amino]phenyl}ethyl (2R)-2-[(tertiarybutoxy)-carbonylamino]propanoate (201)

Compound (**201**) was prepared from the reaction of 1-{[4-(2-hydroxyethyl)phenyl]amino}anthraquinone (**152**) (0.50g, 1.46 mmol) with N-^{t}Boc-D-alanine (0.28g, 1.48 mmol) [Method E]. T.l.c. of the crude product (solvent system 2): R_f 0.25 (red) spacer, 0.60 (red) product, 0.95 (yellow) 1-chloroanthraquinone. Yield [from ethyl acetate/ pentane (1:50)] (0.06g)(8%).

Found: mp 124 °C.

FABMS(+) m/z: 537 (25%)$(M+Na)^+$, 514 (64%)$(MH)^+$, 459 (15%), 415 (5%), 326 (100%), 312 (25%). M, 513.

9.5.22 2-{4-[(9,10-Dioxoanthryl)amino]phenyl}ethyl (2R)-2-propanoate trifluoro-acetate (202) (NU:UB 157)

Deprotection of the ^{t}Boc-D-alanine conjugate (**201**)(0.055g) using TFA [following method F] afforded a red solid of the title compound (0.035g)(63%). T.l.c. (solvent system 3): R_f 0.75 (red) product.

Found: mp 168 °C.

ESMS(+)(Cone 50V) m/z: 433 (1%), 415 (40%)$(RNH_3)^+$, 326 (100%), 182 (15%), 104 (10%).

ESMS(–)(Cone 20V) m/z: 113 (100%). M, 528.

9.5.23 2-[(9,10-Dioxoanthryl)amino]-2-methylpropyl (2S)-2-[(tertiarybutoxy)-carbonylamino]propanoate (203)

Compound (**203**) was prepared from the reaction of 1-[(2-hydroxytertiarybutyl)amino]anthraquinone (**151**) (1.00g, 3.39 mmol) with N-^{t}Boc-L-alanine (0.64g, 3.39 mmol) [following method E]. T.l.c. of the crude product (solvent system 1): R_f 0.40 (red) spacer, 0.75 (red) product, 0.85 (red), 0.95 (yellow) 1-chloroanthraquinone. Yield [from methanol/ ether (1:20)](0.59g)(37%).

Found: mp 120 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.35-1.45 (12H, m, unresolved, CH_3-ala and ^{t}Boc); 1.55 (6H, s, Aq-NH-C(CH_3)$_2$); 4.20-4.40 (3H, m, unresolved, α-CH_2 and CH_2-OCO); 5.10 (1H, d, NH-^{t}Boc); 7.25 (1H, dd, H-2); 7.40-7.65 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 10.25 (1H, s, Aq-NH).

$C_{26}H_{30}N_2O_6$ requires: C 66.94, H 6.48, N 6.01 %. Found C 66.92, H 6.54, N 5.98 %.

CIMS(+) m/z: 467 (90%)$(MH)^+$, 453 (25%), 367 (12%), 264 (50%), 244 (50%), 144 (95%), 61 (100%). M, 466.

9.5.24 2-[(9,10-Dioxoanthryl)amino]-2-methylpropyl (2S)-2-propanoate trifluoro-acetate (204) (NU:UB 128)

The ^{t}Boc-L-alanine conjugate (**203**)(0.20g) was deprotected using TFA [following method F] to give a red solid of the title compound (0.13g)(62%). T.l.c. [chloroform : methanol (2:1)]: R_f 0.75 (red) product, 0.95 (red) AT124.

Found: mp 90 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.40 (3H, d, CH_3-ala, J_{HCCH} 8Hz); 1.55 (6H, m, Aq-NH-C(CH_3)$_2$); 4.15 (1H, q, α-CH, J_{HCCH} 8Hz); 4.35 (1H, d, CH-OCO, J_{GEM} 10Hz); 4.55 (1H, d, CH′-OCO, J_{GEM} 10Hz); 7.45-7.65 (3H, m, unresolved, H-2, H-3 and H-4); 7.80-7.95 (2H, m, H-6 and H-7); 8.10-8.40 (5H, m, , H-5, H-8 and NH_3^+); 10.15 (1H, s, Aq-NH).

ESMS(+)(Cone 20V) m/z: 399 (2%), 367 (100%)$(RNH_3)^+$, 278 (55%), 144 (35%).

ESMS(–)(Cone 20V) m/z: 113 (100%).M, 480.

9.5.25 4,8-Dihydroxy-1[-3-(N-tertiarybutoxycarbonyl-D-alanylamino)propylamino] anthraquinone (205)

N-Boc-D-alanine-N-hydroxysuccinimide ester (0.37g, 1.29 mmol) was reacted with 4,8-dihydroxy-1-[(3-aminopropyl)amino]anthraquinone trifluoroacetate (**158**) (0.50g, 1.17 mmol) in THF (70 cm^3) and triethylamine (2 cm^3)[following method C]. Recrystallisation from ethyl acetate afforded the title compound as a dark purple solid (0.40g)(70%). T.l.c. (solvent system 1): R_f 0.50 (purple) product.

Found: mp 167 °C.

$C_{25}H_{29}N_3O_7$ requires: C 62.10, H 6.05, N 8.69 %. Found C 61.66, H 6.13, N 8.34 %.

EIMS m/z: 483 (95%)$(M)^+$, 427 (90%), 339 (90%), 41 (100%). M, 483.

9.5.26 4,8-Dihydroxy-1-[3-(D-alanylamino)propylamino]anthraquinone trifluoro-acetate (206) (NU:UB 118)

The ^{t}Boc-D-alanine conjugate (**205**)(0.35g) was deprotected using TFA [following method F]. The crude product was purified by column chromatography eluting with chloroform : methanol (20:1 → 5:1) to give the title compound as a purple solid (0.11g)(31%). T.l.c. (solvent system 3): R_f 0.60 (purple) product.

Found: mp 164 °C.

ESMS(+)(Cone 50V) m/z: 406 (3%), 384 (100%)$(RNH_3)^+$, 128 (55%).

ESMS(−)(Cone 50V) m/z: 113 (80%), 69 (100%). M, 497.

9.6 PROLINE-CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

9.6.1 1-[3-(N-Tertiarybutoxycarbonyl-L-prolylamino)propylaminoanthraquinone /(207)

N-^{t}Boc-L-Proline-pentafluorophenolate ester (5.30g, 13.9 mmol) was reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(3.54g, 12.6 mmol) [following method C]. T.l.c. of the crude product (solvent system 1): R_f 0.05 (red) spacer, 0.50 (purple), 0.60 (red) product, 0.85 (red). The title compound was obtained as bright red crystals from ethanol (3.98g)(66%).

Found: mp 140 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.75-2.40 (6H, m, unresolved, Aq-NH-CH_2-CH_2, β-CH_2 and γ-CH_2); 3.25-3.55 (6H, m, unresolved, ArNH-CH_2-CH_2-CH_2 and δ-CH_2); 4.25 (1H, t, α-CH); 7.00-7.10 (2H, m, unresolved, NHCO and H-2); 7.50-7.65 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.25 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH, exchangeable).

$C_{27}H_{31}N_3O_5$ requires: C, 67.90, H,6.56, N,8.80%. Found C, 68.07, H,6.60, N,8.76%.

FABMS(+) m/z: 478 (7%)$(MH)^+$, 378 (24%), 114 (17%), 70 (100%). M, 477.

9.6.2 1-[3-(L-Prolylamino)propylamino]anthraquinone trifluoroacetate (208) /(NU:UB 31)

The ^{t}Boc proline conjugate (**207**)(0.19g) was deprotected using trifluoroacetic acid [following method F], to give a red solid of the title compound (0.12g)(62%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 176 °C.

^{1}H nmr spectrum (300MHz, d_6-DMSO) δ: 1.80-2.05 (4H, unresolved, γ-CH_2 and Aq-NH-CH_2-CH_2); 2.30 (2H, m, β-CH_2); 3.10-3.60 (6H, unresolved, δ-CH_2 and ArNH-CH_2-CH_2-CH_2); 4.10 (1H, t, α-CH); 7.25 (1H, d, H-2); 7.45 (1H, d, H-4); 7.65 (1H, t, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.10-8.20 (2H, m, H-5 and H-8); 8.70 (1H, t, NHCO); 9.00 (2H, br s, NH_2^+); 9.70 (1H, t, ArNH).

FABMS(+) m/z 378 (12%)$(RNH_2)^+$, 176 (31%), 89 (51%), 77 (80%), 31 (100%). M, 491.

9.6.3 1-[3-(N-Tertiarybutoxycarbonyl-D-prolylamino)propylamino]anthraquinone (209)

N-^{t}Boc-D-Proline-pentafluorophenolate ester (1.00g, 3.57 mmol) was reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(0.85g, 3.04 mmol)[following method C]. T.l.c. of the crude product (solvent system 1): R_f 0.00 (red) spacer, 0.45 (purple), 0.55 (red) product, 0.95 (red). Recrystallisation from ethyl acetate/ethanol (1:1) afforded the title compound as a red solid (0.57g)(39%).

Found: mp 138 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.80-2.30 (6H, m, unresolved, Aq-NH-CH_2-CH_2, β-CH_2 and γ-CH_2); 3.30-3.55 (6H, m, unresolved, ArNH-CH_2-CH_2-CH_2 and δ-CH_2); 4.30 (1H, t, α-CH); 7.00-7.10 (2H, m, unresolved, NHCO and H-2); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

$C_{27}H_{31}N_3O_5$ requires: C 67.91, H 6.54, N 8.80 %. Found C 67.68, H 6.57, N 8.73 %.

FABMS(+) m/z: 478 (14%)$(MH)^+$, 378 (22%), 263 (35%), 114 (20%), 57 (100%). M,477.

9.6.4 1-[3-(D-Prolylamino)propylamino]anthraquinone trifluoroacetate (210) /(NU:UB 46)

Compound (**209**)(0.48g) was deprotected using trifluoroacetic acid [following method F] to afford an analytically pure sample of the title compound (**210**)(0.44g)(90%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 177 °C.

^{1}H nmr spectrum (300MHz, d_6-DMSO) δ: 1.70-2.00 (5H, m, unresolved, β-CH, γ-CH_2 and Aq-NH-CH_2-CH_2); 2.20 (1H, m, β-CH′); 3.20-3.50 (6H, unresolved, δ-CH_2 and Aq-

NH-C$\underline{H}_2$-CH_2-C$\underline{H}_2$); 4.05 (1H, t, α-C$\underline{H}$); 7.25 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.05-8.10 (2H, m, H-5 and H-8); 8.60 (1H, t, N$\underline{H}$CO); 9.00 (2H, br. s, N$\underline{H}_2^+$); 9.75 (1H, t, Aq-N$\underline{H}$).

$\underline{C_{24}H_{24}N_3O_5F_3 \text{ requires}}$: C 58.65, H 4.92, N, 8.55 %. Found C 57.84, H 4.76, N 8.35 %.

$\underline{\text{FABMS(+)}}$ m/z: 400 (4%), 378 (36%)$(RNH_3)^+$, 263 (10%), 89 (30%), 70 (100%). M, 491.

9.6.5 1-[3-(N-Fluorenylmethoxycarbonyl-O-tertiarybutyl-L-hydroxyprolylamino)-propylamino]anthraquinone (211)

N-α-Fmoc-O-^{t}Bu-trans-4-hydroxyproline was converted to its pentafluorophenolate ester (2.80g, 4.87 mmol) and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(1.24g, 4.43 mmol) [following methods C and D]. The title compound was obtained as fine deep red crystals from ethyl acetate/ ether (1:10). Yield (1.27g)(43%). T.l.c. (solvent system 1): R_f 0.65 (red) product.

Found: $\underline{\text{mp}}$ 112 °C.

$\underline{^1\text{H nmr spectrum (CDCl}_3\text{, 200MHz)}}$ δ: 1.20 (9H, s, ^{t}Bu); 1.65-1.90 (3H, m, unresolved, Aq-NH-CH_2-C$\underline{H}_2$- and β-CH); 2.50 (1H, m, β-CH′); 3.20-3.50 (5H, m, unresolved, Aq-NH-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$ and H-9-Fmoc); 3.65 (1H, m, δ-C$\underline{H}$); 4.20 (1H, m, α-C$\underline{H}$); 4.25-4.50 (4H, m, unresolved, C$\underline{H}_2$-Fmoc, γ-C$\underline{H}$, and δ-C$\underline{H}$′); 6.80-7.05 (2H, m, unresolved, N$\underline{H}$CO and H-2-Aq); 7.20-7.40 [5H, m, unresolved, H-3-Aq, (H-1, H-2 H-7 and H-8)-Fmoc]; 7.45-7.55 [3H, m, H-4-Aq, (H-4 and H-5)-Fmoc]; 7.60-7.75 [4H, m, (H-6 and H-7)-Aq, (H-3 and H-6)-Fmoc]; 8.15-8.25 (2H, m, H-5 and H-8); 9.65 (1H, t, Aq-N$\underline{H}$).

$\underline{\text{FABMS(+)}}$ m/z: 694 (35%), 673 (92%)$(MH)^+$, 391 (8%), 263 (52%), 179 (100%). M, 672

9.6.6 1-[3-(O-Tertiarybutyl-L-hydroxyprolylamino)propylamino]anthraquinone /(212)

The doubly protected compound (**211**) (1.17g) was dissolved in DMF/piperidine (4:1) and stirred at room temperature for 5 minutes, selectively removing the N-α-Fmoc protecting group, to give the title compound (0.38g)(48%).[following method G]. T.l.c. of the crude product (solvent system 1): R_f 0.10 (red), 0.25 (red) product, 0.40 (red), 0.80 (u.v. active) Fmoc-piperidine adduct.

Found: mp 106 °C.

$C_{26}H_{31}N_3O_4$ requires: C, 69.46, H, 6.95, N, 9.34 %. Found C, 68.88, H, 6.80, N, 9.20 %.

9.6.7 1-[3-(L-Hydroxyprolylamino)propylamino]anthraquinone trifluoroacetate (213) (NU:UB 50)

The partially deprotected compound (**212**)(0.27g) was dissolved in trifluoroacetic acid for 24 h at room temperature to remove the O-tertiarybutyl protecting group. The solvent was evaporated and the solid re-evaporated with ethanol (3×10 cm^3) and dissolved in a minimum volume of ethanol (2 cm^3). Addition of a large excess of ether (150 cm^3) gave a red precipitate of the title compound which was filtered off and dried (0.26g)(85%). T.l.c. [chloroform : methanol (3:1)]: R_f 0.40 (red).

Found: mp 164 °C.

^{1}H nmr spectrum (200MHz, d_6-DMSO) δ: 1.75-2.00 (3H, m, unresolved, β-CH and Aq-NH-CH_2-CH_2); 2.25 (1H, m, β-CH′); 3.10 (1H, m, δ-CH); 3.25-3.50 (5H, m, unresolved δ-CH′ and Aq-NH-CH_2-CH_2-CH_2); 4.30 (1H, m, α-CH); 4.45 (1H, m, γ-CH) 5.60 (1H, m, OH); 7.35 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.75-7.95 (2H, m, H-6

and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 8.70 (1H, t, NHCO); 9.25 (2H, br. s, NH_2^+); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 416 (2%), 394 (68%)$(RNH_3)^+$, 131 (10%), 86 (100%). M, 507.

9.6.8 1-[4-(N-Tertiarybutoxycarbonyl-L-prolylamino)butylamino]anthraquinone /(214)

N-^{t}Boc-proline-N-hydroxysuccinimide ester (0.88g, 2.81 mmol) was reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(0.75g, 2.55 mmol) [following method C]. T.l.c. of the crude product (solvent system 1): R_f 0.05 (red) spacer, 0.55 (purple), 0.65 (red) product, 0.90 (red). Recrystallisation from ethyl acetate/ethanol afforded the title compound as a red solid (0.46g)(37%).

Found: mp 110 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.60-2.00 (8H, unresolved, Aq-NH-CH_2-CH_2-CH_2, β-CH_2-pro and γ-CH_2-pro); 3.30-3.50 (6H, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2 and δ-CH_2 pro); 4.30 (1H, br. s, α-CH); 7.00-7.10 (2H, m, unresolved, NHCO-pro and H-2); 7.50-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

$C_{28}H_{33}N_3O_5$ requires: C 68.41, H 6.77, N 8.55 %. Found C 67.94, H 6.70, N 8.49 %.

FABMS(+) m/z: 492 (5%)$(MH)^+$, 392 (10%), 114 (15%), 70.0 (100%), 57 (55%). M,491.

9.6.9 1-[4-(L-Prolylamino)butylamino]anthraquinone trifluoroacetate (215) /(NU:UB 43)

Treatment of the N-^{t}Boc protected proline conjugate (**214**) (0.39g) with TFA [following method F] gave the title compound in an analytically pure form (0.21g)(53%). T.l.c. (solvent system 3): R_f 0.50 (red).

Found: mp 104 °C.

^{1}H nmr spectrum (300MHz, d_6-DMSO) δ: 1.55-1.90 (6H, m, unresolved, γ-C$\underline{H}_2$ and Aq-NH-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$); 2.40 (2H, m, β-C$\underline{H}_2$); 3.05-3.55 (6H, unresolved, δ-C$\underline{H}_2$ and Aq-NH-C$\underline{H}_2$-CH$_2$-CH$_2$-C$\underline{H}_2$); 4.05 (1H, t, α-C$\underline{H}$); 7.25 (1H, d, H-2); 7.45 (1H, d, H-4); 7.65 (1H, t, H-3); 7.80-8.00 (2H, m, H-6 and H-7); 8.10-8.20 (2H, m, H-5 and H-8); 8.55 (1H, t, N$\underline{H}$CO); 8.80 (2H, br s, N$\underline{H}_2^+$); 9.70 (1H, t, Aq-N$\underline{H}$).

FABMS(+) m/z: 392 (30%)$(RNH_2)^+$, 149 (10%), 136 (10%), 105 (12%), 70 (100%). M, 505.

9.6.10 1-[2-(N-Tertiarybutoxycarbonyl-L-prolyloxy)ethylamino]anthraquinone (216)

Compound (**216**) was prepared from the reaction of 1-[(2-hydroxyethyl)amino]anthraquinone (**148**) (0.70g, 2.62 mmol) with N-^{t}Boc-L-proline (0.64g, 2.61 mmol) [following method E]. Yield [precipitated from chloroform/ methanol (1:100)] (0.50g)(41%). T.l.c. (solvent system 1): R_f 0.55 (red).

Found: mp 120 °C.

FABMS(+) m/z: 465 (100%)$(MH)^+$. M, 464.

9.6.11 1-[2-(L-Prolyloxy)ethylamino]anthraquinone trifluoroacetate (217) /(NU:UB 115)

Deprotection of the ^{t}Boc-L-proline conjugate (**216**)(0.46g) using TFA [following method F] gave the title compound as deep orange crystals (0.36g)(77%). T.l.c.(solvent system 1): R_f 0.65 (red).

Found: mp 137 °C.

^{1}H nmr spectrum (200MHz, d_6-DMSO) δ: 1.85 (2H, m, γ-CH_2); 2.05 (1H, m, β-CH); 2.20 (1H, m, β-CH'); 3.20 (2H, m, δ-CH_2); 3.70 (2H, q, Aq-NH-CH_2); 4.30-4.60 (3H, m, unresolved, α-CH and CH_2OCO); 7.35 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.00-8.20 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 729 (4%), 365 (100%)$(RNH_2)^+$, 130 (85%). M, 478.

9.6.12 1-[3-(N-Tertiarybutoxycarbonylprolyloxy)propylamino]anthraquinone /(218)

Compound (**218**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (0.50g, 1.78 mmol) with N-^{t}Boc-L-proline (0.38g, 1.77 mmol) [following method E]. T.l.c. of the crude product [chloroform : methanol (6:1)]: R_f 0.70 (red) spacer, 0.90 (red) product. Yield (from methanol) (0.42g)(49%).

Found: mp 121 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, ^{t}Boc); 1.85-2.35 (6H, m, unresolved, CH_2-CH_2-CH_2, β-CH_2 and γ-CH_2); 3.40-3.60 (4H, unresolved, Aq-NHCH_2 and δ-CH_2); 4.20-4.40 (3H, unresolved, α-CH and CH_2OCO); 7.10 (1H, m, H-3); 7.50-7.60 (2H, m, H-

2 and H-4); 7.70-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-NH).

$C_{27}H_{30}N_2O_6$ requires: C 67.77, H 6.32, N 5.85 %. Found C 67.14, H 6.49, N 5.99 %.

FABMS(+) m/z: 501 (40%), 479 (86%)$(MH)^+$, 422 (37%), 379 (47%), 263 (65%), 97 (100%). M, 478.

9.6.13 1-[3-(L-Prolyloxy)propylamino]anthraquinone trifluoroacetate (219) /(NU:UB 111)

The ^{t}Boc-L-proline conjugate (**218**) (0.36g) was deprotected using TFA [following method F] to give the title compound as an orange/ brown powder (0.32g)(86%). T.l.c. [chloroform : methanol (6:1)]: R_f 0.50 (red) product.

Found: mp 66 °C.

^{1}H nmr spectrum(200MHz, d_6-DMSO) δ: 1.80-2.10 (5H, m, unresolved, β-CH, γ-CH_2 and CH_2-CH_2-CH_2); 2.30 (1H, m, β-CH′); 3.25 (2H, m, δ-CH_2); 3.40 (2H, q, Aq-NH-CH_2); 4.25-4.55 (3H, m, unresolved, α-CH and CH_2-OCO); 7.25 (1H, dd, H-2); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.75-8.00 (2H, m, H-6 and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 9.65 (1H, t, Aq-NH).

ESMS(+)(Cone 50V) m/z: 401 (2%), 379 (100%)$(RNH_3)^+$, 264 (50%), 236 (10%), 69 (30%).

ESMS(−)(Cone 20V) m/z: 113 (100%).

9.6.14 1-[3-(N-Tertiarybutoxycarbonyl-D-prolyloxy)propylamino]anthraquinone /(220)

Compound (**220**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (0.50g, 1.78 mmol) with N-^{t}Boc-D-proline (0.38g, 1.77 mmol) [following method E]. Yield (from methanol) (0.39g)(46%). T.l.c. (solvent system 1): R_f 0.70 (red) product.

Found: mp 120 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, ^{t}Boc); 1.85-2.35 (6H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2, β-CH_2 and γ-CH_2); 3.35-3.65 (4H, m, unresolved, Aq-NH-CH_2 and δ-CH_2); 4.25-4.40 (3H, unresolved, α-CH and CH_2OCO); 7.10 (1H, m, H-3); 7.55-7.65 (2H, m, H-2 and H-4); 7.70-7.85 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-NH).

$C_{27}H_{30}N_2O_6$ requires: C 67.77, H 6.32, N 5.84 %. Found C 67.54, H 6.50, 5.93 %.

FABMS(+) m/z: 501 (20%), 479 (100%)$(MH)^+$, 423 (35%), 379 (65%), 197 (85%). M, 478.

9.6.15 1-[3-(D-Prolyloxy)propylamino]anthraquinone trifluoroacetate (221) /(NU:UB 112)

Deprotection of the ^{t}Boc-L-proline conjugate (**220**)(0.35g) with TFA [following method F] afforded the title compound in an analytically pure form (0.31g)(86%).

Found: mp 66 °C.

^{1}H nmr spectrum (200MHz, d_6-DMSO) δ: 1.80-2.10 (5H, m, unresolved, β-CH, γ-CH_2 and CH_2-CH_2-CH_2); 2.30 (1H, m, β-CH′); 3.25 (2H, m, δ-CH_2); 3.40 (2H, q, Aq-NH-CH_2); 4.30-4.50 (3H, unresolved, α-CH and CH_2-OCO); 7.25 (1H, dd, H-2); 7.40 (1H, dd,

H-4); 7.60 (1H, m, H-3); 7.80-8.00 (2H, m, H-6 and H-7); 8.10-8.20 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

ESMS(+)(Cone 50V) m/z: 757 (10%), 657 (15%), 379 (100%)$(RNH_3)^+$, 130 (30%).

ESMS(−)(Cone 20V) m/z: 113 (100%). M, 492.

9.6.16 4,8-Dihydroxy-1-[3-(N-tertiarybutoxycarbonyl-L-prolylamino)propylamino]-anthraquinone (222)

N-^{t}Boc-L-proline-N-hydroxysuccinimide ester (0.66g, 2.12 mmol) was reacted with 4,8-dihydroxy-1-[(3-aminopropyl)amino]anthraquinone trifluoroacetate (**158**) (0.75g, 1.76 mmol) in THF (100 cm^3) and triethylamine (3.5 mmol)[following method C]. T.l.c. of the crude product [chloroform : methanol (3:1)]: R_f 0.00 (purple) (Aq-spacer-TFA), 0.10 (purple) (Aq-spacer-NH_2), 0.75 (purple) product. Recrystallisation from ethyl acetate afforded the title compound as a dark purple solid (0.57g)(63%).

Found: mp 131 °C.

FABMS(+) m/z: 532 (36%), 510 (100%)$(MH)^+$, 410 (31%), 295 (37%), 107 (98%). M, 509.

9.6.17 4,8-Dihydroxy-1-[3-(L-prolylamino)propylamino]anthraquinone trifluoro-acetate (223)

The ^{t}Boc-L-proline conjugate (**222**) (0.50g) was deprotected using TFA [following method F]. The crude product was purified by column chromatography eluting with chloroform: ethanol (2:1) to give a purple crystalline solid of the title compound (**223**)(0.40g)(78%). T.l.c. [chloroform : methanol (3:1)]: R_f 0.30 (purple) product.

Found: mp 104 °C.

^{1}H nmr spectrum (200MHz, d_6-DMSO) δ: 1.70-1.95 (5H, m, unresolved, β-CH-pro, γ-CH_2-pro and CH_2-CH_2-CH_2); 2.25 (1H, m, β-CH'-pro); 3.15 (2H, t, δ-CH_2-pro); 3.25 (2H, q, CH_2-NHCO); 3.45 (2H, q, Aq-NH-CH_2); 4.05 (1H, m, α-CH); 7.20-7.35 (1H, m, H-2 and H-3); 7.45 (1H, d, H-7, $J_{6,7}$ 8Hz); 7.60-7.70 (2H, m, H-5 and H-6); 8.60 (1H, t, NHCO); 9.95 (1H, t, Aq-NH).

ESMS(+) m/z: 819 (3%), 410 (95%)$(RNH_3)^+$, 155 (15%), 119 (35%), 87 (100%).

ESMS(–) m/z: 113 (62%), 69 (100%)$(CF_3)^-$. M, 523.

9.6.18 4,8-Dihydroxy-1-[4-(N-tertiarybutoxycarbonyl-L-prolylamino)butylamino]-anthraquinone (224)

N-Boc-L-proline-N-hydroxysuccinimide ester (0.55g, 1.76 mmol) was reacted with 4,8-dihydroxy-1-[(4-aminobutyl)amino]anthraquinone trifluoroacetate (**160**) (0.65g, 1.47 mmol) in THF (100 cm^3) and triethylamine (3.0 mmol) [following method C]. T.l.c. of the crude product [chloroform : methanol (3:1)]: R_f 0.10 (purple) (Aq-spacer-NH_2), 0.75 (purple) product. Recrystallisation from ethyl acetate afforded the title compound (0.61g)(86%).

Found: mp 128 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.65-2.00 (8H, unresolved, CH_2-CH_2-CH_2-CH_2, β-CH_2-pro and γ-CH_2-pro); 3.30-3.50 (6H, q, Ar-NH-CH_2-CH_2-CH_2-CH_2 and δ-CH_2-pro); 4.25 (1H, br. s, α-CH-ala); 7.00-7.10 (1H, br.s, NH-^{t}Boc); 7.20-7.30 (2H, m, H-2 and H-3); 7.60 (1H, t, H-6); 7.80 (1H, d, H-5); 9.90 (1H, t, Aq-NH); 13.25 (1H, s, OH); 13.85 (1H, s, OH).

FABMS(+) m/z: 546 (35%), 524 (87%)$(MH)^+$, 424 (100%), 257 (48%), 149 (66%), 114 (54%). M, 523.

9.6.19 4,8-Dihydroxy-1-[4-(L-prolylamino)butylamino]anthraquinone trifluoroacetate (225) (NU:UB 85)

The ^{t}Boc-L-proline conjugate (**224**)(0.50g) was deprotected using TFA [following method F]. The crude product was purified by column chromatography eluting with chloroform : ethanol (2:1) to give a purple solid of the title compound (0.49g)(84%).

Found: mp 178 °C.

^{1}H nmr spectrum (200MHz, d_6-DMSO) δ: 1.45-1.70 (4H, m, unresolved, CH_2-CH_2-CH_2-CH_2); 1.75-1.95 (3H, m, unresolved, β-CH and γ-CH_2); 2.25 (1H, m, β-CH′); 3.15-3.55 (6H, m, unresolved, CH_2-CH_2-CH_2-CH_2 and δ-CH_2); 4.15 (1H, m, α-CH); 7.25-7.35 (1H, m, H-2 and H-3); 7.45 (1H, d, H-7); 7.60-7.75 (2H, m, H-5 and H-6); 8.60 (1H, t, NHCO); 9.85 (1H, t, Aq-NH).

ESMS(+)(Cone 50V) m/z: 424 (100%)$(RNH_3)^+$, 105 (54%).

ESMS(−)(Cone 90V) m/z: 113 (65%), 69(100%)$(CF_3)^-$. M, 537.

9.7 LYSINE AND ORNITHINE CONTAINING SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

9.7.1 1-[3-(N-α-Tertiarybutoxycarbonyl-N-ε-benzyloxycarbonyl-L-lysylamino)-propylamino]anthraquinone (226)

N-α-^{t}Boc-N-ε-Z-L-lysine was converted to its O-pentafluorophenolate ester (5.75g, 10.5 mmol) [t.l.c. (solvent system 1): R_f 0.30 (u.v. active) pentafluorophenol, 0.80 (u.v. active) ester] and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(2.68g, 9.60 mmol) [following methods C and D]. The title compound was obtained as bright red crystals from ethanol (3.50g)(57%). T.l.c. (solvent system 1): R_f 0.50 (red) product.

Found: mp 148 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.35-1.60 (13H, m, unresolved, ^{t}Boc, γ-CH_2 and δ-CH_2); 1.85 (2H, m, β-CH_2); 2.00 (2H, qn, Aq-NH-CH_2-CH_2-CH_2-); 3.15 (2H, q, ε-CH_2); 3.35-3.60 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 4.05 (1H, q, α-CH); 4.85 (1H, br.s, NH-Z); 5.10 (2H, s, O-CH_2-Ph); 5.30 (1H, br. d, NH-^{t}Boc); 6.50 (1H, t, spacer-NHCO-lys); 7.05 (1H, dd, H-2); 7.35 (5H, br.s, Ph); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

$C_{36}H_{42}N_4O_7$ requires; C 67.27, H 6.59, N 8.71 %. Found; C 66.95, H 6.60, N 8.61 %.

FABMS(+) m/z; 665 (6%)$(M+Na)^+$, 643 (8%)$(MH)^+$, 91 (100%)$(PhCH_2)^+$, 77 (33%)$(Ph)^+$. M, 642.

9.7.2 1-[3-(N-ε-Benzyloxycarbonyl-L-lysylamino)propylamino]anthraquinone trifluoroacetate (227) (NU:UB 8)

Compound (**226**)(3.40g) was dissolved in trifluoroacetic acid for exactly 0.25h to selectively remove only the N-α-^{t}Boc group [following Method F]. Precipitation with ether afforded the title compound (**227**) (2.42g)(70%).]. T.l.c. (solvent system 1): R_f 0.15 (red) product.

Found: mp 161 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.15-1.45 (4H, m, unresolved, γ-CH_2 and δ-CH_2); 1.65 (2H, m, β-CH_2); 1.85 (2H, qn, Aq-NH-CH_2-CH_2-CH_2-); 2.95 (2H, q, ε-CH_2); 3.20-3.50 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-); 3.70 (1H, m, α-CH); 4.95 (2H, s, OCH_2-Ph); 7.15-7.25 (2H, m, unresolved, H-2 and NHCO-Z); 7.30 (5H, br.s, Ph); 7.40 (1H, d, H-4); 7.65 (1H, t, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.05-8.20 (5H, unresolved, RNH_3^+, H-5 and H-8); 8.55 (1H, t, NHCO-lys); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 565(5%)(RNH_2+Na)$^+$, 543 (21%)(RNH_3)$^+$, 91 (100%)($PhCH_2$)$^+$. M, 656.

9.7.3 1-[3-(N-α-Tertiarybutoxycarbonyl-N-δ-benzyloxycarbonyl-L-ornithylamino)-propylamino]anthraquinone (228)

N-α-^{t}Boc-N-δ-Z-ornithine was converted to its O-pentafluorophenolate ester (1.45g, 2.73 mmol) and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(0.69g, 2.46 mmol) [following methods C and D] to give the title compound as bright crystals from ethanol (0.76g)(49%). T.l.c. (solvent system 1): R_f 0.40 (red) product.

Found: mp 159 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40 (9H, s, ^{t}Boc); 1.50-1.90 (4H, m, unresolved, β-CH_2 and γ-CH_2); 1.95 (2H, qn, AQ-NH-CH_2-CH_2-CH_2-); 3.10-3.60 (6H, m, unresolved, δ-CH_2 and Aq-NHCH_2-CH_2-CH_2); 4.25 (1H, br.s, α-CH); 5.05 (3H, m, unresolved, NH-COOCH_2-Ph); 5.35 (1H, d, NHCO-^{t}Boc); 6.75 (1H, t, spacer-NHCO-orn); 7.00 (1H, dd, H-2); 7.25 (5H, br.s, Ph); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.75 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, ArNH).

FABMS(+) m/z: 630 (4%)(MH)$^+$, 529 (5%), 149 (94%), 91 (60%)($PhCH_2$)$^+$, 57 (100%)[$(CH_3)_3C$]$^+$. M, 629.

9.7.4 1-[3-(N-δ-Benzyloxycarbonyl-L-ornithylamino)propylamino]anthraquinone acetate (229) (NU:UB 47)

Compound (**228**) (0.50g) was dissolved in TFA for 0.25h to selectively remove the ^{t}Boc protecting group [following method F]. Treatment with triethylamine followed by column chromatography gave the free amine, 1-[3-(N-δ-Z-ornithylamino)propylamino]anthra-

quinone, which was dissolved in glacial acetic acid and evaporated to dryness to give the title compound (**229**)(0.28g)(60%). T.l.c. (solvent system 3): R_f 0.60 (red) product.

Found: mp 144 °C.

$C_{32}H_{36}N_4O_7$ requires: C 65.29, H 6.16, N 9.52 %. Found C 64.60, H 5.82, N 9.47 %.

FABMS(+) m/z: 551 (2%), 529 (20%)$(RNH_3)^+$, 279 (35%), 149 (100%). M, 588.

9.7.5 1-[3-N-α-Tertiarybutoxycarbonyl-N-ε-benzyloxycarbonyl-L-ornithyloxy)-propylamino]anthraquinone (230)

Compound (**230**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (1.50g, 5.34 mmol) with N-α-^{t}Boc-N-δ-Z-ornithine (1.96g, 5.36 mmol) [following method E]. Yield [from ethyl acetate/ methanol (1:50)](0.48g)(15%). T.l.c. (solvent system 1): R_f 0.70 (red) product.

Found: mp 130 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40 (9H, s, ^{t}Boc); 1.45-1.95 (4H, m, unresolved, β-CH_2 and γ-CH_2); 2.10 (2H, qn, Aq-NH-CH_2-CH_2); 3.20 (2H, q, δ-CH_2); 3.40 (2H, q, Aq-NH-CH_2); 4.25-4.45 (3H, m, unresolved, CH_2-OCO and α-CH); 4.95 (1H, t, NHCO-Z); 5.25 (2H, s, O-CH_2-Ph); 5.25 (1H, d, NH-^{t}Boc); 7.05 (1H, dd, H-2); 7.35 (5H, br.s, Ph); 7.55-7.65 (2H, m, H-3 and H-4); 7.70-7.75 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-NH).

$C_{35}H_{39}N_3O_8$ requires: C 66.76, H 6.24, N 6.67 %. Found; C 66.83, H 6.30, N 6.64 %.

FABMS(+) m/z: 652 (27%), 630 (51%)$(MH)^+$, 530 (8%), 413 (10%), 391 (25%), 291 (10%), 107 (100%)$(PhCH_2)^+$. M, 629.

9.7.6 1-[3-N-δ-Benzyloxycarbonyl-L-ornithyloxy)propylamino]anthraquinone trifluoroacetate (231) (NU:UB 120)

Compound (**230**) (0.30g) was dissolved in trifluoroacetic acid for precisely 0.25h to selectively remove only the N-α-^{t}Boc group [following method F]. The crude product was purified by column chromatography eluting with chloroform : methanol (20:1→10:1). Precipitation with ether afforded the title compound (**231**) (0.16g)(52%). T.l.c. [chloroform : methanol (6:1)]: R_f 0.65 (red) product.

Found: mp 80 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.40-1.70 (4H, unresolved, γ-CH_2 and β-CH_2); 2.00 (2H, qn, Aq-NH-CH_2-CH_2-CH_2-); 3.00 (2H, q, δ-CH_2); 3.45 (2H, m, Aq-NH-CH_2); 3.60 (1H, m, α-CH); 4.20 (2H, t, CH_2-OCO, J_{HCCH} 4Hz); 5.00 (2H, s, CH_2-Z); 7.20-7.35 (6H, unresolved, H-2 and C_6H_5); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.10-8.20 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH, J_{HNCH} 4Hz).

ESMS(+)(Cone 20V) m/z: 552 (10%), 530 (100%)$(RNH_3)^+$, 129 (25%), 97 (45%).

ESMS(−)(Cone -20V) m/z: 113 (100%). M, 643.

9.7.7 1-[3-(N-α-Fluorenylmethoxycarbonyl-N-ε-tertiarybutoxycarbonyl-L-lysyl-amino)propylamino]anthraquinone (232)

N-α-Fmoc-N-ε-^{t}Boc-lysine pentafluorophenolate ester (1.00g, 1.57 mmol) was reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**) (0.40g, 1.43 mmol) to give the title compound [following method C]. Yield [from ethyl acetate/ ether (1:50)] (0.80g)(77%). T.l.c. (solvent system 1): R_f 0.70 (red) product.

Found: mp 176 °C

$C_{43}H_{46}N_4O_7$ requires: C 70.67, H 6.34, N 7.67 %. Found C 69.95, H 6.18, N 7.54 %.

FABMS(+) m/z: 732 (3%)$(MH)^+$, 632 (2%), 434 (2%), 279 (33%), 149 (100%). M, 731.

9.7.8 1-[3-(N-ε-Tertiarybutoxycarbonyl-L-lysylamino)propylamino]anthraquinone acetate (233) (NU:UB 45)

Compound (**232**) was partially deprotected to selectively remove the N-α-Fmoc protecting group. T.l.c. of the crude product (solvent system 1): R_f 0.50 (red) amine, 0.70 (red), 0.90 (u.v. active) [following method G]. 1-[3-(N-ε-tertiarybutoxycarbonyl-L-lysylamino)propylamino]anthraquinone was dissolved in glacial acetic acid and evaporated to dryness to give the title compound (0.28g)(59%). T.l.c. (solvent system 3): R_f 0.55 (red) product.

Found: mp 106 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.20-1.50 (14H, m, unresolved, ^{t}Boc, β-C$\underline{H}$, γ-C$\underline{H_2}$ and δ-C$\underline{H_2}$); 1.55 (1H, m, β-C$\underline{H}$′); 1.80 (2H, qn, Aq-NH-CH_2-C$\underline{H_2}$-CH_2-); 1.90 (3H, s, C$\underline{H_3}$$COO^-$); 2.85 (2H, q, ε-C$\underline{H_2}$); 3.15-3.30 (3H, m, unresolved, C$\underline{H_2}$-spacer and α-C$\underline{H}$); 3.40 (2H, q, C$\underline{H_2}$-spacer); 6.80 (1H, t, N$\underline{H}$CO-Boc); 7.20 (1H, d, H-2); 7.20 (1H, d, H-4); 7.65 (1H, t, H-3); 7.80-8.00 (2H, m, H-6 and H-7); 8.05-8.20 (5H, unresolved, H-5 and H-8 and N$\underline{H_3}^+$); 9.70 (1H, t, Aq-N$\underline{H}$).

FABMS(+) m/z: 531 (2%), 509 (7%)$(RNH_3)^+$, 409 (8%), 84 (60%), 57 (100%). M, 568.

9.7.9 1-[3-(L-Lysylamino)propylamino]anthraquinone bis trifluoroacetate (234) /(NU:UB 16)

Compound (**233**)(0.25g) was dissolved in trifluoroacetic acid for 48h. The bis salt (**234**) was precipitated as a purple solid (0.1g)(42%) by addition of a large excess of ether (200 cm^3). T.l.c. (solvent system 3): R_f 0.25 (red) product.

Found: mp 202 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.30 (2H, m, γ-CH_2); 1.50 (2H, m, δ-CH_2); 1.65 (2H, m, β-CH_2); 1.75 (2H, qn, Aq-NH-CH_2-CH_2-CH_2-); 2.70 (2H, t, ε-CH_2, J_{HCCH} 7Hz); 3.30-3.60 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.65 (1H, t, α-CH, J_{HCCH} 7Hz); 7.25 (1H, d, H-2, $J_{2,3}$ 10Hz); 7.45 (1H, d, H-4); 7.65 (1H, t, H-3); 7.75-8.20 (10H, m, unresolved, H-5, H-6, H-7, H-8 and NH_3^+×2); 8.60 (1H, t, NHCO); 9.75 (1H, t, Aq-NH).

$C_{27}H_{30}N_4O_7F_6$, requires: C 50.95, H 4.75, N 8.80 %. Found C 49.85, H 4.76, N 8.47 %.

FABMS(+) m/z: 431 (7%)[$(R(NH_2)_2+Na]^+$, 409 (69%) [$(R(NH_2)NH_3]^+$, 236 (13%)$(ArNHCH_2)^+$, 84 (100%). M, 636.

9.7.10 1-[3-(N-α-Fluorenylmethoxycarbonyl-N-δ-tertiarybutoxycarbonyl-L-ornithylamino)propylamino]anthraquinone (235)

N-α-Fmoc-N-δ-^{t}Boc-ornithine was converted to its O-pentafluorophenolate ester, (2.04g, 3.29 mmol) using ethyl acetate/DMF (5:1) as the solvent, [t.l.c. (solvent system 1): R_f 0.35 (u.v. active) PfpOH, 0.80 (u.v. active) ester] and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(0.84g, 3.00 mmol) [following methods C and D] to give the title compound. Yield [from ethanol/ ether (1:50)] (0.52g)(24%). T.l.c. (solvent system 1): R_f 0.40 (red) product.

Found: mp 179 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40 (9H, s, ^{t}Boc); 1.45-1.90 (6H, unresolved, β-CH_2, γ-CH_2 and δ-CH_2); 2.00 (2H, qn, Aq-NH-CH_2-CH_2); 3.0-3.65 (7H, m, unresolved, ε-CH_2, Aq-NH-CH_2-CH_2-CH_2-NH and H-9-Fmoc); 4.15 (1H, t, α-CH); 4.40 (2H, d, CH_2-Fmoc); 4.70 (1H, m, NH-^{t}Boc); 5.80 (1H, br. d, NH-Fmoc); 6.80 (1H, m, NHCO-Orn); 7.05 (1H, d, H-2); 7.20-7.45 [4H, m, unresolved, (H-3, H-4)-AQ and (H-1, H-8)-Fmoc];

7.50-7.65 [4H, unresolved, (H-2, H-4 H-5 and H-7)-Fmoc]; 7.70-7.80 [4H, m, (H-6, H-7)-AQ, (H-3 H-6)-Fmoc); 8.20-8.30 [2H, m, (H-5 and H-8)-AQ]; 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 717 (8%)$(MH)^+$, 617 (3%), 263 (40%), 155 (96%), 44 (100%). M,716.

9.7.11 1-[3-(L-Ornithylamino)propylamino]anthraquinone bis trifluoroacetate (236) /(NU:UB 24)

The doubly protected compound (**235**) was dissolved in DMF/ piperidine (4:1) to remove the N-α-Fmoc group. T.l.c. of the crude product (solvent system 1): R_f 0.25 (red) product, 0.40 (red), 0.95 (u.v. active) [following method G]. The resultant 1-[3-(N-δ-^{t}Boc-L-ornithylamino)propyl-amino]anthraquinone compound was deprotected using TFA [Method F] to give purple crystals of the title compound(0.15g)(43%). T.l.c. (solvent system 1): R_f 0.05 (red) product.

Found: mp 144 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.50-2.00 (6H, unresolved, β-CH_2, γ-CH_2 and Aq-NH-CH_2-CH_2); 2.80 (2H, m, δ-CH_2); 3.30-3.60 (4H, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.70 (1H, t, α-CH); 7.25 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.75-8.10 (5H, unresolved, H-6 and H-7 and NH_3^+×2); 8.10-8.20 (2H, m, H-5 and H-8); 8.60 (1H, t, NHCO); 9.75 (1H, t, Aq-NH).

ESMS(+)(Cone 8V) m/z: 395 (100%)$[R(NH)_2NH_3]^+$, 87 (25%), 65 (10%).

ESMS(-)(Cone 20V) m/z: 113 (100%)$(CF_3COO)^-$.

9.7.12 1-[4-(N-α-Tertiarybutoxycarbonyl-N-ε-benzyloxycarbonyl-L-lysylamino)-butylamino]anthraquinone.(237)

N-α-^{t}Boc-N-ε-Z-L-lysine-N-hydroxysuccinimide ester (1.25g, 2.60 mmol) was reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(0.7g, 2.40 mmol)[following method C] T.l.c. of the crude product (solvent system 1): R_f 0.05 (red) spacer, 0.20 (purple), 0.45 (red) product, 0.90 (red). Recrystallisation from ethanol afforded the title compound as a bright red solid (0.45g)(29%).

Found: mp 142 °C.

^{1}H nmr spectrum ($CDCl_3$, 400MHz) δ: 1.35 (2H, m, γ-CH_2); 1.45 (9H, s, ^{t}Boc); 1.50 (2H, m, δ-CH_2); 1.60-1.85 (6H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2 and β-CH_2); 3.15 (2H, q, ε-CH_2); 3.30-3.40 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2); 4.05 (1H, m, α-CH); 4.95 (1H, t, NH-Z); 5.10 (2H, s, OCH_2Ph); 5.25 (1H, d, NH^{t}Boc); 6.45 (1H, t, NHCO-lys); 7.00 (1H, d, N-2); 7.30 (5H, brs, C_6H_5); 7.45-7.60 (2H, m, H-3 and H-4); 7.70-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

^{13}C nmr spectrum ($CDCl_3$, 100MHz) δ: 22.56 (–ve, γ-CH_2); 26.32 (–ve); 27.24 (–ve); 28.32 [+ve, C(CH_3)$_3$]; 29.49 (–ve); 31.85 (–ve); [38.96 (–ve), 40.38 (–ve), 42.43 (–ve), Aq-NH-CH_2-CH_2-CH_2-CH_2 and ε-CH_2]; 54.48 (+ve, α-CH); 66.64 (–ve, O-CH_2-Ph); 80.12 [ab, C(CH_3)$_3$]; 112.91 (ab, aromatic b); 115.73 [+ve, aromatic (Aq) CH]; 117.83 [+ve, aromatic (Aq) CH]; [126.64 (+ve, aromatic CH), 126.72 (+ve, aromatic CH), (AQ-6 and 7)]; [128.08 (+ve, aromatic CH), 128.50 (+ve, aromatic CH), Z-ortho, meta and para]; 132.95 [+ve, aromatic (Aq) CH]; 133.05 (ab, aromatic c); 133.94 [+ve, aromatic (Aq) CH]; [134.63 (ab), 134.96 (ab), aromatic e and d]; 135.38 [+ve, aromatic (Aq) CH]; 136.58 [ab, aromatic (Z) C-1]; 151.67 [ab, aromatic (Aq) C-1]; 155.90 (ab, NHCOO);

156.56 (ab, NHCOO); 172.25 (ab, spacer-NHCO-lys); 183.76 (ab, C=O); 185.04 (ab, C=O).

$C_{37}H_{44}N_4O_7$ requires: C 67.66, H 6.77, N 8.53 %. Found C 67.44, H 6.78, N 8.24 %.

FABMS(+) m/z: 680 (2%), 658 (5%)$(MH)^+$, 557 (5%), 149 (48%)$(PhCH_2OOCN)^+$; 91 (97%)$(PhCH_2)^+$, 70 (54%); 57 (100%) $[(CH_3)_3C]^+$. M, 657.

9.7.13 1-[4-(N-ε-Benzyloxycarbonyl-L-lysylamino)butylamino]anthraquinone acetate (238) (NU:UB 19)

Compound (**237**)(0.35g) was dissolved in trifluoroacetic acid for 0.25h [following method F] to give 1-[4-(N-ε-Z-L-lysylamino)butylamino]anthraquinone trifluoroacetate salt as the major product which was treated with triethylamine prior to column chromatography, eluting with chloroform : methanol (20:1→10:1). 1-[4-(N-ε-Z-lysylamino)butylamino]anthraquinone was dissolved in glacial acetic acid and evaporated to dryness to give the title compound (0.09g)(26%). T.l.c. (solvent system 3): R_f 0.50 (red) product.

Found: mp 104 °C.

^{1}H nmr spectrum (d_6-DMSO, 400MHz) δ: 1.40-1.70 (10H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2, β-CH_2, γ-CH_2 and δ-CH_2); 2.85 (2H, q, ε-CH_2); 3.15 (2H, qn, CH_2-NHCO); 3.25-3.40 (3H, m, unresolved, Aq-NH-CH_2 and α-CH); 4.95 (2H, s, OCH_2-Ph); 7.15 (1H, t, NHCO-Z); 7.25-7.35 (6H, unresolved, H-2 and C_6H_5); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.75-7.90 (3H, m, H-6 and H-7 and spacer-NHCO-lys); 8.10-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

^{13}C nmr spectrum (d_6-DMSO, 100MHz) δ: 23.48 (–ve, γ-CH_2); 26.89 (–ve); 27.65 (–ve); 30.21 (–ve); 35.75 (–ve); [38.71 (–ve), 41.10 (–ve), 42.72 (–ve), Aq-NH-CH_2-CH_2-CH_2-

$\underline{C}H_2$ and ε-$\underline{C}H_2$]; 55.55 (+ve, α-$\underline{C}$H); 65.92 (–ve, O-$\underline{C}H_2$-Ph); 112.74 (ab, aromatic b); 115.85 [+ve, aromatic (Aq) $\underline{C}$H]; 119.52 [+ve, aromatic (Aq) $\underline{C}$H]; 127.12 [+ve, aromatic (Aq) $\underline{C}$H]; 127.26 [+ve, aromatic (Aq) $\underline{C}$H]; [128.54 (+ve, aromatic $\underline{C}$H), 129.18 (+ve, aromatic) Z, ortho, meta and para]; 133.22 (ab, aromatic c); 134.30 [+ve, aromatic (Aq) $\underline{C}$H]; [134.79 (ab), 135.22 (ab), aromatic e and f]; [135.36 (+ve, aromatic, Aq-$\underline{C}$H), 136.52 (+ve, aromatic, Aq-$\underline{C}$H), 5 and 8]; 138.13 (ab); 152.25 [ab, aromatic(Aq) $\underline{C}$-1]; 156.90 (ab, NH$\underline{C}$OO); 175.91 (ab, spacer-NH$\underline{C}$O-lys); 183.75 (ab, $\underline{C}$=O); 184.86 (ab, $\underline{C}$=O).

$\underline{C_{34}H_{40}N_4O_7 \text{ requires}}$: C 66.21, H 6.55, N 9.09 %. Found C 65.91, H 6.32, N 9.33 %.

$\underline{\text{ESMS(+)(Cone 50V)}}$ m/z: 579 (15%), 557 (100%)$(RNH_3)^+$, 513 (10%).

$\underline{\text{ESMS(-)(Cone 50 V)}}$ m/z: 59 (100%)(CH_3COO^-). M, 616.

9.7.14 1-[4-(N-α-Tertiarybutoxycarbonyl-N-δ-benzyloxycarbonyl-L-ornithyl-amino)butylamino]anthraquinone (239)

N-α-^{t}Boc-N-δ-Z-L-ornithine was converted to its O-pentafluorophenolate ester (1.45g, 2.73 mmol), using ethyl acetate/ DMF (5:1) as the solvent, and reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(0.73g, 2.48 mmol) [following methods C and D] to give the title compound. Yield [from ethyl acetate/ hexane (1:100)] (0.69g)(43%). T.l.c. (solvent system 1): R_f 0.40 (red) product.

Found: $\underline{\text{mp}}$ 130 °C.

$\underline{^1\text{H nmr spectrum (CDCl}_3\text{, 200MHz)}}$ δ: 1.40 (9H, s, ^{t}Boc); 1.50-1.85 (8H, m, unresolved, Aq-NH-CH_2-$\underline{C}H_2$-$\underline{C}H_2$, β-$\underline{C}H_2$ and γ-$\underline{C}H_2$); 3.05-3.25(2H, m, δ-$\underline{C}H_2$); 3.25-3.45 (4H, m, unresolved, Aq-NH$\underline{C}H_2$-CH_2-CH_2-$\underline{C}H_2$); 4.20 (1H, d, α-$\underline{C}$H); 5.05 (3H, m, N$\underline{H}$CO-$\underline{C}H_2$-Z); 5.25 (1H, d, N$\underline{H}$^{t}Boc); 6.55 (1H, t, spacer-N$\underline{H}$CO-orn); 7.00 (1H, dd, N-2); 7.25 (5H,

brs, $C_6\underline{H}_5$); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.75 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-N<u>H</u>).

<u>$C_{36}H_{42}N_4O_7$ requires</u>: C 67.27, H 6.59, N 8.72 %. Found C 67.22, H 6.44, N 8.48 %.

<u>FABMS(+)</u> m/z: 644 (1%)$(MH)^+$, 542 (2%), 91 (95%), 70 (57%), 57 (100%). M, 643.

9.7.15 1-[4-(N-δ-Benzyloxycarbonyl-L-ornithylamino)butylamino]anthraquinone acetate (240) (NU:UB 48)

Compound (**239**) was selectively deprotected in the same manner as compound (**230**), the analogous propyl spaced ornithine conjugate, to give a red solid of the title compound (0.31g)(61%). T.l.c. (solvent system 3): R_f 0.65 (red) product.

Found: <u>mp</u> 98 °C.

<u>^{1}H nmr spectrum (d_6-DMSO, 200MHz)</u> δ: 1.30-1.80 (8H, unresolved, Aq-NH-CH_2-$C\underline{H}_2$-$C\underline{H}_2$, β-$C\underline{H}_2$ and γ-$C\underline{H}_2$); 1.85 (3H, s, $C\underline{H}_3COO^-$); 3.00 (2H, q, δ-$C\underline{H}_2$); 3.15 (3H, unresolved, $C\underline{H}_2$-NHCO and α-C<u>H</u>); 3.35 (2H, m, Aq-NH-$C\underline{H}_2$); 4.95 (2H, s, OCH_2-Ph); 7.15-7.35 (7H, unresolved, H-2, N<u>H</u>CO-Z and $C_6\underline{H}_5$); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.00 (1H, t, spacer-N<u>H</u>CO-orn); 8.10-8.20 (2H, m, H-5 and H-8); 9.65 (1H, t, Aq-N<u>H</u>).

<u>FAB(+)</u> m/z: 543 (15%)$(RNH_3)^+$, 421 (1%), 318 (5%), 176 (19%), 91 (100%). M, 602.

9.7.16 1-[4-(N-α-Benzyloxycarbonyl-N-ε-tertiarybutoxycarbonyl-L-lysylamino)-butylamino]anthraquinone (241)

N-α-Z-N-ε-^{t}Boc-lysine-N-hydroxysuccinimide ester (1.25g, 2.62 mmol) was reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**) (0.70g, 2.38 mmol)[following method C].

Recrystallisation from ethanol afforded the title compound as a bright red solid (0.77g)(49%). T.l.c. (solvent system 1): R_f 0.45 (red) product.

Found: mp 126 °C.

^{1}H nmr spectrum ($CDCl_3$, 400MHz) δ: 1.35-1.55 (13H, m, unresolved, ^{t}Boc γ-CH_2 and δ-CH_2); 1.60-1.80 (5H, m, unresolved, NH-CH_2-CH_2-CH_2-CH_2-NH and β-CH); 1.90 (1H, m, β-CH′); 3.10 (2H, m, ε-CH_2); 3.25-3.40 (4H, m, unresolved, NH-CH_2-CH_2-CH_2-CH_2-NH); 4.15 (1H, m, α-CH_2); 4.65 (1H, t, NH-^{t}Boc); 5.10 (2H, s, CH_2-Ph); 5.60 (1H, d, NH-Z); 6.40 (1H, t, spacer-NHCO-Lys); 7.0 (1H, d, H-2); 7.30 (5H, br s, C_6H_5); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.75 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

^{13}C nmr spectrum ($CDCl_3$, 100MHz) δ: 22.54 (–ve, γ-CH_2); 26.31 (–ve); 27.19 (–ve); 28.41 [+ve, $C(CH_3)_3$]; 29.65 (–ve); 31.98 (–ve); [39.02 (–ve), 39.77 (–ve), 42.42 (–ve), Aq-NH-CH_2-CH_2-CH_2-CH_2 and ε-CH_2]; 55.03 (+ve, α-CH); 67.07 (–ve, O-CH_2-Ph); 79.21 [ab, $C(CH_3)_3$]; 112.90 (ab, aromatic b); 115.73 [+ve, aromatic (Aq) CH]; 117.83 [+ve, aromatic (Aq) CH]; 126.64 [+ve, aromatic (Aq) CH]; 126.72 [+ve, aromatic (Aq) CH]; [128.08 (+ve, aromatic, CH), 128.34 (+ve, aromatic, CH), 128.50 (+ve, aromatic, CH), Z-ortho, meta and para]; 132.95 [+ve, aromatic (Aq) CH]; 133.05 (ab, aromatic c); 133.93 [+ve, aromatic (Aq) CH]; [134.62 (ab), 134.94 (ab) aromatic e and f]; 135.38 [+ve, aromatic (Aq) CH]; 136.17 [ab, aromatic (Z) C-1]; 151.65 [ab, aromatic (Aq) C-1]; 156.27 (ab, NHCOO); 156.42 (ab, NHCOO); 171.91 (ab, spacer-NHCO-lys); 183.74 (ab, C=O); 185.04 (ab, C=O).

$C_{37}H_{44}N_4O_7$ requires: C 67.66, H 6.75, N 8.53 %. Found C 67.55, H 6.89, N 8.57 %.

FABMS(+) m/z: 680 (1%), 658 (1%)$(RNH_3)^+$, 558 (6%), 277 (5%), 91 (100%). M, 657

9.7.17 1-[4-(N-α-Benzyloxycarbonyl-L-lysylamino)butylamino]anthraquinone acetate (242) (NU:UB 42)

Compound (**241**) was partially deprotected to selectively remove the N-ε-^{t}Boc protecting group in the same manner as for compound (**237**). 1-[4-(N-α-Z-lysylamino)butylamino]-anthraquinone was dissolved in glacial acetic acid and evaporated to dryness to give the title compound (0.10g)(29%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 124°C.

^{1}H nmr spectrum (d_6-DMSO, 400MHz) δ: 1.15-1.70 (10H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2, β-CH_2, γ-CH_2 and δ-CH_2); 1.75 (3H, s, CH_3COO^-); 3.15 (2H, m, ε-CH_2); 3.35 (2H, m, CH_2-NHCO); 3.60 (2H, m, Aq-NH-CH_2); 4.95 (2H, m, OCH_2-Ph); 7.20-7.40 (6H, unresolved, H-2, and C_6H_5); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.75-7.85 (3H, m, H-6 and H-7); 8.00-8.20 (3H, m, H-5, H-8 and spacer-NHCO-lys); 9.70 (1H, t, Aq-NH).

^{13}C nmr spectrum (d_6-DMSO, 100MHz) δ: 23.47 (–ve, γ-CH_2); 26.83 (–ve); 27.52 (–ve); 29.99 (–ve); 32.35 (–ve); [38.92 (–ve), 39.72 (–ve), 42.73 (–ve), Aq-NH-CH_2-CH_2-CH_2-CH_2 and ε-CH_2]; 55.59 (+ve, α-CH); 66.18 (–ve, O-CH_2-Ph); 112.70 (ab, aromatic b); 115.83 [+ve, aromatic (Aq) CH]; 119.48 [+ve, aromatic (Aq) CH]; 127.08 [+ve, aromatic (Aq) CH]; 127.23 [+ve, aromatic (Aq) CH]; [128.49 (+ve, aromatic CH), 128.57 (+ve, aromatic CH), 129.14 (+ve, aromatic CH), Z ortho, meta and para]; 133.18 (ab, aromatic c); 134.26 [+ve, aromatic (Aq) CH]; [134.74 (ab), 135.19 (ab), aromatic e and f]; 135.31 [+ve, aromatic (Aq) CH]; 136.47 [+ve, aromatic (Aq) CH]; 137.90 (ab); 152.20 [ab, aromatic (Aq) C-1]; 156.81 (ab, NHCOO); 172.75 (ab, spacer-NHCO-lys); 183.70 (ab, C=O); 184.80 (ab, C=O).

CIMS(+) m/z: 557 (20%)$(RNH_3)^+$, 423 (100%). M, 616.

9.7.18 1-{4-[N-(α,ε)-Di-tertiarybutoxycarbonyl-L-lysylamino]butylamino}anthraquinone (243)

N-α,ε-di-^{t}Boc-L-lysine-N-hydroxysuccinimide ester (1.3g, 2.90 mmol) was reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(0.75g, 2.55 mmol)[following method C] to give the title compound. Yield [from ethanol/ ether (1:50)] (0.60g)(38%). T.l.c. (solvent system 1): R_f 0.55 (red) product.

Found: mp 108 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.30-1.55 (20H, m, unresolved, α-^{t}Boc, ε-^{t}Boc and γ-CH_2); 1.60-1.90 (8H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2, β-CH_2 and δ-CH_2); 3.10 (2H, q, ε-CH_2); 3.30-3.40 (4H, m, unresolved, Aq-NHCH_2-CH_2-CH_2-CH_2); 4.05 (1H, q, α-CH); 4.60 (1H, br.s, ε-NHBoc); 5.15 (1H, br.s, α-NHBoc); 6.35 (1H, t, spacer-NHCO-lys); 7.05 (1H, dd, N-2); 7.50-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

$C_{34}H_{46}N_4O_7$, requires: C 65.58, H 7.45, N 9.00%. Found C 65.07, H 7.67, N 8.89 %.

FABMS(+) m/z: 646 (1%)$(M+Na)^+$, 623 (2%)$(MH)^+$, 523 (1%)$(M-^{t}Boc)^+$, 423 (13%)$[R(NH_2)NH_3]^+$ 57 (100%)$[(CH_3)C]^+$. M, 622.

9.7.19 1-[4-(L-Lysylamino)butylamino]anthraquinone bis trifluoroacetate (244) /(NU:UB 20)

The N-protected lysine conjugate (**243**)(0.29g) was deprotected using trifluoroacetic acid [following method F] to give a purple solid of the title compound (0.09g)(30%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 185 °C.

^{1}H nmr spectrum (d_6-DMSO, 400MHz) δ: 1.30 (2H, m, γ-C$\underline{H}_2$); 1.45-1.95 (8H, m, unresolved, Aq-NH-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$, β-C$\underline{H}_2$ and γ-C$\underline{H}_2$); 2.75 (2H, m, ε-C$\underline{H}_2$); 3.15 (2H, q, C$\underline{H}_2$-NHCO); 3.40 (2H, Aq-NH-C$\underline{H}_2$); 3.75 (1H, t, α-C$\underline{H}$); 7.20 (1H, d, H-2); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.70-7.80 (2H, m, H-6 and H-7); 7.85-8.35 (8H,unresolved, 2×N$\underline{H}_3^+$, H-5 and H-8); 8.55 (1H, t, N$\underline{H}$CO); 9.65 (1H, t, Aq-N$\underline{H}$).

^{13}C nmr spectrum (d_6-DMSO, 100MHz) δ: 21.80 (–ve, γ-$\underline{C}$H$_2$); 26.56 (–ve); 27.00 (–ve); 27.04 (–ve); 31.05 (–ve); [38.97 (–ve), 39.39 (–ve), 42.36 (–ve), Aq-NH-$\underline{C}$H$_2$-CH$_2$-CH$_2$-$\underline{C}$H$_2$ and ε-$\underline{C}$H$_2$]; 52.64 (+ve, α-$\underline{C}$H); 112.41 (ab, aromatic b); 115.57 [+ve, aromatic (Aq) $\underline{C}$H]; 119.12 [+ve, aromatic (Aq) $\underline{C}$H]; 126.80 [+ve, aromatic (Aq) $\underline{C}$H]; 126.91 [+ve, aromatic (Aq) $\underline{C}$H]; 132.88 (ab, aromatic c); 133.99 [+ve, aromatic (Aq) $\underline{C}$H]; [134.44 (ab), 134.86 (ab) aromatic e and f]; 135.03 [+ve, aromatic (Aq) $\underline{C}$H]; 135.91 (ab); 136.19 [+ve, aromatic (Aq) $\underline{C}$H]; 151.87 [ab, aromatic (Aq) $\underline{C}$-1]; 168.86 (ab, spacer-NH$\underline{C}$O-lys); 183.38 (ab, $\underline{C}$=O); 184.55 (ab, $\underline{C}$=O).

ESMS(+)(Cone 20V) m/z: 423 (100%)[R(NH)$_2$NH$_3$]$^+$, 161 (30%).

ESMS(-)(Cone 20V) m/z: 113 (100%)(CF$_3$COO)$^-$.

9.7.20 1-[4-(N-α-Fluorenylmethoxycarbonyl-N-δ-tertiarybutoxycarbonyl-L-ornithylamino)butylamino]anthraquinone (245)

N-α-Fmoc-N-δ-^{t}Boc-ornithine was converted to its O-pentafluorophenolate ester (2.04g, 3.29 mmol), using ethyl acetate/DMF (5:1) as the solvent, and reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**)(0.88g, 3.00 mmol) [following methods C and D] to give the title compound. Yield [from ethanol/ ether (1:50)] (0.63g)(29%). T.l.c.(solvent system 1): R_f 0.50 (red) product.

Found: mp 168 °C.

FABMS(+) m/z: 754 (1%), 732 (5%)$(MH)^+$, 631 (9%) 179 (96%), 70 (100%). M, 731.

9.7.21 1-[4-(L-Ornithylamino)butylamino]anthraquinone bis trifluoroacetate (246) /(NU:UB 99)

The doubly protected compound (**245**) was dissolved in DMF/ piperidine (4:1) to selectively remove the N-α-Fmoc group [following method G]. T.l.c. of the crude product (solvent system 1): R_f 0.25 (red) amine, 0.52 (red), 0.95 (u.v. active). The resultant 1-[4-(N-δ-^{t}Boc-ornithyl-amino)butylamino]anthraquinone compound was deprotected using TFA [following method F] to give purple crystals of the title compound (0.22g)(52%).

Found: mp 196 °C.

ESMS(+) (sample acidified with 0.2% formic acid) m/z: 409 (100%)$[R(NH)_2NH_3]^+$, 129 (65%), 97 (87%), 65 (45%).

ESMS(-) m/z: 159, (100%), 113 (28%)$(CF_3COO)^-$. M, 636.

9.7.22 4,8-Dihydroxy-1-[4-N-α-tertiarybutoxycarbonyl-N-ε-benzyloxycarbonyl-L-lysylamino)butylamino]anthraquinone (247)

N-α-^{t}Boc-N-ε-Z-L-lysine-N-hydroxysuccinimide ester (1.20g, 1.76 mmol) was reacted with 4,8-dihydroxy-1-[(4-aminobutyl)amino]anthraquinone trifluoroacetate (**160**) (1.00g, 1.47 mmol) in THF (150 cm ^{3}) and triethylamine (1 cm ^{3})[following method C]. Recrystallisation from ethyl acetate/ ethanol afforded the title compound (0.49g)(31%). T.l.c. [chloroform : methanol (3:1)]: R_f 0.60 (purple) product.

Found: mp 162 °C.

FABMS(+) m/z: 711 (15%), 689 (7%)$(MH)^+$, 664 (10%), 413 (53%), 391 (37%), 107 (100%). M, 688.

9.7.23 4,8-Dihydroxy-1-[4-N-ε-benzyloxycarbonyl-L-lysylamino)butylamino]-anthraquinone trifluoroacetate (248)

Compound (**247**)(0.45g) was dissolved in trifluoroacetic acid for exactly 0.25h to selectively remove only the N-α-^{t}Boc group [following method F]. The crude product was purified by column chromatography eluting with chloroform : methanol (10:1).Precipitation with ether afforded the title compound (**248**) (0.34g)(74%). T.l.c. (solvent system 3): R_f 0.50 (purple) product.

Found: mp 155 °C

ESMS(+)(Cone 50V) m/z: 611 (10%)$(M+Na)^+$, 589 (70%)$(RNH_3)^+$, 545 (20%), 97 (100%).

ESMS(-)(Cone -50V) m/z: 113 (60%)$(CF_3COO)^-$, 69 (100%). M, 702.

9.8 OTHER SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

9.8.1 (a) Attempted synthesis of 1-[3-(N-α-tertiarybutoxycarbonyl-L-asparagylamino)propylamino]anthraquinone

N-α-^{t}Boc-L-asparagine was converted to its pentafluorophenolate ester (1.70g, 4.3 mmol) and reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(1.09g, 3.9 mmol) [following methods C and D]. T.l.c. examination of the crude solution showed a mixture of products (solvent system 1): R_f 0.05 (red) spacer, 0.25 (red) trace, 0.35 (red) major product, 0.45 (purple) trace, 0.75 (red) trace, 0.90 (red). The major product was isolated by column chromatography [chloroform : ethyl acetate (4:1)]. Recrystallisation from ethanol gave a red solid (0.25g). Structural determination of the solid by nmr and mass spectroscopy showed that synthesis of the target compound had not been achieved.

Found:

FABMS(+) m/z: 477 (10%)$(MH)^+$, 421 (20%), 57 (100%). M, 476.

Required for title compound m/z: 495. M, 494.

(b) 1-[3-(N-α-Fluorenylmethoxycarbonyl-N-β-trityl-L-asparagylamino)propyl-amino]anthraquinone (249)

N-α-Fmoc-β-trityl-L-asparagine pentafluorophenolate ester (2.00g, 2.62 mmol) was reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**)(0.68g, 2.43 mmol) [following method C]. A red solid of the title compound was obtained from an ethyl acetate/ ether solution (0.51g)(24%). T.l.c. (solvent system 1): R_f 0.25 (red) product.

Found: mp 138 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.80 (2H, qn, Aq-NH-CH_2-CH_2); 2.60 (2H, m, β-CH_2-asn); 3.20 (2H, q, CH_2-NHCO); 3.30-3.45 (3H, m, unresolved, Aq-NH-CH_2 and H-9-Fmoc); 4.10-4.35 (3H, m, unresolved, α-CH and O-CH_2-Fmoc); 7.05-7.25 (17H, m, unresolved, H-2-Aq, NHCO-Fmoc and C_6H_5×3); 7.25-7.45 [3H, m, unresolved, H-3-Aq (H-1 and H-8)-Fmoc]; 7.50-7.75 [5H, m, unresolved, (H-4)-Aq and (H-2, H-4, H-5 and H-7)-Fmoc]; 7.75-7.95 [4H, m, unresolved, (H-6 and H-7)-Aq, (H-3 and H-6)-Fmoc]; 8.05-8.25 (3H, m, NH-Trt, H-5 and H-8); 8.55 (1H, br. s, spacer-NHCO-asn); 9.70 (1H, t, Aq-NH).

FABMS(+) m/z: 882 (1%)$(M+Na)^+$, 860 (2%)$(MH)^+$, 460 (2%), 243 (100%), 89 (40%), 39 (50%). M,859.

9.8.2 1-[3-(L-Asparagylamino)propylamino]anthraquinone trifluoroacetate (250) /(NU:UB 49)

The doubly protected compound (**249**) (0.45g) was dissolved in DMF/ piperidine (4:1) to selectively remove the N-α-Fmoc group [following method G]. The trityl protecting group was removed by dissolving the partially protected compound, 1-[3-(N-β-Trityl-L-asparagylamino)propylamino]anthraquinone (0.18g) in TFA (5 cm^3) for 24h at room temperature. Addition of water (150 cm^3) gave a precipitate of triphenyl carbinol which was filtered off. The filtrate was evaporated to dryness and re-dissolved in a minimum volume of ethanol (3 cm^3) before addition of ether (150 cm^3) to give a precipitate of the title compound (0.15g)(83%). T.l.c. (solvent system 3): R_f 0.40 (red) product.

Found: mp 197 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.80 (2H, qn, Aq-NH-CH_2-CH_2); 2.65 (2H, m, β-CH_2); 3.20 (2H, q, CH_2-NHCO); 3.45 (2H, m, Aq-NH-CH_2); 4.00 (1H, t, α-CH); 7.25 (1H, d, H-2); 7.45 (1H, d, H-4); 7.65 (1H, t, H-3); 7.85-7.95 (2H, m, H-6 and H-7); 7.95-8.10 (3H, br. s, NH_3^+); 8.15-8.25 (2H, m, H-5 and H-8); 8.50 (1H, t, NHCO); 9.70 (1H, t, Aq-NH).

FABMS(+) m/z: 418 (1%), 395 (25%)$(RNH_3)^+$, 368 (8%), 137 (100%), 77.1 (57%), 29 (38%). M, 508.

9.8.3 1-[3-(N-Tertiarybutoxycarbonyl-O-benzyl-L-serylamino)propylamino] anthraquinone (251)

N-^{t}Boc-O-Bzl-serine was converted to its O-pentafluorophenolate ester (3.12g, 6.77 mmol), using ethyl acetate/DMF (5:1) as the solvent, and reacted with 1-[(3-amino-propyl)amino]anthraquinone (**145**)(1.72g, 6.14 mmol) [following methods C and D].

T.l.c. of the crude product (solvent system 1): R_f 0.30 (pink), 0.40 (purple), 0.55 (red) product, 0.75 (purple). The title compound was obtained as fine red crystals from ethanol (2.65g)(77%).

Found: mp 154 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 2.0 (2H, qn, Aq-NH-CH_2-CH_2); 3.35 (2H, q, CH_2-NHCO); 3.50 (2H, q, Aq-NH-CH_2); 3.65 (1H, m, β-CH-ser); 3.95 (1H, m, β-CH′-ser); 4.35 (1H, m, α-CH); 4.55 (2H, m, CH_2-Ph); 5.55 (1H, d, NH-^{t}Boc); 6.70 (1H, t, NHCO-Ser); 7.00 (1H, dd, H-2); 7.25 (5H, br s, C_6H_5); 7.50 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.70-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

$C_{32}H_{35}N_3O_6$ requires: C 68.92, H 6.33, N 7.54 %. Found C 68.59, H 6.36, N 7.45 %.

FABMS(+) m/z: 558 (4%)$(MH)^+$, 502 (4%), 458 (7%), 91 (100%). M, 557.

9.8.4 1-[3-(O-Benzyl-L-serylamino)propylamino]anthraquinone acetate (252) /(NU:UB 22)

Compound (**251**) was partially deprotected using TFA to remove the N-^{t}Boc group [following method F]. The resultant trifluoroacetate salt was dissolved in water, treated with triethylamine and extracted into chloroform prior to column chromatography eluting with chloroform : ethyl acetate (4:1). 1-[3-(O-benzylserylamino)propylamino]-anthraquinone was dissolved in glacial acetic acid and evaporated to dryness to give the title compound (0.28g)(61%). T.l.c. (solvent system 3): R_f 0.50 (red) product.

Found: mp 96 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.80 (2H, qn, Aq-NH-CH_2-CH_2); 1.85 (3H, s, CH_3COO^-); 3.20 (2H, q, CH_2-NHCO); 3.35 (2H, q, Aq-NH-CH_2); 3.45-3.8 (3H, m,

unresolved, α-CH and β-CH_2-ser); 4.50 (2H, s, CH_2-Ph); 7.15-7.35 (6H, m, H-2 and C_6H_5); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.80-8.00 (2H, m, H-6 and H-7); 7.85-8.35 (3H, m, unresolved, H-5, H-8 and NHCO); 9.65 (1H, t, Aq-NH).

$C_{29}H_{31}N_3O_6$ requires: C 67.30, H 6.04, N 8.12 %. Found C 68.20, H 6.10, N 8.47 %

FABMS(+) m/z: 458 (25%)$(RNH_3)^+$,236 (14%), 91 (70%), 39 (100%). M, 517.

9.8.5 1-[4-(N-Fluorenylmethoxycarbonyl-O-tertiarybutyl-L-serylamino)butyl-amino]anthraquinone (253)

N-α-Fmoc-O-^{t}Bu-serine-N-hydroxysuccinimide ester (1.00g, 2.08 mmol) was reacted with 1-[(4-aminobutyl)amino]anthraquinone (**147**) (0.56g, 1.9 mmol) in THF (100 cm^3) [following method C]. A red solid of the title compound was obtained from an ethyl acetate/ ether solution. Yield (0.62g)(49%). T.l.c. (solvent system 1): R_f 0.70 (red) product.

Found: mp 172 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.15 (9H, s, ^{t}Bu); 1.65-1.90 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.35-3.45 (5H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2 and H-9-Fmoc); 3.85 (1H, q, α-CH); 4.20 (2H, m, β-CH_2-ser); 4.40 (2H, d, CH_2-Fmoc, J_{HCCH} 6Hz); 5.85 (1H, d, NHCO-Fmoc); 6.65 (1H, br.s, spacer-NHCO-Ser); 7.05 (1H, dd, H-2-Aq); 7.20-7.40 [4H, m, unresolved, (H-3 and H-4)-Aq, (H-1 and H-8)-Fmoc]; 7.50-7.65 [4H, m, (H-2, H-4, H-5 and H-7)-Fmoc]; 7.70-7.80 [4H, m, (H-6 and H-7)-Aq, (H-3 and H-6)-Fmoc]; 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 683 (1%), 661 (8%)$(MH)^+$, 605 (6%), 179 (100%). M, 660.

9.8.6 1-[4-(L-Serylamino)butylamino]anthraquinone trifluoroacetate (254) /(NU:UB 44)

The doubly protected compound (**253**) (0.45g) was dissolved in DMF/ piperidine (4:1) to selectively remove the N-α-Fmoc group [following method G]. 1-[4-(O-tertiarybutyl-serylamino)butylamino]anthraquinone was dissolved in trifluoroacetic acid (5 cm^3) at room temperature. After 24h the solvent was evaporated and the residue dissolved in water (150 cm^3) prior to the addition of triethylamine (0.5 cm^3). The resultant 1-[4-(serylamino)butylamino]-anthraquinone was extracted into chloroform (150 cm^3), dried (Na_2SO_4), evaporated to a low volume (5 cm^3) and purified by column chromatography [$CHCl_3$:Ethyl Acetate : MeOH (16:3:1)]. The appropriate fractions were combined, evaporated, filtered, dissolved in TFA (5 cm^3) and evaporated. The title compound (0.22g)(79%) was obtained as a red solid from an ethanol/ether (1:50) solution. T.l.c. (solvent system 3): R_f 0.35 (red) product.

Found: mp 162 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.45-1.80 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.25 (2H, q, CH_2-NHCO); 3.35 (2H, q,Aq-NH-CH_2); 3.70-3.85 (3H, m, unresolved, CH_2-OH and α-CH); 5.55 (1H, br. s, OH); 7.20 (1H, d, H-2); 7.40 (1H, d, H-4); 7.60 (1H, t, H-3); 7.75-7.85 (2H, m, H-6 and H-7); 7.90-8.30 (5H,unresolved, NH_3^+, H-5 and H-8); 8.45 (1H, br. s, NHCO); 9.65 (1H, t, Aq-NH).

$C_{23}H_{24}N_3O_6F_3$ requires: C 55.76, H 4.88, N 8.48 %. Found C 55.19, H 4.87, N 8.34 %.

FABMS(+) m/z: 421 (2%)$(RNH_2+K)^+$, 382 (60%)$(RNH_3)^+$, 176 (30%), 136 (32%), 39 (100%). M, 495.

9.8.7 1-[3-(N-α-Fluorenylmethoxycarbonyl-N-im-trityl-L-histidylamino)propyl-amino]anthraquinone (255)

N-α-Fmoc-N-im-trityl-L-histidine pentafluorophenolate ester (1.00g, 1.27 mmol) was reacted with 1-[(3-aminopropyl)amino]anthraquinone (**145**) (0.32g, 1.14 mmol)[following method C]. A red solid of the title compound was obtained from ethyl acetate (0.35g)(35%). T.l.c. (solvent system 1): R_f 0.55 (red) product.

Found: mp 137 °C.

$C_{57}H_{47}N_5O_5$ requires: C 77.62, H 5.37, N 7.94 %. Found C 76.91, H 5.20, N 7.84 %.

FABMS(+) m/z: 904 (100%)$(M+Na)^+$, 882 (20%)$(MH)^+$, 638 (21%), 337 24%). M, 882.

9.8.8 1-[3-(N-im-trityl-L-histidylamino)propylamino]anthraquinone (256)

Compound (**255**)(0.34g) was partially deprotected [following method G], removing the N-α-Fmoc protecting group, to give the title compound (0.21g)(84%). T.l.c. of the crude product [chloroform : methanol (4:1)]: R_f 0.20 (red) product, 0.75.

Found: mp 146 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.90 (2H, qn, Aq-NH-CH_2-CH_2); 2.10 (2H, br. s, NH_2); 2.80 (1H, m, β-CH_2); 3.05 (1H, m, β-CH'_2); 3.35-3.55 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.70 (1H, q, α-CH); 6.65 (1H, s, NHCO); 7.05 (1H, d, H-2); 7.20-7.45 (16H, m, unresolved, 3×C_6H_5 and N-CR=CH-N); 7.50 (1H, t, H-3); 7.60 (1H, d, H-4); 7.70-7.80 (2H, m, H-6 and H-7); 7.85 [(1H, m, N=CH-N); 8.20-8.30 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-NH).

^{13}C nmr spectrum ($CDCl_3$, 100MHz) δ: 29.31 (–ve, Aq-NH-CH_2-CH_2); [32.92 (–ve), 36.99 (–ve), 40.68 (–ve), Aq-NH-CH_2-CH_2-CH_2 and β-CH_2]; 55.56 (+ve, α-CH); 75.32 [ab, $C(Ph)_3$]; 113.08 (ab, aromatic b); 115.76 [+ve, aromatic (Aq) CH]; 117.77 [+ve,

aromatic (Aq) CH]; 119.51 [+ve, aromatic (Aq) CH]; 126.72 [+ve, aromatic (Aq) CH]; [128.07 (+ve, aromatic CH), 129.71 (+ve, aromatic CH) Trt ortho, meta and para]; 132.94 (+ve); 133.94 (+ve); 134.69 (ab); 134.98 (ab); 135.37 (+ve) 137.90 (ab); 138.51 (ab); 142.34 [ab, C-1-his]; 151.61 [ab, aromatic (Aq) C-1]; 174.70 (ab, NHCO); 183.80 (ab, C=O); 185.04 (ab, C=O).

FABMS(+) m/z: 660 (1%)$(MH)^+$, 243 (100%), 165 (21%). M, 659.

9.8.9 1-[3-(L-Histidylamino)propylamino]anthraquinone trifluoroacetate (257) /(NU:UB 30)

The partially deprotected compound (**256**)(0.205g) was dissolved in TFA (5 cm^3) for 1h at room temperature. Addition of water (150 cm^3) gave a precipitate of triphenyl carbinol which was filtered off. Triethylamine was added to the filtrate (2 cm^3) and the resultant 1-[3-(histidylamino)propylamino]anthraquinone was extracted into chloroform (150 cm^3), dried (Na_2SO_4), evaporated to a low volume (5 cm^3) and purified by column chromatography eluting with $CHCl_3$: Ethyl Acetate : MeOH (16:3:1). 1-[3-(histidylamino)propylamino]-anthraquinone was dissolved in TFA and evaporated to dryness to give the title compound (0.10g)(50%). T.l.c. (solvent system 3): R_f 0.30 (red) product.

Found: mp 172 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.80 (2H, q, Aq-NH-CH_2-CH_2); 3.15 (2H, m, β-CH_2); 3.30-3.65 (4H, m, unresolved, Aq-NHCH_2-CH_2-CH_2); 4.05 (1H, t, α-CH); 7.25 (1H, d, H-2); 7.35 (1H, s, N-CR=CH-NH_2^+); 7.45 (1H, d, H-4); 7.60 (1H, T, H-3); 7.65-8.00 (2H, m, H-6 and H-7); 8.05-8.20 (2H, m, H-5 and H-8); 8.60 (1H, t, NHCO); 8.80 (1H, s, N=CH-NH_2^+); 9.70 (1H, t, Aq-NH).

ESMS(+)(Cone 50V) m/z: 418 (100%) $[R(NH_2)NH_3]^+$, 263 (5%), 110 (25%).

ESMS(-)(Cone 50V) m/z: 113 (100%). M, 645.

9.8.10 1-[4-(N-Tertiarybutoxycarbonylsarcosyloxy)butylamino]anthraquinone (258)

Compound (**258**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (2.00g, 1.78 mmol) with N-^{t}Boc-sarcosine (1.30g, 1.77 mmol) [following method E]. T.l.c. of the crude product [toluene : ethyl acetate (1:1)]: R_f 0.25 (red) spacer, 0.55 (red) product, 0.90 (yellow) 1-chloroanthraquinone. Yield [from methanol/ pentane (1:100)] (0.43g)(14%).

Found: mp 90 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, ^{t}Boc); 1.80-1.90 (4H, m, Aq-NH-CH_2-CH_2-CH_2); 2.90 (3H, N-CH_3); 3.40 (2H, q, Aq-NHCH_2); 3.95 (2H, d, CH_2-sar, J 15Hz); 4.25 (2H, t, CH_2-OCO); 7.05 (1H, dd, H-2); 7.45-7.65 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

FABMS(+) m/z: 489 (30%), 467 (63%)$(MH)^+$, 411 (56%), 278 (100%). M, 466.

9.8.11 1-[4-(Sarcosyloxy)butylamino]anthraquinone trifluoroacetate (259) /(NU:UB 163)

The ^{t}Boc-sarcosine conjugate (**258**) (0.38g) was deprotected using TFA [following method F] to give the title compound as a bright red powder (0.36g)(92%). T.l.c. [toluene : ethyl acetate (1:1)]: R_f 0.45 (red) product.

Found: mp 132 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.65-1.85 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 2.55 (3H, s, N-CH_3); 3.45 (2H, q, Aq-NHCH_2); 3.95 (2H, s, CH_2-sar); 4.30

(2H, t, CH_2-OCO); 7.25 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.05-8.20 (2H, m, H-5 and H-8); 9.00 (2H, br.s, $N\underline{H}_2)^+$; 9.70 (1H, t, Aq-N<u>H</u>).

<u>ESMS(+)(Cone 20V)</u> m/z: 367 (100%)$(RNH_3)^+$, 115 (12%), 60 (4%).

<u>ESMS (-)(Cone 50V)</u> m/z: 113 (12%)$(CF_3COO)^-$, 69 (100%). M, 480.

9.8.12 1-[4-(N-α-Tertiarybutoxycarbonyl-α-methyl-L-alanyloxy)butylamino]anthraquinone (260)

Compound (**260**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (0.50g, 1.78 mmol) with N-α-^{t}Boc-N-methyl-L-alanine (0.34g, 1.77 mmol) [following method E]. Yield [from ethyl acetate/ petroleum ether (1:100)] (0.08g)(10%). T.l.c. [toluene : ethyl acetate (1:1)]: R_f 0.50 (red) product.

Found: <u>mp</u> 62 °C.

<u>^{1}H nmr spectrum ($CDCl_3$, 200MHz)</u> δ: 1.35-1.45 (12H, m, unresolved, ^{t}Boc and α-$C\underline{H}_3$); 1.80-1.95 (4H, m, Aq-NH-CH_2-$C\underline{H}_2$-$C\underline{H}_2$); 2.85 (3H, N-$C\underline{H}_3$); 3.40 (2H, q, Aq-NH-$C\underline{H}_2$); 4.20 (2H, t, CH_2-OCO); 4.45 (0.5H, q, α-H rotamer); 4.80 (0.5H, q, α-H rotamer); 7.05 (1H, dd, H-2); 7.50-7.65 (2H, m, H-3 and H-4); 7.65-7.85 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-N<u>H</u>).

<u>FABMS(+)</u> m/z: 503 (26%)$(M+Na)^+$, 481 (98%)$(MH)^+$, 425 (45%), 278 (100%). M, 480.

9.8.13 1-[4-(Methyl-L-alanyloxy)butylamino]anthraquinone trifluoroacetate (261) (NU:UB 166)

The N-^{t}Boc-protected methyl-L-alanine conjugate (**260**)(0.06g) was deprotected using TFA [following method F] to give the title compound (0.045g)(73%). T.l.c. (solvent system 3): R_f 0.50 (red) product.

Found: mp 84 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.45 (3H, d, α-CH_3); 1.70-1.90 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 2.60 (3H, s, N-CH_3); 3.45 (2H, q, Aq-NHCH_2); 4.10 (1H, q, α-CH); 4.70 (2H, t, CH_2-OCO); 7.30 (1H, d, H-2); 7.45 (1H, d, H-4); 7.70 (1H, t, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 9.05 (2H, br.s, NH_2^+); 9.70 (1H, t, Aq-NH).

ESMS(+)(Cone 8V) m/z: 381 (100%)$(RNH_3)^+$.

ESMS(-)(Cone 50V) m/z: 113 (100%)$(CF_3COO)^-$. M, 494.

9.8.14 (S)-1-{4-[2-(N-Tertiarybutoxycarbonylamino)butanoyloxy]butylamino}-anthraquinone (262)

Compound (**262**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (0.50g, 1.78 mmol) with ^{t}Boc-L-2-aminobutanoic acid (0.34g, 1.77 mmol) [following method E]. Yield [from ethyl acetate/ petroleum ether (1:100)] (0.09g)(11%). T.l.c. (solvent system 1): R_f 0.65 (red) product.

Found: mp 58 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 0.95 (3H, t, CH_3-abu); 1.45 (9H, s, ^{t}Boc); 1.60-1.95 (6H, m, unresolved, NH-CH_2-CH_2-CH_2 and CH_2-abu); 3.40 (2H, q, Aq-NH-CH_2); 4.15-4.35 (3H, m, unresolved, CH_2-OCO and α-H); 5.05 (1H, d, NH-Boc); 7.05 (1H, dd,

H-2); 7.55-7.65 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 503 (17%), 481 (66%)$(MH)^+$, 425 (78%), 278 (100%). M, 480.

9.8.15 (S)-1-[4-(2-Aminobutanoyloxy)butylamino]anthraquinone trifluoroacetate (263) (NU:UB 167)

The ^{t}Boc-protected compound (**262**)(0.08g) was deprotected using TFA [following method F] to give the title compound (0.035g)(43%). T.l.c. (solvent system 3): R_f 0.55 (red) product.

Found: mp 118 °C.

ESMS(+)(Cone 8V) m/z: 381 (100%)$(RNH_3)^+$, 179 (40%).

ESMS(-)(Cone 50V) m/z: 113 (100%)$(CF_3COO)^-$. M, 494.

9.8.16 1-{4-[4-(N-Tertiarybutoxycarbonyl)butanoyloxy]butylamino}anthraquinone /(264)

Compound (**264**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (0.50g, 1.78 mmol) with ^{t}Boc-4-aminobutanoic acid (0.34g, 1.77 mmol) [following method E]. Yield [from ethyl acetate/ petroleum ether (1:100)] (0.15g)(19%). T.l.c. [chloroform : ethyl acetate (4:1)]: R_f 0.55 (red) product.

Found: mp 100 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.45 (9H, s, ^{t}Boc); 1.75-1.95 (6H, m, unresolved, NH-CH_2-CH_2-CH_2-CH_2-OCO-CH_2-CH_2); 2.40 (2H, t, OCO-CH_2); 3.18 (2H, q, CH_2-NH-^{t}Boc); 3.40 (2H, q, Aq-NHCH_2); 4.20 (2H, t, CH_2-OCO); 4.65 (1H, br. s, NH-Boc); 7.05

(1H, dd, H-2); 7.50-7.65 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 503 (38%), 481 (100%)$(MH)^+$, 425 (25%), 278 (40%). M, 480.

9.8.17 1-[4-(Butanoyloxy)butylamino]anthraquinone trifluoroacetate (265) /(NU:UB 168)

Treatment of the N-^{t}Boc protected compound (**264**)(0.12g) with TFA [following method F] gave the title compound (0.06g)(50%). T.l.c. (solvent system 3): R_f 0.50 (red) product.

Found: mp 111 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.65-1.90 (6H, m, unresolved, NH-CH_2-CH_2-CH_2-CH_2-OCO-CH_2-CH_2); 2.40 (2H, t, OCO-CH_2); 2.80 (2H, q, CH_2-NH_3^+); 3.45 (2H, q, Aq-NH-CH_2); 4.10 (2H, t, CH_2-OCO); 7.30 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.55-7.80 (4H, m, unresolved, H-3 and NH_3^+); 7.80-8.00 (2H, m, H-6 and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

9.8.18 1-[4-(N-Tertiarybutoxycarbonylmethylalanyloxy)butylamino]anthraquinone /(266)

Compound (**266**) was prepared from the reaction of 1-[(4-hydroxybutyl)amino]anthraquinone (**150**) (0.50g, 1.78 mmol) with N-α-^{t}Boc-α-methylalanine (0.34g, 1.77 mmol) [following method E]. Yield [from ethyl acetate/ petroleum ether (1:100)] (0.11g)(14%). T.l.c. [chloroform : ethyl acetate (4:1)]: R_f 0.70 (red) product.

Found: mp 112 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40 (9H, s, ^{t}Boc); 1.50 [6H, s, $(CH_3)_2$-aib]; 1.80-1.90 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2); 3.40 (2H, q, Aq-NH-CH_2); 4.20 (2H, t, CH_2-OCO); 5.05 (1H, br. s, NHCO-^{t}Boc); 7.05 (1H, dd, H-2); 7.50-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

FABMS(+) m/z: 503 (30%), 481 (95%)$(MH)^+$, 425 (60%), 278 (100%). M, 480.

9.8.19 1-[4-(Methylalanyloxy)butylamino]anthraquinone trifluoroacetate (267) /(NU:UB 169)

Treatment of the N-^{t}Boc-protected conjugate (**266**)(0.095g) with TFA [following method F] gave the title compound (0.085g)(87%). T.l.c. (solvent system 3): R_f 0.60 (red) product. Found: mp 112 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.40 [6H, s, $(CH_3)_2$-aib]; 1.75-1.85 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2-CH_2); 3.40 (2H, q, Aq-NH-CH_2); 4.25 (2H, t, CH_2-OCO); 7.30 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.10-8.25 (2H, m, H-5 and H-8); 8.50 (3H, br. s, NH_3^+); 9.70 (1H, t, ArNH).

ESMS(+)(Cone 8V) m/z: 783 (1%), 761 (2%), 413 (1%), 381 (100%)$(RNH_3)^+$, 119 (20%).

ESMS(-)(Cone 8V) m/z: 113 (100%)$(CF_3COO)^-$. M, 494.

9.9 CHIRAL SPACER-LINKED ANTHRAQUINONE AMINO ACID CONJUGATES

9.9.1 (2S)-2-[(9,10-Dioxoanthryl)amino]propyl (2S)-2-[(tertiarybutoxy)carbonyl-amino]propanoate (268)

Compound (**268**) was prepared from the reaction of 1-[((S)-2-hydroxy-isopropyl)amino]anthraquinone (**154**) (0.20g, 0.71 mmol) with N-^{t}Boc-L-alanine (0.14g, 0.74 mmol) [following method E]. Yield [from ethyl acetate/ pentane (1:20)](0.24g)(75%). T.l.c. (solvent system 1): R_f 0.75 (red) product.

Found: mp 127 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.30-1.45 (15H, m, unresolved, CH_3-ala, CH_3-spacer and ^{t}Boc); 4.00-4.15 (2H, m, unresolved, Aq-NH-CH and α-CH-ala); 4.40 (2H, m, CH_2-OCO); 5.05 (1H, d, NHCO-^{t}Boc); 7.20 (1H, dd, H-2); 7.55-7.65 (2H, m, H-3 and H-4); 7.65-7.85 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.85 (1H, d, Aq-NH).

$C_{25}H_{28}N_2O_6$ requires: C 66.35, H 6.24, N 6.19 %. Found; C 66.29, H 5.86, N 6.14 %.

FABMS(+) m/z: 475 (35%), 453 (66%)$(MH)^+$, 397 (70%), 251 (100%). M, 452.

9.9.2 (2S)-2-[(9,10-Dioxoanthryl)amino]propyl (2S)-2-propanoate trifluoroacetate (269) (NU:UB 170)

Treatment of the N-^{t}Boc protected alanine conjugate (**268**) (0.19g) with trifluoroacetic acid [following method F] gave a red solid of the title compound (0.09g)(47%). T.l.c. (solvent system 1): R_f 0.40 (red) product.

Found: mp 122 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.30-1.45 (6H, m, unresolved, CH_3-ala, CH_3-spacer); 4.05-4.30 (3H, unresolved, Aq-NH-CH and CH_2OCO); 4.40 (1H, q, α-CH-ala); 7.40-7.50 (2H, unresolved, H-2 and H-4); 7.70 (1H, t, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.05-8.20 (2H, m, H-5 and H-8); 8.45 (3H, br.s, NH_3^+); 9.00 (1H, d, Aq-NH).

ESMS(+)(Cone 20V) m/z: 375 (2%), 353 (100%)$(RNH_3)^+$, 264 (60%), 149 (2%), 65 (2%).

ESMS(-)(Cone -20V) m/z: 113 (62%)$(CF_3COO)^-$, 69 (100%). M, 466.

9.9.3 (2S)-2-[(9,10-Dioxoanthryl)pyrrolidin-2-yl]methyl (2S)-2-[(tertiarybutoxy)-carbonylamino]propanoate (270)

Compound (**270**) was prepared from the reaction of 1-[(2S)-2-(hydroxymethyl)pyrrolidinyl]anthraquinone (**155**) (0.50g, 1.63 mmol) with N-^{t}Boc-L-alanine (0.31g, 1.64 mmol) [following method E]. Yield [from ethyl acetate/ pentane (1:100)](0.40g)(51%). T.l.c. [chloroform : ethyl acetate (4:1)]: R_f 0.75 (red) product.

Found: mp 110 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.25 (3H, d, CH_3-ala); 1.45 (9H, s, ^{t}Boc); 1.75 (1H, m, β-CH); 2.00 (2H, m, γ-CH_2); 2.35 (1H, m, β-CH′); 2.55 (1H, dd, δ-CH); 3.80 (1H, m, δ-CH′); 4.20-4.45 (4H, m, unresolved, α-CH-pro, α-CH-ala and CH_2-OCO); 5.40 (1H, d, NHCO-^{t}Boc); 7.45-7.60 (2H, m, H-3 and H-4); 7.65-7.80 (3H, m, H-2, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8).

$C_{27}H_{30}N_2O_6$ requires: C 67.77, H 6.32, N 5.85 %. Found; C 68.27, H 6.00, N 5.78 %.

FABMS(+) m/z: 501 (15%), 479 (100%)$(MH)^+$, 423 (62%), 276 (96%). M, 478.

9.9.4 (2S)-2-[(9,10-Dioxoanthryl)pyrrolidin-2-yl]methyl (2S)-2-aminopropanoate trifluoroacetate salt (271) (NU:UB 171)

Deprotection of the Boc-L-alanine conjugate (**270**) (0.34g) using trifluoroacetic acid [following method F] afforded a red solid of the title compound (0.24g)(69%). T.l.c. (solvent system 3): R_f 0.65 (red) product.

Found: mp 130 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.35 (3H, d, C$\underline{H}_3$-ala); 1.60 (1H, m, β-C$\underline{H}$); 1.95 (2H, m, γ-C$\underline{H}_2$); 2.25 (1H, m, β-C$\underline{H}'$); 2.40 (1H, m, δ-C$\underline{H}$); 3.60 (1H, m, δ-C$\underline{H}'$); 4.10 (1H, q, α-C$\underline{H}$-ala); 4.30 (2H, m, C$\underline{H}_2$-OCO); 4.45 (1H, m, α-C$\underline{H}$-pro); 7.65 (3H, s, H-2, H-3 and H-4); 7.80-7.95 (3H, m, H-6 and H-7); 8.05-8.20 (2H, m, H-5 and H-8); 8.40 (3H, br.s, N$\underline{H}_3^+$).

$\underline{C_{24}H_{23}N_2O_6F_3}$ requires: C 58.54, H 64.71, N 5.69 %. Found; C 58.39, H 4.41, N 5.63 %.

ESMS(+)(Cone 50V) m/z: 401 (1%), 379 (38%)(RNH_3^+), 308 (100%) 104 (70%).

ESMS(-)(Cone 20V) m/z: 113 (100%)$(CF_3COO)^-$. M, 492.

9.9.5 (2S)-2-[(4,8-Dihydroxy-9,10-dioxoanthryl)amino]-3-phenylpropyl (2S)-1-{2-[(tertiarybutoxy)carbonylamino]acetyl}pyrrolidine-2-carboxylate (272)

Compound (**272**) was prepared from the reaction of 4,8-dihydroxy-1-{[(S)-2-hydroxy-1-benzylethyl]amino}anthraquinone (**162**) (0.35g, 0.90 mmol) with N-^{t}Boc-glycyl-L-proline (0.46g, 0.92 mmol) [following method E]. Yield [from ethyl acetate/ hexane (1:50)](0.46g)(79%). T.l.c. (solvent system 2): R_f 0.20 (purple) product.

Found: mp 100 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.40 (9H, s, ^{t}Boc); 1.90-2.25 (4H, m, unresolved, β-C$\underline{H}_2$ and γ-C$\underline{H}_2$); 3.00 (2H, m, C$\underline{H}_2$-phe); 3.60 (2H, m, δ-C$\underline{H}_2$-pro); 3.95 (2H, d, C$\underline{H}_2$-

gly); 4.05-4.35 (3H, unresolved, Aq-NH-CH and CH_2-OCO); 4.55 (1H, m, α-CH-pro); 5.45 (1H, d, NHCO-^{t}Boc); 7.15-7.35 (8H, unresolved, H-2, H-3, H-7 and C_6H_5); 7.60 (1H, m, H-6); 7.85 (1H, dd, H-5, $J_{5,6}$ 8Hz, $J_{5,7}$ 1Hz); 10.15 (1H, d, Aq-NH, J_{HNCH} 8Hz); 13.20 (1H, s, 4-OH); 13.85 (1H, s, 8-OH).

$C_{35}H_{37}N_3O_9$ requires: C 65.31, H 5.79, N 6.53 %. Found; C 65.10, H 5.60, N 6.25 %.

FABMS(+) m/z: 666 (17%), 644 (54%)$(MH)^+$, 413 (26%), 391 (85%), 149 (100%). M, 643.

9.9.6 (2S)-2-[(4,8-Dihydroxy-9,10-dioxoanthryl)amino]-3-phenylpropyl (2S)-1-(2-aminoacetyl)pyrrolidine-2-carboxylate trifluoroacetate (273) (NU:UB 159)

The N-^{t}Boc compound (**272**)(0.30g) was deprotected using trifluoroacetic acid [following method F] to give a purple solid of the title compound (0.25g)(81%). T.l.c. (solvent system 1): R_f 0.15 (red) product. T.l.c. (solvent system 3): R_f 0.60 (red) product.

Found: mp 135 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.75-2.00 (3H, m, unresolved, β-CH-pro and γ-CH_2-pro); 2.15 (1H, m, β-CH′-pro); 3.00 (2H, m, CH_2-phe); 3.50 (2H, m, δ-CH_2-pro); 3.85 (2H, s, CH_2-gly); 4.25 (2H, d, CH_2-OCO); 4.35-4.60 (2H, m, unresolved, Aq-NH-CH and α-CH-pro); 7.15-7.40 (7H, unresolved, H-2 and H-3 and C_6H_5); 7.60 (1H, d, H-7); 7.75 (2H, m, H-5 and H-6); 10.15 (1H, d, Aq-NH).

ESMS(+)(Cone 50V) m/z: 566 (5%), 544 (60%)$(RNH_3)^+$, 87 (100%), 119 (95%).

ESMS(-)(Cone 20V) m/z: 113 (100%)$(CF_3COO)^-$. M, 657.

9.10 SPACER-LINKED-ANTHRAQUINONE DIPEPTIDES

9.10.1 1-[3-(N-α-^{t}Boc-N-ε-Z-lysyl-N-ε-Z-L-lysylamino)propylamino]anthraquinone /(274)

N-α-^{t}Boc-N-ε-Z-L-Lysine-N-Hydroxysuccinimide ester (0.35g, 0.70 mmol) in THF (15cm^3) was added drop-wise to a cooled (0-5°C), stirred solution of 1-[3-(N-ε-Z-lysylamino)propylamino]anthraquinone trifluoroacetate (**227**) (0.38g, 0.60 mmol) and triethylamine (0.2cm^3) [following method C]. The title compound was obtained as a bright red solid from ethanol/ ether (1:100). Yield (0.15g)(30%). T.l.c. (solvent system 1): R_f 0.40 (red) product.

Found: mp 188 °C.

$C_{50}H_{60}N_6O_{10}$ requires: C 66.34, H 6.70, N 9.29%. Found C 65.97 H 6.53, N 9.91%.

FABMS(+) m/z: 928 (35%)$(M+Na)^+$, 906 (60%)$(MH)^+$, 806 (25%), 263 (100%). M, 905.

9.10.2 1-[3-{N-(α,ε)-Di-tertiarybutoxycarbonyl-L-lysyl-glycylamino}propylamino]-anthraquinone (275)

N-α,ε-di-^{t}Boc-L-lysine-N-hydroxysuccinimide ester (2.16g, 4.88 mmol) was reacted with 1-[3-(glycylamino)propylamino]anthraquinone trifluoroacetate (**164**)(2.00g, 4.43 mmol) in THF (150 cm^3) and triethylamine (2 cm^3) [following method C]. T.l.c. of the crude product (solvent system 1): R_f 0.05 (red) AT7, 0.60 (red) product. A red solid of the title compound was obtained from an ethanol/ether solution. Yield (1.10g)(49%).

Found: mp 160 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 1.30-1.45 (20H, m, unresolved, α-^{t}Boc, ε-^{t}Boc and γ-CH_2); 1.65-1.90 (4H, m, unresolved, β-CH_2 and δ-CH_2); 2.05 (2H, qn, Aq-NH-CH_2-CH_2); 3.10 (2H, q, ε-CH_2); 3.20-3.70 (4H, m, unresolved, Aq-NH-CH_2-CH_2-CH_2); 3.90-

4.05 (3H, m, unresolved, C$\underline{H}_2$-gly and α-C$\underline{H}$-lys); 4.65 (1H, t, N$\underline{H}$CO-ε-^{t}Boc); 5.55 (1H, t, N$\underline{H}$CO-α-^{t}Boc); 7.05 (1H, dd, H-2); 7.20 (1H, t, spacer-N$\underline{H}$CO-gly); 7.30 (1H, br.s, gly-N$\underline{H}$CO-lys); 7.50-7.60 (2H, m, H-3 and H-4); 7.70-7.85 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.80 (1H, t, Aq-N$\underline{H}$, exchangeable).

$\underline{C_{35}H_{47}N_5O_8}$ requires: C 63.14, H 7.12, N 10.52 %. Found; C 63.01, H 7.46, N 10.42 %.

FABMS(+) m/z: 666 (3%)$(MH)^+$, 566 (3%), 466 (10%), 263 (20%), 84 (45%), 57 (100%). M, 665.

9.10.3 1-[3-(L-Lysylglycylamino)propylamino]anthraquinone bis trifluoroacetate (276) (NU:UB 23)

Treatment of the N-^{t}Boc protected dipeptide (**275**)(1.00g) with trifluoroacetic acid [following method F] gave a purple solid of the title compound (0.60g)(58%). T.l.c. (solvent system 3): R_f 0.20 (red) product.

Found: mp 147 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.25-1.95 8H, m, unresolved Aq-NH-CH_2-C$\underline{H}_2$, β-C$\underline{H}_2$, γ-C$\underline{H}_2$, δ-C$\underline{H}_2$); 2.75 (2H, t, ε-C$\underline{H}_2$); 3.25 (2H, q, CH_2-C$\underline{H}_2$-NHCO); 3.40 (2H, q, Aq-NH-C$\underline{H}_2$); 3.70-3.90 (3H, m, unresolved, C$\underline{H}_2$-gly and α-C$\underline{H}$-lys); 7.25 (1H, d, H-2); 7.45 (1H, m, H-4); 7.60 (1H, m, H-3); 7.75-8.35 (11H, m, unresolved H-5, H-6, H-7, H-8, N$\underline{H}$CO and 2×RNH_3^+); 8.80 (1H, t, N$\underline{H}$CO); 9.75 (1H, t, Aq-N$\underline{H}$).

FABMS(+) m/z: 488 (1%)$[R(NH_2)_2+Na]^+$, 466 (65%)$[R(NH_2)NH_3]^+$, 263 (20%), 84 (100%). M, 693.

9.10.4 1-[3-(N-Tertiarybutoxycarbonyl-O-benzyl-L-seryl-L-prolylamino)propyl-amino]anthraquinone (277)

N-^{t}Boc-O-benzyl-L-serine was converted to its O-pentafluorophenolate ester (1.03g, 2.23 mmol) and added to a cooled, stirred solution of 1-[3-(prolylamino)propylamino]-anthraquinone trifluoroacetate (**208**)(1.00g, 2.04 mmol) and triethylamine (1 cm^3) [following method C] in DMF. A red solid of the title compound was obtained from an ethanol/ether solution (1.02g)(77%). T.l.c. (solvent system 1): R_f 0.35 (red) product.

Found: mp 128 °C.

^{1}H nmr spectrum ($CDCl_3$, 400MHz) δ: 1.40 (9H, ^{t}Boc); 1.65 (1H, m, β-CH-pro); 1.80 (2H, m, γ-CH_2-pro); 1.85-2.10 (3H, m, unresolved, β-CH′-pro and Aq-NH-CH_2-CH_2); 2.25 (1H, m, δ-CH-pro); 2.90 (1H, m, δ-CH′-pro); 3.25 (2H, m, CH_2-NHCO); 3.60 (2H, m, Aq-NH-CH_2); 3.80 (2H, m, β-CH_2-ser); 4.50 (2H, m, CH_2-Ph); 4.65 (1H, t, α-CH-pro); 4.75 (1H, q, α-CH-ser); 5.45 (1H, d, NH-Boc); 6.80 (1H, t, spacer-NHCO-pro); 7.05 (1H, dd, H-2); 7.15-7.35 (5H, m, C_6H_5); 7.50-7.60 (2H, m, H-3 and H-4); 7.70-7.80 (2H, m, H-6 and H-7); 8.20-8.30 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

^{13}C nmr spectrum ($CDCl_3$, 100MHz) δ: 24.62 (–ve, γ-CH_2); 28.35 [+ve, C(CH_3)$_3$]; 28.46 (–ve); 29.17 (–ve); [36.83 (–ve), 39.98 (–ve), 47.69 (–ve), (Aq-NH-CH_2-CH_2-CH_2-CH_2 and δ-CH_2)]; [51.31 (+ve), 60.54 (+ve), (α-CH-pro and α-CH-ser)]; [71.36 (–ve), 73.82 (–ve), CH_2-O-CH_2-Ph); 80.18 [ab, C(CH_3)$_3$] 112.98 (ab, aromatic b); 115.69 [+ve, aromatic (Aq) CH]; 117.96 [+ve, aromatic (Aq) CH]; 126.70 [+ve, aromatic (Aq) CH); 127.34 [+ve, aromatic (Aq) CH]; 128.21 [+ve, aromatic (Bz) CH); 128.65 [+ve, aromatic (Bz) CH]; 132.90 [+ve, aromatic (Aq) CH]; 133.02 (ab, aromatic c); 133.91 [+ve, aromatic (Aq) CH]; [134.66 (ab), 134.99 (ab) aromatic e and f]; 135.32 [+ve, aromatic (Aq) CH]; 136.91 [ab, aromatic (Bz) C-1]; 151.65 [ab, aromatic (Aq) C-1]; 155.15 (ab,

NHCOO); [170.44 (ab), 171.04 (ab), NHCO and NCO]; 183.79 (ab, C=O); 184.91 (ab, C=O).

$C_{37}H_{42}N_4O_7$ requires: C 67.87, H 6.47, N 8.56 %. Found; C 68.18, H 6.71, N 8.60 %.

FABMS(+) m/z: 677 (3%)$(M+Na)^+$, 655 (4%)$(MH)^+$, 577 (1%), 555 (5%), 378 (5%), 263 (20%), 70 (100%). M, 654.

9.10.5 1-[3-(O-Benzyl-L-seryl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (278) (NU:UB 41)

The above compound (**277**)(0.50g) was partially deprotected using trifluoroacetic acid, removing the ^{t}Boc group, [method F] to give a red solid of the title compound (0.23g)(45%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 118 °C.

^{1}H nmr spectrum (d_6-DMSO)(200MHz) δ: 1.65-2.25 (6H, m, unresolved, β-CH-pro, γ-CH_2- and Aq-NH-CH_2-CH_2); 3.15 (2H, q, δ-CH-pro); 3.50 (2H, m, CH_2-spacer); 3.60-3.90 (4H, m, unresolved, CH_2-spacer and β-CH_2-ser); 4.30 (1H, m, α-CH); 4.45 (1H, m, α-CH); 4.55 (2H, m, CH_2-Ph); 7.20-7.45 (7H, m, unresolved, H-2, H-4 and C_6H_5); 7.65 (1H, m, H-3); 7.80-7.95 (2H, m, H-6 and H-7); 8.00 (1H, t, NHCO); 8.05-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

FABMS(+) m/z: 578 (2%), 556 (22%)$(RNH_3)^+$, 236 (25%), 91 (100%). M, 669.

9.10.6 1-[3-(N-α-tertiarybutoxycarbonyl-ε-benzyloxycarbonyl-L-lysyl-L-alanylamino)propylamino]anthraquinone (279)

N-α-^{t}Boc-N-ε-Z-L-lysine-N-hydroxysuccinimide ester (0.65g, 1.25 mmol) was added to a cooled, stirred solution of 1-[3-(L-alanylamino)propylamino]anthraquinone

trifluoroacetate (**182**) (0.50g, 1.08 mmol) and triethylamine (1 cm^3)[following method C] in THF. A red solid of the title compound was obtained from an ethanol/ether solution (0.25g)(35%). T.l.c. (solvent system 1): R_f 0.35 (red) product.

Found: mp 158 °C.

$C_{39}H_{47}N_5O_8$ requires: C 65.62, H 6.64, N 9.81 %. Found; C 65.26, H 6.73, N 9.62 %.

FABMS(+) m/z: 736 (12%), 714 (5%)$(MH)^+$, 636 (5%), 109 (100%). M, 713.

9.10.7 1-[3-(N-ε-Benzyloxycarbonyl-L-lysyl-L-alanylamino)propylamino]-anthraquinone trifluoroacetate (280)

Compound (**279**)(0.15g) was dissolved in trifluoroacetic acid for exactly 0.5h to selectively remove only the N-α-^{t}Boc group [following method F]. Precipitation with ether afforded the title compound (**280**) (0.065g)(43%). T.l.c. (solvent system 3): R_f 0.65 (red) product.

Found: mp 150 °C.

ESMS(+)(Cone 50V) m/z: 636 (30%), 614 (100%)$(RNH_3)^+$, 570 (10%), 119 (70%).

ESMS(-)(Cone 50V) m/z: 113 (50%)$(CF_3COO)^-$, 69 (100%). M, 727.

9.10.8 1-[3-(Glycyl-L-prolyloxy)propylamino]anthraquinone trifluoroacetate (281) /(NU:UB 116)

1-[3-(N-α-Tertiarybutoxycarbonylglycyl-L-prolyloxy)propylamino]anthraquinone was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (0.52g, 1.78 mmol) with N-^{t}Boc-glycyl-L-proline (0.50g, 1.77 mmol) [Method E] and was deprotected in full [T.l.c. (solvent system 1): R_f 0.40 (red) product] using trifluoroacetic

acid [following method F] gave a red solid of the title compound (0.21g)(37%). T.l.c. (solvent system 3): R_f 0.65 (red) product.

Found: mp 146 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 1.60-2.00 (5H, m, unresolved, β-CH, γ-CH_2 and -CH_2-CH_2-CH_2-); 2.15 (1H, m, β-CH′); 3.25-3.45 (4H, m, unresolved, Aq-NH-CH_2 and δ-CH_2); 3.80 (2H, s, CH_2-gly); 4.25 (2H, t, CH_2OCO); 4.45 (1H, m, α-CH-pro); 7.25 (1H, dd, H-2); 7.40 (1H, dd, H-4); 7.60 (1H, m, H-3); 7.80-8.00 (2H, m, H-6 and H-7); 8.05-8.25 (2H, m, H-5 and H-8); 9.75 (1H, t, Aq-NH).

$C_{26}H_{26}N_3O_7F_3$ requires: C 56.83, H 4.78, N 7.67 %. Found; C 65.16, H 4.60, N 7.54 %.

ESMS(+)(Cone 50V) m/z: 871 (1%), 458 (2%), 436 (100%)$(RNH_3)^+$, 97 (45%).

ESMS(-)(Cone 20V) m/z: 113 (100%)$(CF_3COO)^-$. M, 549.

9.10.9 1-[3-(N-Tertiarybutoxycarbonyl-L-leucylglycyloxy)propylamino]-anthraquinone (282)

Compound (**282**) was prepared from the reaction of 1-[(3-hydroxypropyl)amino]anthraquinone (**149**) (0.49g, 1.74 mmol) with N-^{t}Boc-L-lycylglycine (0.50g, 1.74 mmol) [following method E]. Yield [from methanol/ ether (1:100)](0.29g)(30%). T.l.c. (solvent system 1): R_f 0.65 (red) product.

Found: mp 86 °C.

^{1}H nmr spectrum ($CDCl_3$, 200MHz) δ: 0.85 [6H, m, $(CH_3)_2$-leu]; 1.35 (9H, s, ^{t}Boc); 1.50-1.70 (3H, m, unresolved, β-CH_2 and γ-CH); 2.05 (2H, qn, Aq-NH-CH_2-CH_2-CH_2-); 3.40 (2H, q, Aq-NH-CH_2); 4.10 (2H, d, CH_2-gly); 4.15 (1H, m, α-CH-val); 4.40 (2H, t, CH_2-OCO); 4.80 (1H, d, NHCO-^{t}Boc); 6.80 (1H, t, gly-NHCO-leu); 7.00 (1H, dd, H-2); 7.45-

7.60 (2H, m, H-3 and H-4); 7.60-7.80 (2H, m, H-6 and H-7); 8.15-8.25 (2H, m, H-5 and H-8); 9.70 (1H, t, Aq-NH).

$C_{30}H_{37}N_3O_7$ requires: C 65.32, H 6.76, N 7.62 %. Found; C 65.35, H 6.89, N 7.71 %.

FABMS(+) m/z: 575 (65%), 553 (45%)$(MH)^+$, 497 (8%), 475 (17%), 453 (25%), 236 (100%). M, 552.

9.10.10 1-[3-(L-Leucylglycyloxy)propylamino]anthraquinone trifluoroacetate (283) /(NU:UB 127)

The N-^{t}Boc-lysyl glycine conjugate (**282**)(0.25g) was deprotected using trifluoroacetic acid [following method F] to give a red solid of the title compound (0.19g)(73%). T.l.c. (solvent system 1): R_f 0.35 (red) product.

Found: mp 100 °C.

^{1}H nmr spectrum (d_6-DMSO, 200MHz) δ: 0.90 [6H, m, $(CH_3)_2$-leu]; 1.45-1.85 (3H, m, unresolved, β-CH_2, γ-CH); 2.00 (2H, qn, Aq-CH_2-CH_2-CH_2-); 3.45 (2H, q, Aq-NH-CH_2); 3.80 (1H, t, α-CH-leu); 4.00 (2H, m, CH_2-gly); 4.25 (2H, t, CH_2OCO); 7.30 (1H, dd, H-2); 7.45 (1H, dd, H-4); 7.65 (1H, m, H-3); 7.75-7.95 (2H, m, H-6 and H-7); 8.00-8.30 (5H, unresolved, NH_3^+, H-5 and H-8); 8.95 (1H, t, NHCO); 9.70 (1H, t, Aq-NH).

$C_{27}H_{30}N_3O_7F_3$ requires: C 67.34, H 5.35, N 7.43 %. Found; C 56.85, H 5.24, N 7.26 %.

ESMS(+)(Cone 20V) m/z: 474 (4%), 452 (100%)$(RNH_3)^+$, 119 (2%).

ESMS(-)(Cone 20V) m/z: 113 (100%)$(CF_3COO)^-$. M, 565.

PART B

9.11 SPACER-LINKED-ANTHRAQUINONE OLIGOPEPTIDES

9.11.1 1-[3-(N-Tertiarybutoxycarbonyl-L-leucyl-L-prolylamino)propylamino] anthraquinone (284)

N-^{t}Boc-L-leucine-N-hydroxysuccinimide ester (1.60g, 4.88 mmol) was added to a cooled, stirred, solution of 1-[3-(L-prolylamino)propylamino]anthraquinone trifluoroacetate (**208**) (2.00g, 4.07 mmol) and triethylamine (2 cm^3) in THF (150 cm^3)[following method C]. T.l.c. of the crude product (solvent system 1): R_f 0.25 (red) (NU:UB 31), 0.35 (red) free-NU:UB31, 0.75 (red) product. A red solid of the title compound was precipitated from a chloroform/ hexane solution (1:100). Yield (1.79g)(75%).

Found: mp 78 °C.

FABMS(+) m/z: 613 (70%)$(M+Na)^+$, 591 (100%)$(MH)^+$, 513 (10%), 491 (17%), 378 (35%), 263 (75%). M, 590.

9.11.2 1-[3-(L-Leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (285) (NU:UB 184)

The N-^{t}Boc derivative (**284**)(1.66g) was deprotected using trifluoroacetic acid [following method F] to give a red solid of the title compound. Yield (1.62g)(95%). T.l.c. (solvent system 3): R_f 0.25 (red) product.

Found: mp 126 °C.

ESMS(+)(Cone 20V) m/z: 513 (4%)$(RNH_2+Na)^+$, 491 (100%)$(RNH_3)^+$, 119 (10%), 87 (30%), 55 (2%).

ESMS(-)(Cone 50V) m/z: 113 (70%)$(CF_3COO)^-$, 69 (100%). M, 604.

9.11.3 1-[3-(Glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoro-acetate (286) (NU:UB 185)

N-^{t}Boc-glycine-N-hydroxysuccinimide ester (0.05g, 0.18 mmol) was reacted with 1-[3-(L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (**285**) (0.10g, 0.17 mmol) in THF (20 cm^3) and triethylamine (0.2 cm^3)[following method C] to give 1-[3-(N-tertiarybutoxycarbonylglycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone (0.090g) (82%) [T.l.c. (solvent system 1): R_f 0.40 (red) product]. The Boc-protected compound was deprotected in full using trifluoroacetic acid [following method F] to give a red solid of the title compound. Yield (0.065g)(64%). T.l.c. (solvent system 3): R_f 0.30 (red) product.

Found: mp 144-148 °C.

ESMS(+)(Cone 20V) m/z: 570 (15%)$(RNH_2+Na)^+$, 548 (100%)$(RNH_3)^+$, 239 (10%), 87 (75%), 55 (5%). M, 661.

9.11.4 1-[3-(N-Tertiarybutoxycarbonyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)-propylamino]anthraquinone (287)

N-^{t}Boc-L-leucylglycine (1.0g, 3.47 mmol) was converted to its pentafluorophenolate ester and added to a cooled, stirred, solution of 1-[3-(L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (**284**) (1.0g, 1.66 mmol) and triethylamine (0.5 cm^3) in DMF (80 cm^3) [following methods C and D]. The title compound was precipitated from a chloroform/ hexane solution (1:100). Yield (0.95g)(75%). T.l.c. (solvent system 3): R_f 0.75 (red) product.

Found: mp 112 °C.

FABMS(+) m/z: 783 (95%)$(M+Na)^+$, 761 (50%)$(MH)^+$, 683 (15%), 661 (4%), 378 (100%), 263 (25%). M, 760.

9.11.5 1-[3-(L-Leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate. (288)(NU:UB 186)

The N-^{t}Boc-protected derivative (**287**) (0.95g) was deprotected using trifluoroacetic acid [following method F] to give a red solid of the title compound. Yield (0.92g)(95%). T.l.c. (solvent system 3): R_f 0.45 (red) product.

Found: mp 144 °C.

ESMS(+)(Cone 50V) m/z: 683 (10%)$(RNH_2+Na)^+$, 661 (50%)$(RNH_3)^+$, 491 (2%), 378 (100%), 155 (5%), 87 (35%). M, 774.

9.11.6 1-[3-(L-Alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (289) (NU:UB 204)

N-^{t}Boc-L-alanine-N-hydroxysuccinimide ester (0.02g, 0.07 mmol) in THF (5 cm^3) was reacted with 1-[3-(L-leucyl-glycyl-L-leucyl-L-prolyl-amino)propylamino]anthraquinone trifluoroacetate (**288**) (0.05g, 0.065 mmol) in THF (20 cm^3) and triethylamine (0.2 cm^3) [following method C] to give 1-[3-(N-Tertiarybutoxycarbonyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolyl-amino)propylamino]anthraquinone [(0.055g)(70%), T.l.c. (solvent system 3): R_f 0.75 (red) product]. The Boc-protected compound was deprotected in full using trifluoroacetic acid [following method F] to give a red solid of the title compound (0.035g)(63%). T.l.c. (solvent system 3): R_f 0.25 (red) product.

Found: mp 168 °C.

ESMS(+)(Cone 20V) m/z: 754 (5%)$(RNH_2+Na)^+$, 732 (45%)$(RNH_3)^+$, 239 (15%), 119 (15%), 87 (100%). M, 846.

9.11.7 1-[3-(N-Tertiarybutoxycarbonyl-L-alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone (290)

N-^{t}Boc-L-alanyl alanine (0.25g, 0.95 mmol) was converted to its pentafluorophenolate ester and reacted with 1-[3-(L-Leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone trifluoroacetate (**288**) (0.35g, 4.45 mmol) in DMF (50 cm^3) and triethylamine (0.5 cm^3) [following methods C and D]. The title compound was precipitated from a chloroform/ hexane solution (1:100). Yield (0.27g)(68%). T.l.c. (solvent system 3): R_f 0.75 (red) product.

Found: mp 138 °C.

9.11.8 1-[3-(L-Alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propyl-amino]anthraquinone trifluoroacetate (291) NU:UB 205

The N-^{t}Boc derivative (**290**)(0.27g) was deprotected using trifluoroacetic acid [following method F] to give a red solid of the title compound (0.22g)(82%). T.l.c. (solvent system 3): R_f 0.20 (red) product.

Found: mp 132 °C.

ESMS(+)(Cone 50V) m/z: 825 (12%)$(RNH_2+Na)^+$, 803 (25%)$(RNH_3)^+$, 378 (75%), 239 (5%), 119 (20%), 87 (100%).

ESMS(-)(Cone 50V) m/z: 113 $(CF_3COO)^-$, 69 (100%). M, 917.

9.11.9 1-[3-(N-Tertiarybutoxycarbonyl-D-alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)propylamino]anthraquinone (292)

N-^{t}Boc-D-alanine-N-hydroxysuccinimide ester (0.08g, 0.28 mmol) in THF (5 cm^3) was reacted with 1-[3-(L-alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolyl-amino)propylamino]anthraquinone trifluoroacetate (**291**) (0.21g, 0.23 mmol)) in THF (20 cm^3) and triethylamine (0.2 cm^3) [following method C]. The title compound was precipitated from a chloroform/ hexane solution (1:100). Yield (0.135g)(60%). T.l.c. (solvent system 3): R_f 0.60 (red) product.

Found: mp 126 °C

9.11.10 1-[3-(D-alanyl-L-Alanyl-L-alanyl-L-leucyl-glycyl-L-leucyl-L-prolylamino)-propylamino]anthraquinone trifluoroacetate (293) (NU:UB 187) PL 1

The N-^{t}Boc-protected conjugate (**292**)(0.11g) was deprotected using trifluoroacetic acid [following method F] to give a red solid of the title compound (0.10g)(90%). T.l.c. (solvent system 3): R_f 0.20 (red) product.

Found: mp 166 °C.

ESMS(+)(Cone 50V) m/z: 896 (25%)$(RNH_2+Na)^+$, 874 (80%)$(RNH_3)^+$, 497 (65%), 378 (100%)$(Aq\text{-}Spacer\text{-}Pro\text{-}NH_2)^+$, 239 (10%), 87 (100%).

ESMS(-)(Cone 50V) m/z: 113 (100)$(CF_3COO)^-$. M, 988.

9.12 *In-vitro* Chemosensitivity: MTT Assay Protocol

MAC15A murine colon adenocarcinoma cell line

MAC15A cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum containing a 1% antibiotic mixture under standard tissue culture conditions, and were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested from a stock culture in exponential growth phase and plated in 96-well flat-bottomed plates (180µl per well, lanes 2→12; 180µl medium, lane 1) to achieve a final density of 2×10^3 cells per well. After 2hr incubation 20µl of either fresh medium containing 0.5% DMSO (control, lanes 1 and 2) or medium containing NU:UB compounds (diluted in DMSO and medium) was added to yield a range of final drug concentrations from 100 to 0.01µM.

Following 96hr continuous exposure to drug at 37 °C (to allow sufficient time for cell replication, drug induced death and loss of enzymatic activity), cells were incubated with fresh drug-free medium immediately prior to addition of MTT solution (5mg/ ml). Medium and MTT were removed after 4 hours and 150 µl of DMSO was added. For each plate, the aborbance of the resulting solution was measured at the analytical wave length 580nm of formazan product, using a Labsystem Multiskan. IC_{50} values were obtained from growth curves of drug concentration against % survival and are expressed in µM.

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Appendix 1

Biochemical and Biological Evaluation Protocols

(i) NCI *In Vitro* Anticancer Drug Screen: Chemosensitivity Assay

Cell suspensions (diluted according to the target cell densities of 5000-40,000 cells per well, based on cell growth characteristics of the particular cell type) were added to 96 well plates (100 µl) and preincubated for 24 h at 37 °C for. Test agents were added at twice the intended test concentration in five 10-fold dilutions (highest well concentration 100 µM) and incubated for 48 h in 5% CO_2 atmosphere and 100% humidity. Chemosensitivity was determined using the sulphorhodamine B (SRB) assay (SRB, a protein binding dye, binds to the basic amino acids of cellular macromolecules). A plate reader was used to read the optical densities which were processed to give the special concentration parameters GI_{50}, TGI and LC_{50} and hence generation of mean graphs (Monks *et al* 1991).

(ii) Topoisomerase I and Topoisomerase II[#] (α And β) Relaxation Assays

Stock solution, 100µM and 1000µM of NU:UB compound was made up in DMSO/dH_2O. Plasmid pBR322 DNA, was diluted in dH_2O to 40ng/µl. To make up a total volume of 20µl, buffer, DNA, dH_2O and compound solution were added to Eppendorf tubes as in **Appendix Table 1**.

	DNA Control	**Topoisomerase Control**	**10µM Compound**	**25µM Compound**	**50µM Compound**
Buffer (I or II)	2	2	2	2	2
DNA	10	10	10	10	10
dH_2O	8	7.8	5.8	2.8	6.8
Topoisomerase (I, IIα or IIβ)	-	0.2	0.2	0.2	0.2

Appendix Table 1 Topoisomerase I and II relaxation assay components (volumes in µl)
[#] Topoisomerase II (α And β) were a gift from Caroline Austin, University of Newcastle.

Finally, topoisomerase I (3 units) was added and the contents of the tubes were mixed. Samples were incubated for 30 mins in a waterbath at 37°C. The reaction was terminated by addition of 4μl loading buffer. The plasmid samples were separated on an agarose gel (0.8%) by 1 x TBE at 50V for 2h, or overnight at 16V. DNA was stained in ethidium bromide (1μg/ml in 1 x TBE) for 1h and was then destained in dH_2O water for another hour to reduce background fluorescence. The gel was viewed in UV light and photographed.

The topoisomerase II relaxation assay protocol was the same as the above topoisomerase I relaxation assay protocol, but the topoisomerase I buffer was exchanged for the topoisomerase II buffer and topoisomerase II (α or β, 5 units) replaced topoisomerase I.

(iii) Topoisomerase II (α And β) Decatenation Assays

The topoisomerase II decatenation assay protocol was the same as the above topoisomerase I relaxation assay protocol, but the topoisomerase I buffer was exchanged for the topoisomerase II buffer, topoisomerase II (α or β, 5 units) replaced topoisomerase II and kinetoplast DNA (k-DNA) replaced pBR322 plasmid DNA

(iv)Topoisomerase I and Topoisomerase II (α And β) Cleavage Assays

NU:UB compound stock solutions were prepared. Plasmid pBR322 DNA was prepared at 40ng/μl. Buffer, DNA, dH_2O and compound were added to an Eppendorf as depicted in **Appendix Table 2.**

	DNA Control	**Topoisomerase Control**	**10μM Compound**	**50μM Compound**	**100μM Compound**
Buffer (I or II)	2	2	2	2	2
DNA	10	10	10	10	10
dH_2O	8	4	2	3	2
Compound	-	-	2 (at 100μM)	1 (at 1000μM)	2 (at 1000μM)
Topoisomerase (I, IIα or IIβ)	-	4	4	4	4

Appendix Table 2 Topoisomerase I and II cleavage assay components (volumes in μl)

Topoisomerase I (40units) was added and samples were left in a waterbath at 37°C for 45mins. SDS (10%), (2.2μl) was added and samples left for 30 sec. Proteinase K (5mg/ml), (2.4μl) was added and the samples were incubated in the waterbath, at 37°C for 1h. Loading buffer, 4μl was added to each sample. Samples were loaded onto an agarose gel (0.8%). The gel was electrophoresed in 1 x TBE buffer with ethidium bromide, 0.5μg/ml at 50V for 2h or overnight at 16V. The gel was de-stained in dH_2O for 1h in darkness, viewed in UV light and photographed.

The topoisomerase II (α and β) cleavage assay was performed by a similar procedure to the topoisomerase I cleavage assay. The topoisomerase II buffer was used and suitable

topoisomerase II enzyme. Furthermore, following the addition of SDS, the tubes were left for 30 seconds followed by the addition of 1.5µl of 250mM Na_2EDTA. Proteinase K, 2µl was then added at 0.8mg/ml and the tubes were incubated for 1h at 45° C. Loading buffer, 4µl was added to each sample to terminate the reaction. The samples were then separated on an agarose gel as described above. The electrophoresis was performed in the absence of ethidium bromide and, under these conditions, evidence of cleavable complex formation was detected by the formation of a band of linear DNA that has previously been shown to run between the supercoiled plasmid and the retarded nicked/relaxed DNA bands. The methods were adapted from Austin (1995).

(v)Topoisomerase I and II Immunoband Depletion Assays

The method was adapted from Boege (1996); briefly, 10^6 HL-60 cells were cultivated for 45min with or without drugs. Reactions were terminated by sedimentation of the cells (1000xg, 5min, 4°C) and subsequent lysis in PBS/NP40, RIPA buffer containing pepstatin and leupeptin. Samples were subjected to SDS-polyacrylamide (8%) gel electrophoresis and proteins that had entered the gel were electrophoretically transferred to nitro-cellulose sheets by the semi-dry method. Immunstaining of immobilised proteins was carried out using a polyclonal Ab of human topo I [Topogen], and subsequently anti-human Ig biotinylated whole Ab (sheep)[Amersham], streptavidin horse-radish peroxidase and the ECL system [Amersham]. When large proteins, such as topoisomerase IIα and topoisomerase IIβ were transferred, a greater transfer was achieved by the addition of SDS (0.02%) to the transfer buffer. Western blotting using human anti-human topoisomerase I antibody, mouse monoclonal anti-topoisomerase IIα (Novocastra, Newcastle UK) or mouse monoclonal anti-topoisomerase IIβ (Novocastra,

Newcastle UK) were performed and topoisomerase bands photographed and identified. Band densities were quantified using a Syngene [Cambridge, UK] digital imaging system and GeneSnap software.

(vi) MAC15A *In Vivo* Chemosensitivity

MAC15A cells were obtained from the peritoneum of a donor ascitic mouse. 0.2ml volumes of the cell suspension were then injected subcutaneously into the lower flank region of each mouse. Approximately 10 mice were set up per treatment or control group. Solid, measurable tumours developed after 3 days, at which point treatments were commenced (Day 0). Tumours were measured daily using callipers and volumes calculated from the formula: $(A^2 \times b)/2$. Where A is the smaller diameter and b is the larger. Mice were given a single dose of each compound at its pre-determined (by dose escalation) MTD (Maximum Tolerated Dose).

Growth curves were plotted for each group to compare mean relative tumour volume (RTV) against time in days, the control experiment showing the uninhibited tumour growth pattern without the addition of cytotoxic agent. Statistical analyses were also carried out using the Mann Whitney Test which compares the time taken for each tumour to reach RTV × 2 between control and treated cells.

(vii) HPLC Analysis MMP-9 Incubations and Tissue Metabolism of Prodrugs

Separation was achieved using gradient reversed phase HPLC on a Lichrospher (25 x 4 mm) column. Mobile phase A consisted of 10% acetonitrile: 90% TFA (0.05%) and mobile phase B of 60% acetonitrile: 40% TFA (0.05%). Optimum detection was at the λmax of 248 nm using a flow rate of 1.2 ml/min. The gradient profile was from 60%A

to 5% A over 25 min. Sample preparation for HPLC was by simple protein precipitation. Three volumes of methanol were added to one volume of sample (typically 100µl) which was centrifuged at 3000g for 5 min. The supernatant was injected directly onto the HPLC column.

(viii) *In vitro* Purified Enzyme Incubations

Compounds were incubated *in vitro* with purified recombinant enzyme (either MMP-9 or MMP-2) (Calbiochem, Nottingham, UK). Typically, compounds (drugs) were incubated at 5µM concentrations using 10µl of enzyme, 10µl of drug and 90µl of buffer at 25 °C. The buffer used consisted of 200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 20 µM $ZnSO_4$ and 0.05% Brij35 at pH 7.6.

At various time points 20µl samples were taken and 60µl of methanol added to precipitate proteins and the metabolism of the compounds was analysed by HPLC (as above).

(ix) *In vitro* tumour incubations

Solid tumour (HT1080 – excised from NCI-Nu mice under a Home Office Licence) was homogenised (1:4) in MMP buffer. Tumour homogenates were incubated at 25 °C and compounds (drugs) were added to the homogenate to give a final concentration of 10µM or 100µM. Samples were taken at timed intervals and prepared for HPLC analysis as described above.

Where metabolism was so rapid tumour homogenates required dilution (to 1:500) before analysis.

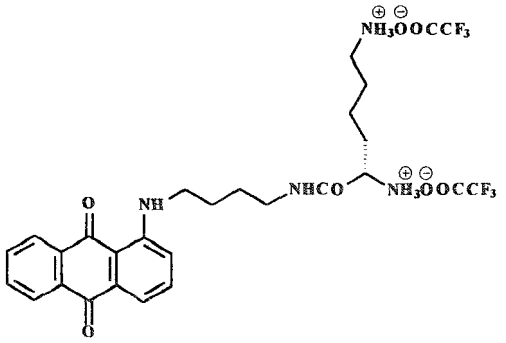
Appendix 2

Mean Graphs and *In Vitro* Test Data for Selected NU:UB Conjugates at the GI_{50}, TGI and LC_{50} Levels

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709764 -Q / 1	**Experiment ID:** 9907MD58-30	**Test Type:** 08	**Units:** Molar
Report Date: August 5, 1999	**Test Date:** July 6, 1999	**QNS:**	**MC:**
COMI: NU:UB 20	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
HL-60(TB)	0.246	0.773	0.977	1.040	0.962	0.412	0.048	139	150	136	31	-80	6.65E-06	1.91E-05	5.34E-05
K-562	0.157	1.310	1.268	1.228	1.156	0.938	0.162	96	93	87	68	0	1.83E-05	>1.00E-04	>1.00E-04
MOLT-4	0.149	0.662	0.705	0.613	0.446	0.314	0.099	108	90	58	32	-34	2.00E-06	3.06E-05	>1.00E-04
SR	0.136	0.461	0.398	0.341	0.357	0.331	0.207	80	63	68	60	22	1.82E-05	>1.00E-04	>1.00E-04
Non-Small Cell Lung Cancer															
A549/ATCC	0.198	0.937	0.917	0.931	0.920	0.579	0.022	97	99	98	52	-89	1.03E-05	2.33E-05	5.27E-05
EKVX	0.630	1.332	1.327	1.344	1.364	1.340	0.116	99	102	104	101	-82	1.90E-05	3.58E-05	6.72E-05
HOP-62	0.199	0.474	0.497	0.478	0.492	0.365	-0.002	108	102	107	60	-100	1.16E-05	2.38E-05	4.88E-05
HOP-92	0.394	0.930	0.968	0.953	0.976	1.008	0.064	107	104	109	114	-84	2.11E-05	3.78E-05	6.75E-05
NCI-H23	0.301	0.907	0.881	0.862	0.849	0.553	0.008	96	93	90	42	-97	6.73E-06	1.99E-05	4.56E-05
NCI-H322M	0.427	1.278	1.263	1.261	1.261	0.890	-0.007	98	98	98	54	-100	1.07E-05	2.25E-05	4.74E-05
NCI-H460	0.312	1.670	1.513	1.648	1.657	0.830	0.006	88	98	99	38	-98	6.38E-06	1.90E-05	4.43E-05
NCI-H522	0.262	0.614	0.627	0.662	0.649	0.537	-0.005	104	114	110	78	-100	1.44E-05	2.75E-05	5.24E-05
Colon Cancer															
COLO 205	0.406	1.335	1.340	1.360	1.470	0.455	0.020	101	103	115	5	-95	3.90E-06	1.13E-05	3.55E-05
HCC-2998	0.360	0.674	0.591	0.654	0.650	0.338	-0.026	74	94	92	-6	-100	2.69E-06	8.67E-06	2.93E-05
HCT-116	0.254	1.353	1.432	1.389	1.509	1.204	-0.005	107	103	114	86	-100	1.57E-05	2.91E-05	5.39E-05
HCT-15	0.169	1.072	1.063	1.101	1.102	0.786	0.001	99	103	103	68	-100	1.28E-05	2.55E-05	5.06E-05
HT29	0.162	0.982	0.981	0.964	0.949	0.771	-0.017	100	98	96	74	-100	1.38E-05	2.67E-05	5.16E-05
KM12	0.286	1.103	1.167	1.123	1.205	0.894	0.001	108	102	112	74	-100	1.38E-05	2.68E-05	5.19E-05
CNS Cancer															
SF-268	0.250	0.972	0.989	1.009	0.993	0.861	0.018	102	105	103	85	-93	1.57E-05	3.00E-05	5.74E-05
SF-295	0.454	1.262	1.268	1.232	1.243	0.869	0.027	101	96	98	51	-94	1.02E-05	2.25E-05	4.98E-05
SF-539	0.568	0.915	0.905	0.925	1.019	0.747	0.013	97	103	130	52	-98	1.02E-05	2.21E-05	4.79E-05
SNB-19	0.335	0.958	0.965	0.950	0.934	0.757	0.022	101	99	96	68	-93	1.29E-05	2.63E-05	5.38E-05
SNB-75	0.265	0.586	0.594	0.571	0.556	0.509	-0.008	102	95	90	76	-100	1.40E-05	2.70E-05	5.20E-05
U251	0.161	0.903	0.838	0.900	0.859	0.715	-0.027	91	100	94	75	-100	1.38E-05	2.68E-05	5.17E-05
Melanoma															
LOX IMVI	0.085	0.772	0.780	0.791	0.795	0.548	0.005	101	103	103	67	-95	1.28E-05	2.61E-05	5.30E-05
MALME-3M	0.367	0.815	0.876	0.914	0.935	0.378	0.008	114	122	127	2	-98	4.14E-06	1.06E-05	3.33E-05
M14	0.295	0.918	0.930	0.903	0.795	0.545	0.020	102	98	80	40	-93	5.67E-06	2.00E-05	4.74E-05
SK-MEL-2	0.257	0.671	0.633	0.660	0.687	0.542	-0.023	91	97	104	69	-100	1.29E-05	2.56E-05	5.06E-05
SK-MEL-28	0.267	0.893	0.898	0.861	0.951	0.976	0.019	101	95	109	113	-93	2.03E-05	3.54E-05	6.19E-05
SK-MEL-5	0.428	1.461	1.466	1.466	1.334	0.513	-0.028	100	100	88	8	-100	2.98E-06	1.19E-05	3.45E-05
UACC-257	0.554	1.173	1.037	1.132	1.187	1.047	0.027	78	93	102	80	-95	1.48E-05	2.86E-05	5.52E-05
UACC-62	0.700	1.654	1.646	1.571	1.660	1.432	-0.008	99	91	101	77	-100	1.42E-05	2.72E-05	5.21E-05
Ovarian Cancer															
IGROV1	0.110	0.744	0.799	0.761	0.746	0.582	0.041	109	103	100	74	-63	1.51E-05	3.48E-05	8.02E-05
OVCAR-3	0.446	0.757	0.769	0.767	0.758	0.675	0.082	104	103	100	73	-82	1.42E-05	2.97E-05	6.25E-05
OVCAR-4	0.265	0.710	0.714	0.726	0.741	0.659	0.062	101	104	107	88	-77	1.71E-05	3.43E-05	6.88E-05
OVCAR-5	0.774	1.690	1.698	1.689	1.734	1.767	0.040	101	100	105	108	-95	1.94E-05	3.41E-05	6.02E-05
OVCAR-8	0.121	0.845	0.832	0.869	0.851	0.679	0.024	98	103	101	77	-81	1.48E-05	3.08E-05	6.40E-05
SK-OV-3	0.281	0.507	0.533	0.507	0.525	0.474	0.038	111	100	108	85	-87	1.60E-05	3.13E-05	6.12E-05
Renal Cancer															
786-0	0.226	0.846	0.898	0.796	0.847	0.604	-0.004	108	92	100	61	-100	1.17E-05	2.39E-05	4.89E-05
A498	0.848	1.356	1.409	1.392	1.493	1.410	0.044	110	107	127	111	-95	1.97E-05	3.45E-05	6.05E-05
ACHN	0.237	0.868	0.919	0.868	0.856	0.653	-0.027	108	100	98	66	-100	1.25E-05	2.50E-05	5.00E-05
CAKI-1	0.454	0.999	1.016	0.989	1.045	1.026	0.009	103	98	108	105	-98	1.86E-05	3.29E-05	5.79E-05
RXF 393	0.359	0.849	0.853	0.809	0.779	0.618	0.104	101	92	86	53	-71	1.05E-05	2.66E-05	6.75E-05
SN12C	0.377	0.920	0.933	0.951	0.994	0.785	0.037	102	106	114	75	-90	1.42E-05	2.85E-05	5.71E-05
TK-10	0.529	1.098	1.061	1.165	1.184	1.082	0.031	93	112	115	97	-94	1.76E-05	3.22E-05	5.88E-05
UO-31	0.242	0.626	0.634	0.573	0.531	0.657	0.012	102	86	75	108	-95	1.93E-05	3.40E-05	6.00E-05
Prostate Cancer															
PC-3	0.189	0.659	0.636	0.654	0.651	0.579	-0.026	95	99	98	83	-100	1.51E-05	2.84E-05	5.33E-05
DU-145	0.211	0.772	0.805	0.762	0.776	0.622	-0.005	106	98	101	73	-100	1.36E-05	2.65E-05	5.15E-05
Breast Cancer															
MCF7	0.245	0.851	0.866	0.882	0.797	0.617	-0.026	102	105	91	61	-100	1.18E-05	2.40E-05	4.90E-05
NCI/ADR-RES	0.299	0.752	0.770	0.756	0.710	0.620	0.030	104	101	91	71	-90	1.35E-05	2.76E-05	5.64E-05
MDA-MB-231/ATCC	0.322	0.900	0.867	0.889	0.819	0.771	0.005	94	98	86	78	-99	1.44E-05	2.76E-05	5.30E-05
MDA-MB-435	0.279	0.918	0.982	0.980	0.974	0.404	-0.011	110	110	109	20	-100	4.56E-06	1.46E-05	3.82E-05
MDA-N	0.352	1.200	1.323	1.293	1.385	0.564	0.025	114	111	122	25	-93	5.51E-06	1.63E-05	4.32E-05
BT-549	0.502	1.080	1.084	1.053	1.108	1.019	-0.025	101	95	105	90	-100	1.62E-05	2.97E-05	5.45E-05
T-47D	0.300	0.743	0.742	0.703	0.684	0.633	0.151	100	91	87	75	-50	1.59E-05	4.00E-05	>1.00E-04



NU:UB 20

National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709764 -Q / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD58-30

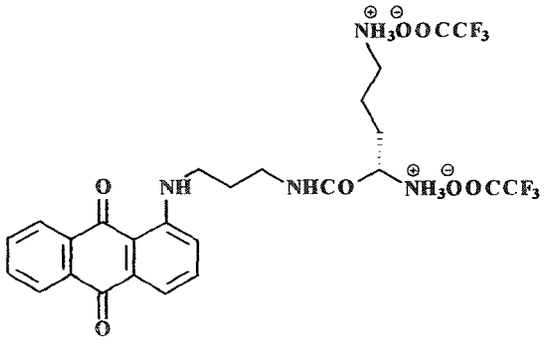
Report Date: August 5, 1999 | Test Date: July 6, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
HL-60(TB)	-5.18	-4.72	-4.27
K-562	-4.74	> -4.00	> -4.00
MOLT-4	-5.70	-4.51	> -4.00
SR	-4.74	> -4.00	> -4.00
Non-Small Cell Lung Cancer			
A549/ATCC	-4.99	-4.63	-4.28
EKVX	-4.72	-4.45	-4.17
HOP-62	-4.94	-4.62	-4.31
HOP-92	-4.68	-4.42	-4.17
NCI-H23	-5.17	-4.70	-4.34
NCI-H322M	-4.97	-4.65	-4.32
NCI-H460	-5.20	-4.72	-4.35
NCI-H522	-4.84	-4.56	-4.28
Colon Cancer			
COLO 205	-5.41	-4.95	-4.45
HCC-2998	-5.57	-5.06	-4.53
HCT-116	-4.80	-4.54	-4.27
HCT-15	-4.89	-4.59	-4.30
HT29	-4.86	-4.57	-4.29
KM12	-4.86	-4.57	-4.28
CNS Cancer			
SF-268	-4.80	-4.52	-4.24
SF-295	-4.99	-4.65	-4.30
SF-539	-4.99	-4.66	-4.32
SNB-19	-4.89	-4.58	-4.27
SNB-75	-4.85	-4.57	-4.28
U251	-4.86	-4.57	-4.29
Melanoma			
LOX IMVI	-4.89	-4.58	-4.28
MALME-3M	-5.38	-4.97	-4.48
M14	-5.25	-4.70	-4.32
SK-MEL-2	-4.89	-4.59	-4.30
SK-MEL-28	-4.69	-4.45	-4.21
SK-MEL-5	-5.53	-4.92	-4.46
UACC-257	-4.83	-4.54	-4.26
UACC-62	-4.85	-4.57	-4.28
Ovarian Cancer			
IGROV1	-4.82	-4.46	-4.10
OVCAR-3	-4.85	-4.53	-4.20
OVCAR-4	-4.77	-4.46	-4.16
OVCAR-5	-4.71	-4.47	-4.22
OVCAR-8	-4.83	-4.51	-4.19
SK-OV-3	-4.80	-4.50	-4.21
Renal Cancer			
786-0	-4.93	-4.62	-4.31
A498	-4.71	-4.46	-4.22
ACHN	-4.90	-4.60	-4.30
CAKI-1	-4.73	-4.48	-4.24
RXF 393	-4.98	-4.58	-4.17
SN12C	-4.85	-4.55	-4.24
TK-10	-4.75	-4.49	-4.23
UO-31	-4.71	-4.47	-4.22
Prostate Cancer			
PC-3	-4.82	-4.55	-4.27
DU-145	-4.87	-4.58	-4.29
Breast Cancer			
MCF7	-4.93	-4.62	-4.31
NCI/ADR-RES	-4.87	-4.56	-4.25
MDA-MB-231/ATCC	-4.84	-4.56	-4.28
MDA-MB-435	-5.34	-4.84	-4.42
MDA-N	-5.26	-4.79	-4.36
BT-549	-4.79	-4.53	-4.26
T-47D	-4.80	-4.40	> -4.00
MG_MID	-4.94	-4.58	-4.26
Delta	0.76	0.48	0.27
Range	1.02	1.06	0.53

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709765 -R / 1	**Experiment ID:** 9907MD58-31	**Test Type:** 08	**Units:** Molar
Report Date: August 5, 1999	**Test Date:** July 6, 1999	**QNS:**	**MC:**
COMI: NU:UB 24	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

		Log10 Concentration												
	Time	Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.107	0.456	0.396	0.365	0.368	0.235	0.022	83	74	75	37	-79	4.44E-06	2.06E-05	5.57E-05
HL-60(TB)	0.246	1.197	1.021	1.090	1.145	0.778	0.048	81	89	95	56	-80	1.11E-05	2.57E-05	5.98E-05
K-562	0.157	1.369	1.279	1.465	1.366	0.842	0.085	93	108	100	56	-46	1.16E-05	3.56E-05	>1.00E-04
MOLT-4	0.149	0.640	0.635	0.644	0.653	0.281	0.062	99	101	103	27	-58	4.96E-06	2.07E-05	7.97E-05
SR	0.136	0.415	0.410	0.456	0.405	0.284	0.208	98	115	96	53	26	1.29E-05	>1.00E-04	>1.00E-04
Non-Small Cell Lung Cancer															
A549/ATCC	0.198	1.042	1.063	1.016	1.034	0.776	0.030	103	97	99	69	-85	1.32E-05	2.79E-05	5.91E-05
EKVX	0.630	1.291	1.253	1.241	1.238	1.031	0.056	94	92	92	61	-91	1.17E-05	2.51E-05	5.35E-05
HOP-62	0.199	0.476	0.458	0.483	0.447	0.345	0.028	93	102	89	52	-86	1.04E-05	2.39E-05	5.48E-05
NCI-H23	0.301	0.903	0.914	0.915	0.886	0.760	0.044	102	102	97	76	-86	1.45E-05	2.96E-05	6.03E-05
NCI-H322M	0.427	1.290	1.270	1.147	1.278	0.911	-0.006	98	83	99	56	-100	1.09E-05	2.29E-05	4.78E-05
NCI-H460	0.312	1.646	1.660	1.620	1.632	1.019	0.025	101	98	99	53	-92	1.05E-05	2.32E-05	5.12E-05
NCI-H522	0.262	0.804	0.797	0.822	0.813	0.530	0.021	99	103	102	49	-92	9.72E-06	2.23E-05	5.04E-05
Colon Cancer															
COLO 205	0.406	1.511	1.424	1.481	1.427	0.891	-0.007	92	97	92	44	-100	7.46E-06	2.02E-05	4.49E-05
HCC-2998	0.360	0.772	0.720	0.730	0.697	0.556	-0.017	87	90	82	48	-100	8.46E-06	2.10E-05	4.58E-05
HCT-116	0.254	1.411	1.386	1.570	1.376	0.818	0.006	98	114	97	49	-98	9.42E-06	2.15E-05	4.72E-05
HCT-15	0.169	1.108	1.198	1.159	1.119	0.871	0.015	110	105	101	75	-91	1.41E-05	2.82E-05	5.65E-05
HT29	0.162	0.995	0.992	0.916	0.946	0.583	0.005	100	90	94	50	-97	1.01E-05	2.20E-05	4.79E-05
KM12	0.286	1.186	1.139	1.197	1.150	0.915	0.044	95	101	96	70	-85	1.34E-05	2.83E-05	5.96E-05
SW-620	0.066	0.457	0.430	0.436	0.417	0.245	-0.001	93	95	90	46	-100	8.04E-06	2.06E-05	4.54E-05
CNS Cancer															
SF-268	0.250	1.052	1.058	1.076	1.066	0.816	0.064	101	103	102	71	-74	1.39E-05	3.07E-05	6.79E-05
SF-295	0.454	1.188	1.195	1.231	1.161	0.895	0.081	101	106	96	60	-82	1.18E-05	2.64E-05	5.94E-05
SF-539	0.568	1.051	0.981	1.034	1.063	0.750	-0.008	85	96	102	38	-100	6.45E-06	1.88E-05	4.33E-05
SNB-19	0.335	1.015	1.050	1.082	1.097	0.728	0.038	105	110	112	58	-89	1.13E-05	2.48E-05	5.44E-05
SNB-75	0.265	0.572	0.561	0.581	0.590	0.497	0.035	96	103	106	75	-87	1.44E-05	2.92E-05	5.93E-05
U251	0.161	0.972	0.956	0.934	0.958	0.616	-0.017	98	95	98	56	-100	1.09E-05	2.29E-05	4.78E-05
Melanoma															
MALME-3M	0.367	0.861	0.873	0.898	0.945	0.903	0.036	102	107	117	109	-90	1.97E-05	3.52E-05	6.28E-05
M14	0.295	1.043	1.044	1.058	1.075	0.931	0.031	100	102	104	85	-90	1.59E-05	3.07E-05	5.93E-05
SK-MEL-2	0.257	0.729	0.738	0.702	0.714	0.617	0.006	102	94	97	76	-98	1.42E-05	2.74E-05	5.31E-05
SK-MEL-28	0.267	0.960	0.911	0.953	0.936	0.869	0.010	93	99	97	87	-96	1.59E-05	2.98E-05	5.59E-05
SK-MEL-5	0.428	1.658	1.526	1.514	1.562	1.236	-0.003	89	88	92	66	-100	1.24E-05	2.49E-05	4.99E-05
UACC-257	0.554	1.147	1.184	1.125	1.156	1.030	0.084	106	96	102	80	-85	1.53E-05	3.06E-05	6.15E-05
UACC-62	0.700	1.598	1.514	1.665	1.516	1.405	0.049	91	107	91	79	-93	1.47E-05	2.87E-05	5.61E-05
Ovarian Cancer															
OVCAR-3	0.446	0.779	0.766	0.767	0.743	0.670	0.103	96	96	89	67	-77	1.32E-05	2.92E-05	6.50E-05
OVCAR-4	0.265	0.729	0.639	0.734	0.690	0.592	0.094	81	101	92	70	-65	1.42E-05	3.32E-05	7.78E-05
OVCAR-5	0.774	1.636	1.580	1.155	1.595	1.466	0.040	94	44	95	80	-95	.	2.87E-05	5.54E-05
SK-OV-3	0.281	0.506	0.491	0.496	0.474	0.407	0.030	93	96	86	56	-89	1.10E-05	2.42E-05	5.36E-05
Renal Cancer															
786-0	0.226	0.792	0.740	0.894	0.847	0.560	0.005	91	118	110	59	-98	1.14E-05	2.38E-05	4.96E-05
A498	0.848	1.398	1.347	1.432	1.397	1.240	0.009	91	106	100	71	-99	1.33E-05	2.62E-05	5.15E-05
ACHN	0.237	0.828	0.852	0.867	0.796	0.532	0.002	104	107	95	50	-99	9.97E-06	2.16E-05	4.67E-05
CAKI-1	0.454	1.077	0.950	1.010	0.927	0.756	0.016	80	89	76	48	-96	8.73E-06	2.16E-05	4.78E-05
RXF 393	0.359	0.824	0.788	0.840	0.840	0.598	0.041	92	103	103	51	-89	1.02E-05	2.33E-05	5.30E-05
SN12C	0.377	1.016	1.029	1.025	0.974	0.644	0.056	102	101	94	42	-85	6.94E-06	2.13E-05	5.29E-05
TK-10	0.529	1.129	1.150	1.135	1.135	0.892	-0.002	103	101	101	61	-100	1.16E-05	2.38E-05	4.88E-05
UO-31	0.242	0.694	0.696	0.658	0.618	0.458	0.029	100	92	83	48	-88	8.62E-06	2.25E-05	5.25E-05
Prostate Cancer															
PC-3	0.189	0.725	0.692	0.710	0.707	0.546	0.005	94	97	97	67	-97	1.26E-05	2.55E-05	5.14E-05
DU-145	0.211	0.776	0.712	0.788	0.799	0.651	-0.007	89	102	104	78	-100	1.43E-05	2.74E-05	5.23E-05
Breast Cancer															
MCF7	0.245	0.888	0.832	0.902	0.741	0.616	0.004	91	102	77	58	-98	1.12E-05	2.34E-05	4.90E-05
NCI/ADR-RES	0.299	0.739	0.794	0.812	0.781	0.620	0.076	112	117	109	73	-75	1.43E-05	3.12E-05	6.81E-05
MDA-MB-231/ATCC	0.322	0.937	0.923	0.889	0.839	0.666	-0.003	98	92	84	56	-100	1.09E-05	2.28E-05	4.78E-05
HS 578T	0.106	0.466	0.464	0.466	0.483	0.374	0.055	99	100	105	74	-49	1.58E-05	4.02E-05	>1.00E-04
MDA-MB-435	0.279	0.952	0.860	0.941	0.915	0.770	0.013	86	98	94	73	-95	1.37E-05	2.71E-05	5.38E-05
MDA-N	0.352	1.246	1.233	1.292	1.397	1.154	0.031	99	105	117	90	-91	1.66E-05	3.13E-05	5.91E-05
BT-549	0.502	1.099	1.098	1.127	1.077	0.912	0.036	100	105	96	69	-93	1.30E-05	2.66E-05	5.42E-05
T-47D	0.300	0.716	0.681	0.688	0.701	0.574	0.153	92	93	96	66	-49	1.37E-05	3.73E-05	>1.00E-04



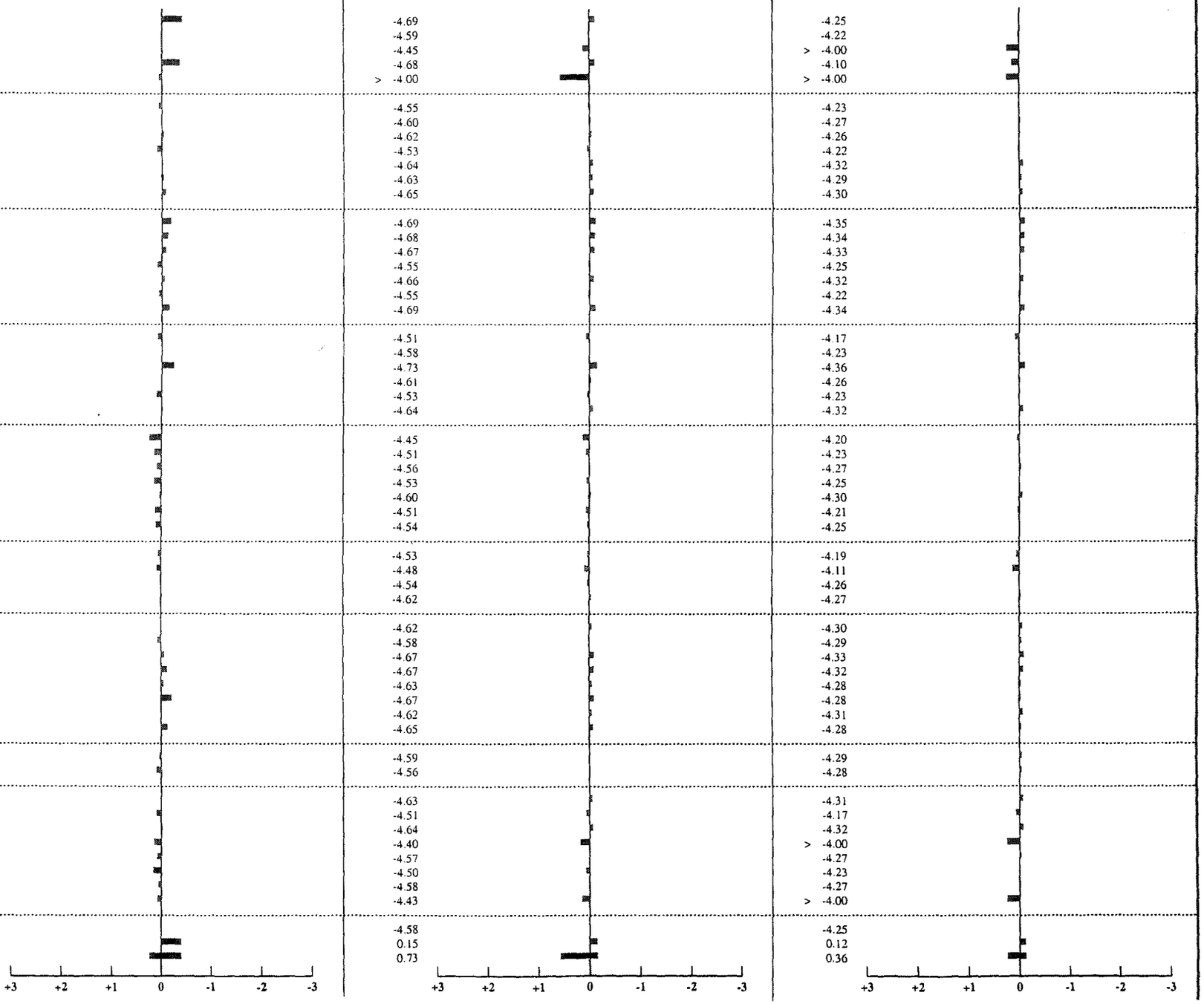
National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709765 -R / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD58-31

Report Date: August 5, 1999 | Test Date: July 6, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-5.35	-4.69	-4.25
HL-60(TB)	-4.95	-4.59	-4.22
K-562	-4.94	-4.45	> -4.00
MOLT-4	-5.30	-4.68	-4.10
SR	-4.89	> -4.00	> -4.00
Non-Small Cell Lung Cancer			
A549/ATCC	-4.88	-4.55	-4.23
EKVX	-4.93	-4.60	-4.27
HOP-62	-4.98	-4.62	-4.26
NCI-H23	-4.84	-4.53	-4.22
NCI-H322M	-4.96	-4.64	-4.32
NCI-H460	-4.98	-4.63	-4.29
NCI-H522	-5.01	-4.65	-4.30
Colon Cancer			
COLO 205	-5.13	-4.69	-4.35
HCC-2998	-5.07	-4.68	-4.34
HCT-116	-5.03	-4.67	-4.33
HCT-15	-4.85	-4.55	-4.25
HT29	-5.00	-4.66	-4.32
KM12	-4.87	-4.55	-4.22
SW-620	-5.09	-4.69	-4.34
CNS Cancer			
SF-268	-4.86	-4.51	-4.17
SF-295	-4.93	-4.58	-4.23
SF-539	-5.19	-4.73	-4.36
SNB-19	-4.95	-4.61	-4.26
SNB-75	-4.84	-4.53	-4.23
U251	-4.96	-4.64	-4.32
Melanoma			
MALME-3M	-4.71	-4.45	-4.20
M14	-4.80	-4.51	-4.23
SK-MEL-2	-4.85	-4.56	-4.27
SK-MEL-28	-4.80	-4.53	-4.25
SK-MEL-5	-4.91	-4.60	-4.30
UACC-257	-4.82	-4.51	-4.21
UACC-62	-4.83	-4.54	-4.25
Ovarian Cancer			
OVCAR-3	-4.88	-4.53	-4.19
OVCAR-4	-4.85	-4.48	-4.11
OVCAR-5		-4.54	-4.26
SK-OV-3	-4.96	-4.62	-4.27
Renal Cancer			
786-0	-4.94	-4.62	-4.30
A498	-4.88	-4.58	-4.29
ACHN	-5.00	-4.67	-4.33
CAKI-1	-5.06	-4.67	-4.32
RXF 393	-4.99	-4.63	-4.28
SN12C	-5.16	-4.67	-4.28
TK-10	-4.94	-4.62	-4.31
UO-31	-5.06	-4.65	-4.28
Prostate Cancer			
PC-3	-4.90	-4.59	-4.29
DU-145	-4.84	-4.56	-4.28
Breast Cancer			
MCF7	-4.95	-4.63	-4.31
NCI/ADR-RES	-4.84	-4.51	-4.17
MDA-MB-231/ATCC	-4.96	-4.64	-4.32
HS 578T	-4.80	-4.40	> -4.00
MDA-MB-435	-4.86	-4.57	-4.27
MDA-N	-4.78	-4.50	-4.23
BT-549	-4.89	-4.58	-4.27
T-47D	-4.86	-4.43	> -4.00
MG_MID	-4.94	-4.58	-4.25
Delta	0.41	0.15	0.12
Range	0.65	0.73	0.36

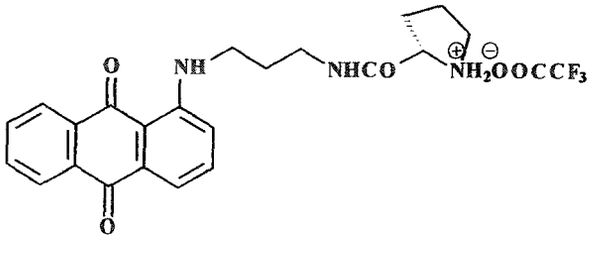


NU:UB 24 (236)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709766 -S / 1	**Experiment ID:** 9907MD59-21	**Test Type:** 08	**Units:** Molar
Report Date: August 6, 1999	**Test Date:** July 12, 1999	**QNS:**	**MC:**
COMI: NU:UB 31	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.300	0.776	0.841	0.816	0.769	0.284	0.083	114	108	99	-6	-72	2.93E-06	8.85E-06	4.63E-05
HL-60(TB)	0.939	2.432	2.420	2.778	2.252	1.808	0.200	99	123	88	58	-79	1.15E-05	2.66E-05	6.17E-05
K-562	0.157	0.916	0.934	0.939	0.890	0.083	0.028	102	103	97	-47	-82	2.11E-06	4.70E-06	1.21E-05
MOLT-4	0.423	1.390	1.382	1.437	1.378	0.411	0.063	99	105	99	-3	-85	3.02E-06	9.38E-06	3.74E-05
SR	1.084	2.934	2.930	2.985	2.998	1.136	0.247	100	103	103	3	-77	3.40E-06	1.08E-05	4.57E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.272	1.342	1.365	1.374	1.314	0.720	0.026	102	103	97	42	-90	7.14E-06	2.07E-05	4.95E-05
EKVX	0.307	0.761	0.771	0.751	0.717	0.537	0.013	102	98	90	51	-96	1.01E-05	2.21E-05	4.86E-05
HOP-62	0.362	0.815	0.829	0.812	0.781	0.529	0.051	103	99	92	37	-86	5.79E-06	1.99E-05	5.10E-05
HOP-92	0.506	0.763	0.751	0.731	0.728	0.569	0.057	95	87	86	25	-89	3.87E-06	1.65E-05	4.54E-05
NCI-H226	0.788	0.987	1.000	1.022	1.017	0.781	0.081	107	118	115	-1	-90	3.65E-06	9.81E-06	3.57E-05
NCI-H23	0.291	0.877	0.902	0.934	0.889	0.614	0.028	104	110	102	55	-91	1.08E-05	2.39E-05	5.27E-05
NCI-H460	0.341	1.989	2.019	1.912	1.981	0.788	0.075	102	95	100	27	-78	4.83E-06	1.81E-05	5.42E-05
NCI-H522	0.267	0.811	0.825	0.853	0.837	0.441	0.024	103	108	105	32	-91	5.66E-06	1.82E-05	4.63E-05
Colon Cancer															
COLO 205	0.553	1.568	1.612	1.637	1.551	0.682	0.027	104	107	98	13	-95	3.66E-06	1.31E-05	3.81E-05
HCC-2998	0.333	0.889	0.900	0.913	0.896	0.487	0.087	102	104	101	28	-74	4.96E-06	1.87E-05	5.80E-05
HCT-116	0.119	0.991	0.948	0.929	0.938	0.218	0.002	95	93	94	11	-98	3.40E-06	1.27E-05	3.63E-05
HCT-15	0.084	0.493	0.523	0.523	0.508	0.143	0.003	107	107	104	14	-97	4.00E-06	1.35E-05	3.79E-05
HT29	0.381	1.555	1.581	1.495	1.526	0.492	0.029	102	95	98	9	-93	3.46E-06	1.24E-05	3.83E-05
KM12	0.279	1.027	1.030	0.936	0.957	0.486	0.029	100	88	91	28	-90	4.41E-06	1.72E-05	4.58E-05
SW-620	0.081	0.497	0.475	0.511	0.456	0.176	0.043	95	103	90	23	-48	3.94E-06	2.11E-05	>1.00E-04
CNS Cancer															
SF-268	0.251	1.025	1.043	1.047	1.043	0.729	0.085	102	103	102	62	-66	1.23E-05	3.03E-05	7.45E-05
SF-295	0.622	1.767	1.829	1.816	1.721	1.146	0.076	105	104	96	46	-88	8.22E-06	2.20E-05	5.21E-05
SNB-19	0.387	1.080	1.067	1.058	1.036	0.665	0.014	98	97	94	40	-97	6.53E-06	1.97E-05	4.57E-05
SNB-75	0.418	0.615	0.660	0.668	0.651	0.476	0.205	123	127	118	29	-51	5.84E-06	2.31E-05	9.70E-05
U251	0.268	1.377	1.304	1.342	1.308	0.807	0.013	93	97	94	49	-95	9.31E-06	2.18E-05	4.85E-05
Melanoma															
LOX IMVI	0.226	1.160	1.136	1.146	1.081	0.100	0.023	97	98	92	-56	-90	1.91E-06	4.18E-06	9.14E-06
MALME-3M	0.319	0.795	0.822	0.811	0.816	0.667	0.031	106	103	104	73	-90	1.39E-05	2.80E-05	5.67E-05
M14	0.402	1.514	1.484	1.408	1.472	0.137	0.043	97	90	96	-66	-89	1.93E-06	3.92E-06	7.96E-06
SK-MEL-28	0.220	0.705	0.693	0.717	0.674	0.148	0.038	97	102	94	-33	-83	2.21E-06	5.49E-06	2.19E-05
SK-MEL-5	0.703	1.609	1.653	1.669	1.610	0.898	.	105	107	100	21	-100	4.34E-06	1.50E-05	3.88E-05
UACC-257	0.615	1.520	1.568	1.497	1.559	0.491	0.096	105	97	104	-20	-84	2.73E-06	6.89E-06	2.91E-05
UACC-62	0.457	1.603	1.587	1.484	1.609	0.349	0.017	99	90	101	-24	-96	2.55E-06	6.45E-06	2.31E-05
Ovarian Cancer															
IGROV1	0.279	0.952	0.930	0.896	0.832	0.449	0.007	97	92	82	25	-97	3.67E-06	1.61E-05	4.10E-05
OVCAR-3	0.534	0.815	0.808	0.822	0.774	0.525	0.072	97	102	85	-2	-87	2.54E-06	9.56E-06	3.71E-05
OVCAR-4	0.510	1.231	1.212	1.244	1.111	0.736	0.037	97	102	83	31	-93	4.37E-06	1.79E-05	4.52E-05
OVCAR-5	0.596	1.300	1.337	1.341	1.315	0.974	0.015	105	106	102	54	-97	1.06E-05	2.27E-05	4.85E-05
OVCAR-8	0.230	0.755	0.753	0.729	0.727	0.395	0.019	100	95	95	31	-92	5.08E-06	1.80E-05	4.57E-05
SK-OV-3	0.356	0.790	0.761	0.766	0.753	0.564	0.052	93	94	91	48	-85	8.90E-06	2.28E-05	5.42E-05
Renal Cancer															
786-0	0.429	1.712	1.689	1.707	1.654	0.904	0.012	98	100	95	37	-97	5.99E-06	1.89E-05	4.45E-05
A498	0.991	1.665	1.634	1.648	1.624	1.308	0.095	95	97	94	47	-90	8.63E-06	2.20E-05	5.08E-05
ACHN	0.341	1.273	1.270	1.260	1.186	0.582	-0.003	100	99	91	26	-100	4.23E-06	1.60E-05	4.00E-05
CAKI-1	0.413	1.112	1.052	1.055	1.021	0.752	0.015	91	92	87	48	-96	9.11E-06	2.16E-05	4.78E-05
RXF 393	0.583	1.244	1.242	1.184	1.152	0.802	0.196	100	91	86	33	-66	4.79E-06	2.15E-05	6.84E-05
SN12C	0.302	0.931	1.002	0.940	0.941	0.511	0.009	111	101	102	33	-97	5.69E-06	1.80E-05	4.36E-05
TK-10	0.520	1.308	1.324	1.287	1.396	0.901	0.092	102	97	111	48	-82	9.41E-06	2.34E-05	5.65E-05
UO-31	0.526	1.220	1.238	1.205	1.147	0.627	0.016	103	98	90	14	-97	3.36E-06	1.35E-05	3.79E-05
Prostate Cancer															
PC-3	0.293	0.977	0.986	1.008	0.948	0.538	0.011	101	104	96	36	-96	5.80E-06	1.87E-05	4.46E-05
DU-145	0.265	0.790	0.800	0.774	0.779	0.466	0.059	102	97	98	38	-78	6.35E-06	2.14E-05	5.76E-05
Breast Cancer															
MCF7	0.316	1.590	1.581	1.490	1.557	0.793	0.059	99	92	97	37	-81	6.17E-06	2.06E-05	5.44E-05
NCI/ADR-RES	0.282	0.759	0.773	0.800	0.785	0.488	0.057	103	109	106	43	-80	7.76E-06	2.24E-05	5.72E-05
MDA-MB-231/ATCC	0.391	0.888	0.909	0.924	0.839	0.548	0.060	104	107	90	32	-85	4.84E-06	1.87E-05	5.03E-05
HS 578T	0.225	0.850	0.890	0.879	0.877	0.691	0.165	106	105	104	74	-27	1.75E-05	5.45E-05	>1.00E-04
MDA-MB-435	0.297	1.132	1.080	1.100	1.067	0.483	0.026	94	96	92	22	-91	4.01E-06	1.57E-05	4.32E-05
MDA-N	0.691	2.289	2.406	2.517	2.470	1.773	0.012	107	114	111	68	-98	1.28E-05	2.56E-05	5.12E-05
BT-549	0.304	0.723	0.760	0.750	0.734	0.560	0.018	109	107	103	61	-94	1.18E-05	2.48E-05	5.19E-05
T-47D	0.433	0.999	1.006	0.969	0.938	0.795	0.226	101	95	89	64	-48	1.33E-05	3.73E-05	>1.00E-04



NU:UB 31

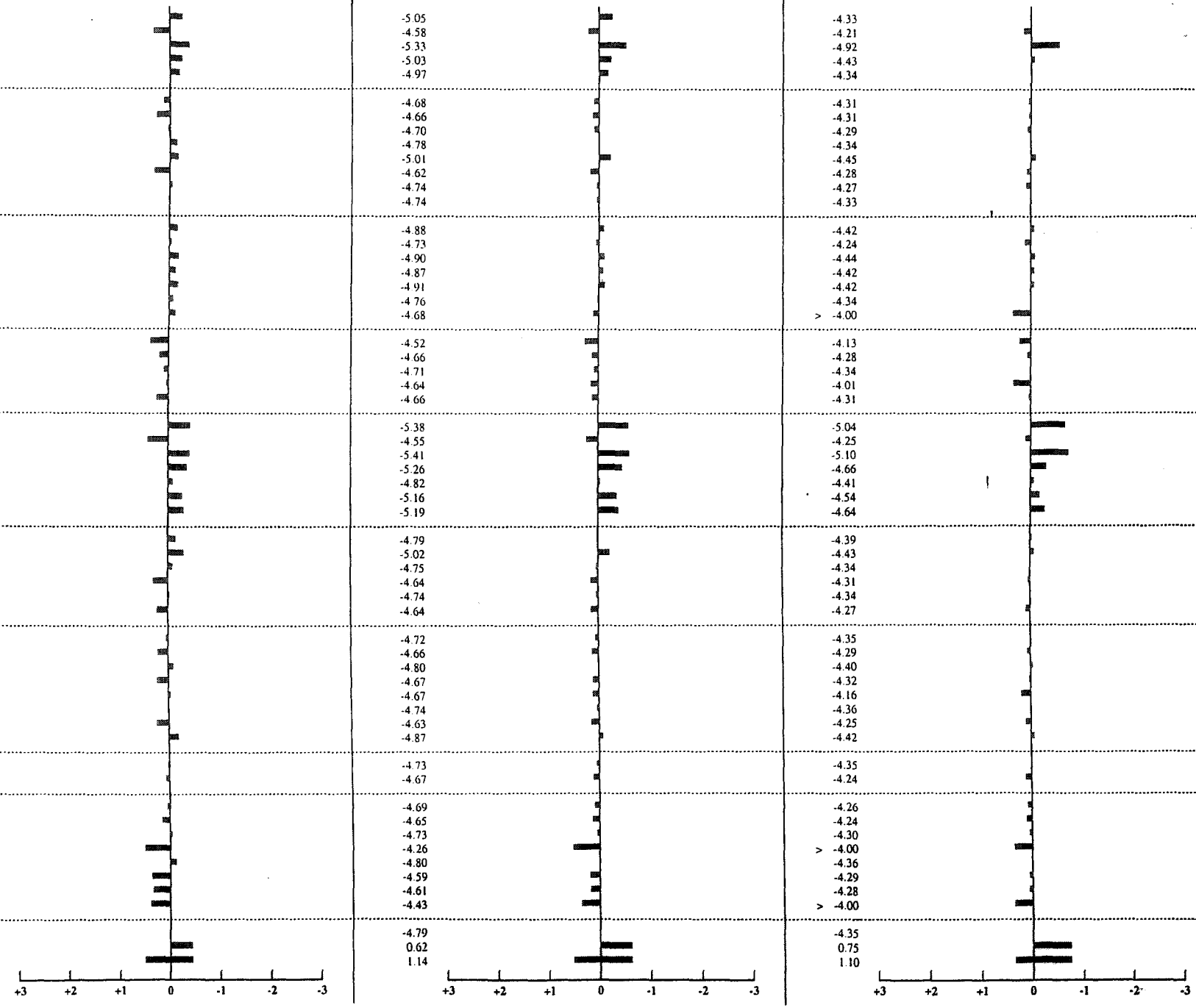
National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709766 -S / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD59-21

Report Date: August 6, 1999 | Test Date: July 12, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-5.53	-5.05	-4.33
HL-60(TB)	-4.94	-4.58	-4.21
K-562	-5.68	-5.33	-4.92
MOLT-4	-5.52	-5.03	-4.43
SR	-5.47	-4.97	-4.34
Non-Small Cell Lung Cancer			
A549/ATCC	-5.15	-4.68	-4.31
EKVX	-5.00	-4.66	-4.31
HOP-62	-5.24	-4.70	-4.29
HOP-92	-5.41	-4.78	-4.34
NCI-H226	-5.44	-5.01	-4.45
NCI-H23	-4.97	-4.62	-4.28
NCI-H460	-5.32	-4.74	-4.27
NCI-H522	-5.25	-4.74	-4.33
Colon Cancer			
COLO 205	-5.44	-4.88	-4.42
HCC-2998	-5.30	-4.73	-4.24
HCT-116	-5.47	-4.90	-4.44
HCT-15	-5.40	-4.87	-4.42
HT29	-5.46	-4.91	-4.42
KM12	-5.36	-4.76	-4.34
SW-620	-5.40	-4.68	> -4.00
CNS Cancer			
SF-268	-4.91	-4.52	-4.13
SF-295	-5.09	-4.66	-4.28
SNB-19	-5.19	-4.71	-4.34
SNB-75	-5.23	-4.64	-4.01
U251	-5.03	-4.66	-4.31
Melanoma			
LOX IMVI	-5.72	-5.38	-5.04
MALME-3M	-4.86	-4.55	-4.25
M14	-5.71	-5.41	-5.10
SK-MEL-28	-5.66	-5.26	-4.66
SK-MEL-5	-5.36	-4.82	-4.41
UACC-257	-5.56	-5.16	-4.54
UACC-62	-5.59	-5.19	-4.64
Ovarian Cancer			
IGROV1	-5.44	-4.79	-4.39
OVCAR-3	-5.60	-5.02	-4.43
OVCAR-4	-5.36	-4.75	-4.34
OVCAR-5	-4.97	-4.64	-4.31
OVCAR-8	-5.29	-4.74	-4.34
SK-OV-3	-5.05	-4.64	-4.27
Renal Cancer			
786-0	-5.22	-4.72	-4.35
A498	-5.06	-4.66	-4.29
ACHN	-5.37	-4.80	-4.40
CAKI-1	-5.04	-4.67	-4.32
RXF 393	-5.32	-4.67	-4.16
SN12C	-5.24	-4.74	-4.36
TK-10	-5.03	-4.63	-4.25
UO-31	-5.47	-4.87	-4.42
Prostate Cancer			
PC-3	-5.24	-4.73	-4.35
DU-145	-5.20	-4.67	-4.24
Breast Cancer			
MCF7	-5.21	-4.69	-4.26
NCI/ADR-RES	-5.11	-4.65	-4.24
MDA-MB-231/ATCC	-5.32	-4.73	-4.30
HS 578T	-4.76	-4.26	> -4.00
MDA-MB-435	-5.40	-4.80	-4.36
MDA-N	-4.89	-4.59	-4.29
BT-549	-4.93	-4.61	-4.28
T-47D	-4.88	-4.43	> -4.00
MG_MID	-5.27	-4.79	-4.35
Delta	0.45	0.62	0.75
Range	0.96	1.14	1.10

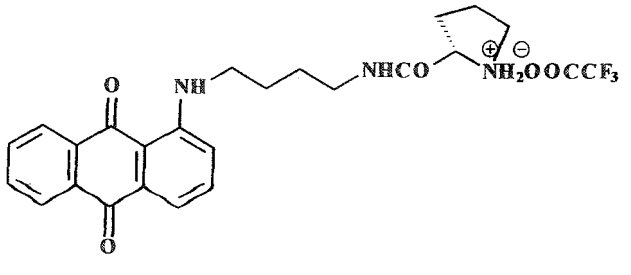


NU:UB 31 (208)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709778 -H / 1	**Experiment ID:** 9908RM73-52	**Test Type:** 08	**Units:** Molar
Report Date: October 7, 1999	**Test Date:** August 30, 1999	**QNS:**	**MC:**
COMI: NU:UB 43	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
K-562	0.076	0.472	0.496	0.462	0.294	0.233	0.188	106	97	55	39	28	2.08E-06	>1.00E-04	>1.00E-04
MOLT-4	0.282	1.050	1.024	0.944	0.581	0.370	0.291	97	86	39	11	1	5.83E-07	>1.00E-04	>1.00E-04
RPMI-8226	0.176	0.785	0.630	0.491	0.353	0.322	0.250	74	52	29	24	12	1.19E-07	>1.00E-04	>1.00E-04
Non-Small Cell Lung Cancer															
A549/ATCC	0.148	1.285	1.309	1.320	1.314	0.566	-0.007	102	103	103	37	-100	6.28E-06	1.86E-05	4.31E-05
EKVX	0.369	0.892	0.876	0.890	0.870	0.679	-0.007	97	100	96	59	-100	1.14E-05	2.36E-05	4.85E-05
HOP-62	0.285	0.551	0.535	0.587	0.561	0.275	0.040	94	114	104	-4	-86	3.17E-06	9.24E-06	3.66E-05
HOP-92	0.219	1.006	1.004	0.972	0.979	0.835	0.038	100	96	97	78	-83	1.50E-05	3.06E-05	6.25E-05
NCI-H23	0.464	1.311	1.320	1.315	1.301	1.031	0.042	101	100	99	67	-91	1.28E-05	2.65E-05	5.50E-05
NCI-H322M	0.378	1.005	1.026	1.048	1.016	0.352	-0.018	103	107	102	-7	-100	2.99E-06	8.62E-06	2.90E-05
NCI-H460	0.232	1.517	1.590	1.442	1.414	0.120	0.019	106	94	92	-48	-92	1.99E-06	4.53E-06	1.10E-05
NCI-H522	0.309	0.920	0.942	0.952	0.967	0.494	0.001	103	105	108	30	-100	5.55E-06	1.71E-05	4.14E-05
Colon Cancer															
COLO 205	0.396	1.586	1.661	1.387	1.530	0.025	-0.001	106	83	95	-94	-100	1.74E-06	3.19E-06	5.87E-06
HCC-2998	0.369	0.983	1.001	1.014	0.928	0.029	0.017	103	105	91	-92	-95	1.67E-06	3.14E-06	5.88E-06
HCT-116	0.145	1.250	1.064	1.153	1.026	0.070	-0.012	83	91	80	-52	-100	1.68E-06	4.04E-06	9.70E-06
HCT-15	0.281	1.661	1.691	1.724	1.642	0.201	0.009	102	105	99	-29	-97	2.41E-06	5.96E-06	2.05E-05
HT29	0.163	0.963	0.954	0.976	0.947	0.121	-0.012	99	102	98	-26	-100	2.44E-06	6.16E-06	2.11E-05
KM12	0.278	1.547	1.519	1.541	1.526	0.488	0.096	98	100	98	17	-66	3.90E-06	1.59E-05	6.45E-05
SW-620	0.118	0.515	0.477	0.554	0.527	0.153	0.029	90	110	103	9	-75	3.66E-06	1.27E-05	4.99E-05
CNS Cancer															
SF-268	0.376	1.473	1.519	1.508	1.481	1.027	0.049	104	103	101	59	-87	1.16E-05	2.54E-05	5.59E-05
SF-295	0.588	1.379	1.393	1.435	1.364	0.352	0.031	102	107	98	-40	-95	2.23E-06	5.12E-06	1.51E-05
SF-539	0.269	0.430	0.408	0.379	0.381	0.004	-0.005	87	69	70	-99	-100	1.31E-06	2.60E-06	5.15E-06
SNB-19	0.279	1.314	1.310	1.294	1.280	0.644	-0.009	100	98	97	35	-100	5.75E-06	1.82E-05	4.27E-05
SNB-75	0.245	0.543	0.511	0.519	0.485	0.391	0.037	89	92	80	49	-85	9.29E-06	2.32E-05	5.49E-05
U251	0.212	0.978	1.019	1.033	0.955	0.261	0.009	105	107	97	6	-96	3.30E-06	1.15E-05	3.55E-05
Melanoma															
LOX IMVI	0.145	1.449	1.439	1.456	1.436	0.065	0.061	99	100	99	-55	-58	2.08E-06	4.39E-06	9.26E-06
MALME-3M	0.489	1.142	1.153	1.178	1.200	0.883	0.007	102	106	109	60	-99	1.16E-05	2.39E-05	4.94E-05
M14	0.543	2.067	2.082	2.151	2.043	0.058	-0.008	101	105	98	-89	-100	1.81E-06	3.34E-06	6.17E-06
SK-MEL-2	0.615	1.248	1.261	1.329	1.219	0.232	0.014	102	113	95	-62	-98	1.94E-06	4.03E-06	8.36E-06
SK-MEL-28	0.464	1.116	1.114	1.140	1.123	0.139	0.031	100	104	101	-70	-93	1.99E-06	3.89E-06	7.63E-06
SK-MEL-5	0.425	1.516	1.469	1.367	1.549	0.091	-0.006	96	86	103	-79	-100	1.96E-06	3.69E-06	6.95E-06
UACC-257	0.665	1.501	1.509	1.474	1.411	0.054	0.009	101	97	89	-92	-99	1.65E-06	3.11E-06	5.87E-06
UACC-62	0.543	1.631	1.660	1.650	1.681	0.073	0.022	103	102	105	-87	-96	1.93E-06	3.52E-06	6.43E-06
Ovarian Cancer															
IGROV1	0.123	1.168	1.180	1.154	1.150	0.792	0.016	101	99	98	64	-87	1.24E-05	2.65E-05	5.69E-05
OVCAR-3	0.482	0.752	0.737	0.771	0.745	0.379	0.031	94	107	97	-21	-94	2.50E-06	6.60E-06	2.48E-05
OVCAR-4	0.446	1.424	1.375	1.366	1.334	0.995	0.041	95	94	91	56	-91	1.10E-05	2.41E-05	5.28E-05
OVCAR-5	0.463	1.144	1.137	1.175	1.146	0.767	-0.008	99	105	100	45	-100	8.01E-06	2.04E-05	4.51E-05
OVCAR-8	0.116	0.931	0.956	0.966	0.953	0.433	0.041	103	104	103	39	-65	6.70E-06	2.37E-05	7.22E-05
SK-OV-3	0.436	0.736	0.716	0.738	0.683	0.513	0.024	93	101	82	26	-94	3.72E-06	1.64E-05	4.26E-05
Renal Cancer															
A498	0.535	1.357	1.351	1.310	1.308	0.472	0.055	99	94	94	-12	-90	2.61E-06	7.74E-06	3.09E-05
ACHN	0.386	1.532	1.538	1.509	1.491	0.840	0.036	100	98	96	40	-91	6.55E-06	2.01E-05	4.87E-05
CAKI-1	0.389	1.868	1.817	1.794	1.757	0.835	0.057	97	95	92	30	-85	4.80E-06	1.82E-05	4.94E-05
RXF 393	0.435	1.110	1.088	1.087	1.057	0.446	0.090	97	96	92	2	-79	2.92E-06	1.05E-05	4.33E-05
SN12C	0.440	1.000	0.902	0.866	0.886	0.557	-0.005	83	76	80	21	-100	3.19E-06	1.49E-05	3.86E-05
TK-10	0.631	1.361	1.340	1.223	1.247	0.981	0.012	97	81	84	48	-98	8.75E-06	2.13E-05	4.68E-05
UO-31	0.159	0.744	0.671	0.748	0.787	0.057	-0.003	88	101	107	-64	-100	2.16E-06	4.22E-06	8.24E-06
Prostate Cancer															
PC-3	0.192	0.959	0.985	1.021	0.904	0.470	0.003	103	108	93	36	-98	5.72E-06	1.86E-05	4.37E-05
DU-145	0.236	1.039	1.050	1.040	1.062	0.739	0.026	101	100	103	63	-89	1.21E-05	2.59E-05	5.53E-05
Breast Cancer															
MCF7	0.233	1.141	1.148	1.079	1.164	0.583	0.053	101	93	103	39	-77	6.62E-06	2.15E-05	5.82E-05
NCI/ADR-RES	0.419	1.081	1.189	1.206	1.136	0.794	0.210	116	119	108	57	-50	1.16E-05	3.40E-05	>1.00E-04
MDA-MB-231/ATCC	0.433	0.954	0.912	0.968	0.922	0.548	0.021	92	103	94	22	-95	4.07E-06	1.54E-05	4.11E-05
HS 578T	0.585	1.380	1.421	1.394	1.380	0.988	0.116	105	102	100	51	-80	1.01E-05	2.44E-05	5.87E-05
MDA-MB-435	0.332	1.578	1.513	1.569	1.572	0.261	0.070	95	99	99	-21	-79	2.57E-06	6.65E-06	3.13E-05
MDA-N	0.210	0.904	0.907	0.904	0.877	0.053	-0.013	101	100	96	-75	-100	1.86E-06	3.64E-06	7.14E-06
BT-549	0.640	1.475	1.515	1.520	1.536	1.280	0.037	105	105	107	77	-94	1.43E-05	2.81E-05	5.51E-05
T-47D	0.379	1.003	0.995	0.971	0.956	0.727	0.113	99	95	92	56	-70	1.11E-05	2.77E-05	6.91E-05



NU:UB 43

National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709778 -H / 1 | Units: Molar | SSPL: 0B62 | Exp. ID: 9908KM75-32

Report Date: October 7, 1999 | **Test Date:** August 30, 1999

Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
K-562	-5.68		> -4.00		> -4.00	
MOLT-4	-6.23		> -4.00		> -4.00	
RPMI-8226	-6.92		> -4.00		> -4.00	
Non-Small Cell Lung Cancer						
A549/ATCC	-5.20		-4.73		-4.37	
EKVX	-4.94		-4.63		-4.31	
HOP-62	-5.50		-5.03		-4.44	
HOP-92	-4.82		-4.51		-4.20	
NCI-H23	-4.89		-4.58		-4.26	
NCI-H322M	-5.52		-5.06		-4.54	
NCI-H460	-5.70		-5.34		-4.96	
NCI-H522	-5.26		-4.77		-4.38	
Colon Cancer						
COLO 205	-5.76		-5.50		-5.23	
HCC-2998	-5.78		-5.50		-5.23	
HCT-116	-5.77		-5.39		-5.01	
HCT-15	-5.62		-5.22		-4.69	
HT29	-5.61		-5.21		-4.68	
KM12	-5.41		-4.80		-4.19	
SW-620	-5.44		-4.90		-4.30	
CNS Cancer						
SF-268	-4.94		-4.60		-4.25	
SF-295	-5.65		-5.29		-4.82	
SF-539	-5.88		-5.59		-5.29	
SNB-19	-5.24		-4.74		-4.37	
SNB-75	-5.03		-4.63		-4.26	
U251	-5.48		-4.94		-4.45	
Melanoma						
LOX IMVI	-5.68		-5.36		-5.03	
MALME-3M	-4.94		-4.62		-4.31	
M14	-5.74		-5.48		-5.21	
SK-MEL-2	-5.71		-5.39		-5.08	
SK-MEL-28	-5.70		-5.41		-5.12	
SK-MEL-5	-5.71		-5.43		-5.16	
UACC-257	-5.78		-5.51		-5.23	
UACC-62	-5.71		-5.45		-5.19	
Ovarian Cancer						
IGROV1	-4.91		-4.58		-4.24	
OVCAR-3	-5.60		-5.18		-4.61	
OVCAR-4	-4.96		-4.62		-4.28	
OVCAR-5	-5.10		-4.69		-4.35	
OVCAR-8	-5.17		-4.63		-4.14	
SK-OV-3	-5.43		-4.79		-4.37	
Renal Cancer						
A498	-5.58		-5.11		-4.51	
ACHN	-5.18		-4.70		-4.31	
CAKI-1	-5.32		-4.74		-4.31	
RXF 393	-5.53		-4.98		-4.36	
SN12C	-5.50		-4.83		-4.41	
TK-10	-5.06		-4.67		-4.33	
UO-31	-5.67		-5.37		-5.08	
Prostate Cancer						
PC-3	-5.24		-4.73		-4.36	
DU-145	-4.92		-4.59		-4.26	
Breast Cancer						
MCF7	-5.18		-4.67		-4.24	
NCI/ADR-RES	-4.94		-4.47		> -4.00	
MDA-MB-231/ATCC	-5.39		-4.81		-4.39	
HS 578T	-5.00		-4.61		-4.23	
MDA-MB-435	-5.59		-5.18		-4.50	
MDA-N	-5.73		-5.44		-5.15	
BT-549	-4.84		-4.55		-4.26	
T-47D	-4.95		-4.56		-4.16	
MG_MID	-5.42		-4.91		-4.53	
Delta	1.51		0.67		0.75	
Range	2.10		1.59		1.29	

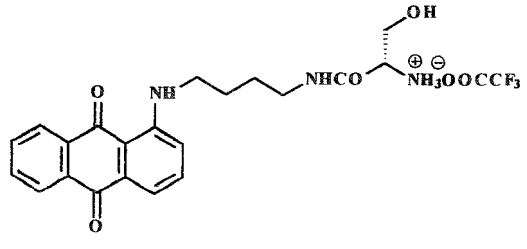
+3 +2 +1 0 -1 -2 -3

NU:UB 43 (215)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709767 -T / 1	**Experiment ID:** 9907MD59-22	**Test Type:** 08	**Units:** Molar
Report Date: August 6, 1999	**Test Date:** July 12, 1999	**QNS:**	**MC:**
COMI: NU:UB 44	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.300	0.776	0.701	0.738	0.683	0.250	0.115	84	92	80	-17	-62	2.05E-06	6.71E-06	5.49E-05
HL-60(TB)	0.939	2.432	2.349	2.526	1.667	1.232	0.202	94	106	49	20	-79	9.52E-07	1.58E-05	5.12E-05
K-562	0.157	0.916	0.884	0.865	0.860	0.180	0.050	96	93	93	3	-68	2.99E-06	1.10E-05	5.52E-05
MOLT-4	0.423	1.390	1.415	1.383	1.375	0.412	0.102	103	99	98	-3	-76	3.01E-06	9.40E-06	4.43E-05
SR	1.084	2.934	2.954	2.864	2.969	1.148	0.411	101	96	102	3	-62	3.37E-06	1.13E-05	6.53E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.272	1.342	1.411	1.375	1.312	0.641	0.027	106	103	97	35	-90	5.66E-06	1.89E-05	4.76E-05
EKVX	0.307	0.761	0.750	0.749	0.754	0.494	0.008	97	97	98	41	-97	6.98E-06	1.98E-05	4.55E-05
HOP-62	0.362	0.815	0.778	0.811	0.830	0.537	0.048	92	99	103	39	-87	6.67E-06	2.03E-05	5.08E-05
HOP-92	0.506	0.763	0.789	0.760	0.757	0.695	0.087	110	99	98	73	-83	1.41E-05	2.95E-05	6.16E-05
NCI-H226	0.788	0.987	0.999	1.011	1.079	0.895	0.228	106	112	146	54	-71	1.07E-05	2.69E-05	6.77E-05
NCI-H23	0.291	0.877	0.860	0.847	0.817	0.529	0.036	97	95	90	41	-88	6.44E-06	2.07E-05	5.09E-05
NCI-H460	0.341	1.989	1.896	1.990	1.925	0.555	0.087	94	100	96	13	-74	3.58E-06	1.41E-05	5.25E-05
NCI-H522	0.267	0.811	0.816	0.808	0.807	0.472	0.025	101	99	99	38	-91	6.29E-06	1.96E-05	4.82E-05
Colon Cancer															
COLO 205	0.553	1.568	1.593	1.574	1.639	0.310	0.046	102	101	107	-44	-92	2.38E-06	5.11E-06	1.34E-05
HCC-2998	0.333	0.889	0.820	0.845	0.705	0.244	0.056	87	92	67	-27	-83	1.51E-06	5.18E-06	2.58E-05
HCT-116	0.119	0.991	1.007	1.001	1.024	0.205	-0.001	102	101	104	10	-100	3.73E-06	1.23E-05	3.50E-05
HCT-15	0.084	0.493	0.488	0.482	0.473	0.119	0.014	99	97	95	9	-83	3.32E-06	1.24E-05	4.34E-05
HT29	0.381	1.555	1.591	1.541	1.588	0.355	0.038	103	99	103	-7	-90	3.03E-06	8.66E-06	3.30E-05
KM12	0.279	1.027	1.024	1.092	1.028	0.364	0.090	100	109	100	11	-68	3.67E-06	1.39E-05	5.94E-05
SW-620	0.081	0.497	0.505	0.487	0.492	0.179	0.053	102	98	99	23	-35	4.45E-06	2.51E-05	>1.00E-04
CNS Cancer															
SF-268	0.251	1.025	1.053	1.026	0.987	0.785	0.090	104	100	95	69	-64	1.39E-05	3.30E-05	7.83E-05
SF-295	0.622	1.767	1.691	1.728	1.602	0.864	0.042	93	97	86	21	-93	3.56E-06	1.53E-05	4.19E-05
SNB-19	0.387	1.080	1.135	1.111	1.117	0.477	0.029	108	104	105	13	-93	3.97E-06	1.33E-05	3.95E-05
SNB-75	0.418	0.615	0.630	0.616	0.624	0.497	0.058	107	101	105	40	-86	7.02E-06	2.08E-05	5.17E-05
U251	0.268	1.377	1.197	1.311	1.333	0.610	0.039	84	94	96	31	-86	5.08E-06	1.84E-05	4.94E-05
Melanoma															
LOX IMVI	0.226	1.160	1.185	1.148	1.104	0.216	0.020	103	99	94	-4	-91	2.80E-06	9.02E-06	3.34E-05
MALME-3M	0.319	0.795	0.827	0.820	0.735	0.229	0.059	107	105	87	-28	-82	2.10E-06	5.70E-06	2.56E-05
M14	0.402	1.514	1.449	1.480	1.363	0.619	0.023	94	97	86	19	-94	3.50E-06	1.48E-05	4.08E-05
SK-MEL-28	0.220	0.705	0.689	0.701	0.730	0.517	0.048	97	99	105	61	-78	1.20E-05	2.75E-05	6.28E-05
SK-MEL-5	0.703	1.609	1.631	1.626	1.219	0.139	0.021	102	102	57	-80	-97	1.12E-06	2.60E-06	6.02E-06
UACC-257	0.615	1.520	1.470	1.504	1.495	1.112	0.057	95	98	97	55	-91	1.08E-05	2.38E-05	5.25E-05
UACC-62	0.457	1.603	1.461	1.631	1.612	0.784	0.048	88	102	101	29	-90	5.05E-06	1.74E-05	4.62E-05
Ovarian Cancer															
IGROV1	0.279	0.952	0.947	0.927	0.872	0.492	0.021	99	96	88	32	-93	4.72E-06	1.80E-05	4.54E-05
OVCAR-3	0.534	0.815	0.772	0.795	0.756	0.386	0.038	85	93	79	-28	-93	1.86E-06	5.49E-06	2.20E-05
OVCAR-4	0.510	1.231	1.169	1.187	1.155	0.907	0.114	91	94	89	55	-78	1.09E-05	2.60E-05	6.18E-05
OVCAR-5	0.596	1.300	1.279	1.259	1.304	1.077	0.002	97	94	101	68	-100	1.28E-05	2.55E-05	5.05E-05
OVCAR-8	0.230	0.755	0.785	0.810	0.800	0.387	0.011	106	110	109	30	-95	5.56E-06	1.73E-05	4.34E-05
SK-OV-3	0.356	0.790	0.714	0.748	0.693	0.589	0.041	82	90	78	54	-88	1.06E-05	2.38E-05	5.36E-05
Renal Cancer															
786-0	0.429	1.712	1.709	1.723	1.771	0.725	0.021	100	101	105	23	-95	4.67E-06	1.57E-05	4.15E-05
A498	0.991	1.665	1.557	1.644	1.640	1.536	0.156	84	97	96	81	-84	1.54E-05	3.09E-05	6.20E-05
ACHN	0.341	1.273	1.231	1.276	1.166	0.690	0.003	95	100	88	37	-99	5.66E-06	1.88E-05	4.36E-05
CAKI-1	0.413	1.112	1.141	1.154	1.173	0.766	0.036	104	106	109	50	-91	1.01E-05	2.27E-05	5.11E-05
RXF 393	0.583	1.244	1.251	1.230	1.204	0.928	0.196	101	98	94	52	-66	1.04E-05	2.76E-05	7.28E-05
SN12C	0.302	0.931	0.954	0.979	0.996	0.563	0.004	104	108	110	41	-99	7.51E-06	1.97E-05	4.49E-05
TK-10	0.520	1.308	1.239	1.348	1.373	1.121	0.045	91	105	108	76	-91	1.43E-05	2.85E-05	5.66E-05
UO-31	0.526	1.220	1.250	1.258	1.228	0.962	0.014	104	105	101	63	-97	1.20E-05	2.47E-05	5.06E-05
Prostate Cancer															
PC-3	0.293	0.977	0.917	0.958	0.942	0.570	0.026	91	97	95	40	-91	6.68E-06	2.03E-05	4.86E-05
DU-145	0.265	0.790	0.823	0.822	0.807	0.472	0.026	106	106	103	39	-90	6.83E-06	2.02E-05	4.90E-05
Breast Cancer															
MCF7	0.316	1.590	1.437	1.507	1.459	0.556	0.091	88	93	90	19	-71	3.63E-06	1.62E-05	5.80E-05
NCI/ADR-RES	0.282	0.759	0.769	0.784	0.775	0.588	0.103	102	105	103	64	-64	1.29E-05	3.18E-05	7.82E-05
MDA-MB-231/ATCC	0.391	0.888	0.813	0.841	0.817	0.578	0.070	85	90	86	38	-82	5.50E-06	2.06E-05	5.39E-05
HS 578T	0.225	0.850	0.889	0.893	0.894	0.630	0.186	106	107	107	65	-17	1.51E-05	6.15E-05	>1.00E-04
MDA-MB-435	0.297	1.132	1.110	1.139	1.134	0.230	0.043	97	101	100	-23	-86	2.56E-06	6.53E-06	2.72E-05
MDA-N	0.691	2.289	2.573	2.435	2.419	0.074	-0.002	118	109	108	-89	-100	1.97E-06	3.53E-06	6.32E-06
BT-549	0.304	0.723	0.729	0.735	0.720	0.595	0.039	101	103	99	70	-87	1.33E-05	2.77E-05	5.78E-05
T-47D	0.433	0.999	0.979	1.009	0.979	0.876	0.224	96	102	97	78	-48	1.67E-05	4.16E-05	>1.00E-04



NU:UB 44

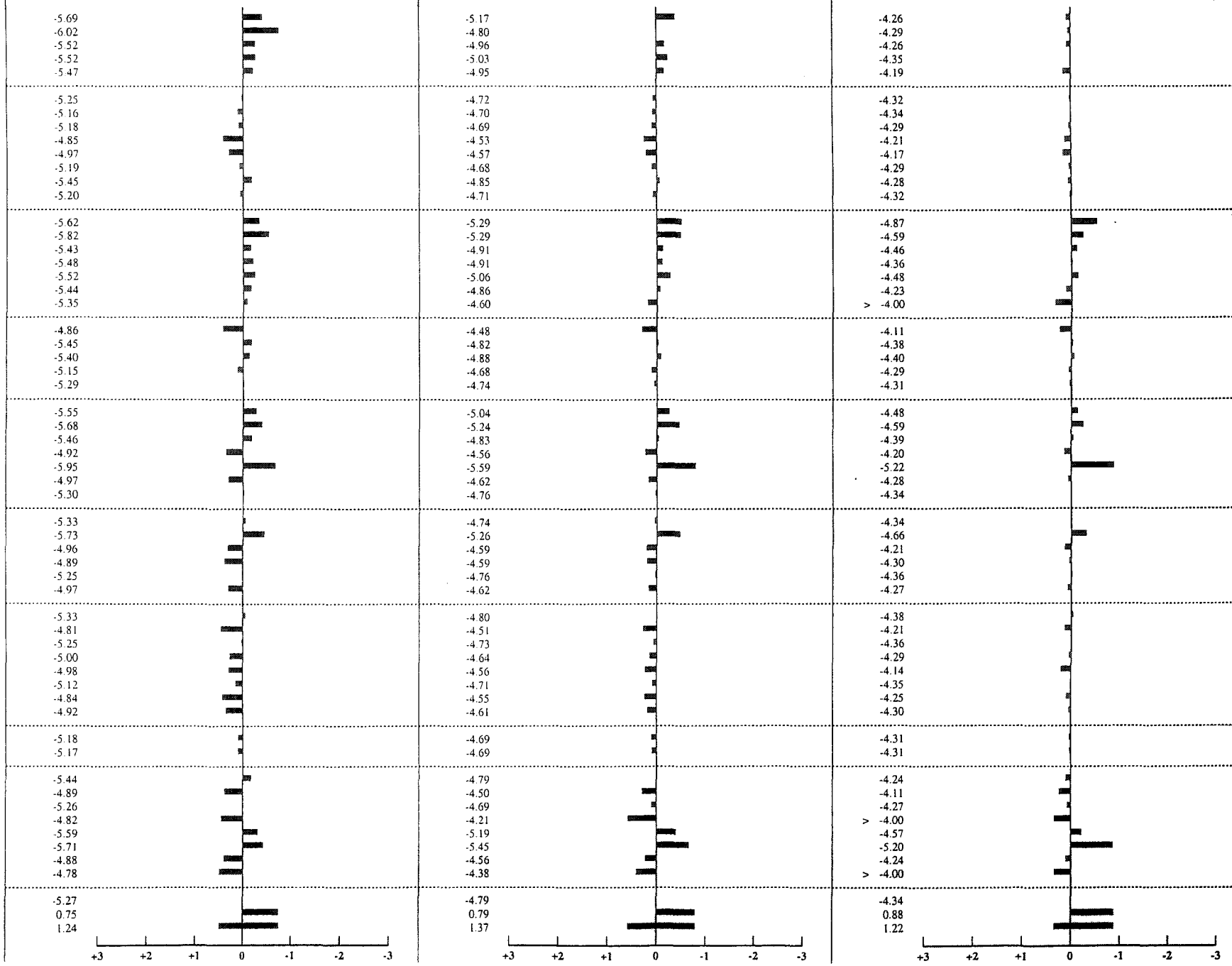
National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709767 -T / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD59-22

Report Date: August 6, 1999 | Test Date: July 12, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-5.69	-5.17	-4.26
HL-60(TB)	-6.02	-4.80	-4.29
K-562	-5.52	-4.96	-4.26
MOLT-4	-5.52	-5.03	-4.35
SR	-5.47	-4.95	-4.19
Non-Small Cell Lung Cancer			
A549/ATCC	-5.25	-4.72	-4.32
EKVX	-5.16	-4.70	-4.34
HOP-62	-5.18	-4.69	-4.29
HOP-92	-4.85	-4.53	-4.21
NCI-H226	-4.97	-4.57	-4.17
NCI-H23	-5.19	-4.68	-4.29
NCI-H460	-5.45	-4.85	-4.28
NCI-H522	-5.20	-4.71	-4.32
Colon Cancer			
COLO 205	-5.62	-5.29	-4.87
HCC-2998	-5.82	-5.29	-4.59
HCT-116	-5.43	-4.91	-4.46
HCT-15	-5.48	-4.91	-4.36
HT29	-5.52	-5.06	-4.48
KM12	-5.44	-4.86	-4.23
SW-620	-5.35	-4.60	> -4.00
CNS Cancer			
SF-268	-4.86	-4.48	-4.11
SF-295	-5.45	-4.82	-4.38
SNB-19	-5.40	-4.88	-4.40
SNB-75	-5.15	-4.68	-4.29
U251	-5.29	-4.74	-4.31
Melanoma			
LOX IMVI	-5.55	-5.04	-4.48
MALME-3M	-5.68	-5.24	-4.59
M14	-5.46	-4.83	-4.39
SK-MEL-28	-4.92	-4.56	-4.20
SK-MEL-5	-5.95	-5.59	-5.22
UACC-257	-4.97	-4.62	-4.28
UACC-62	-5.30	-4.76	-4.34
Ovarian Cancer			
IGROV1	-5.33	-4.74	-4.34
OVCAR-3	-5.73	-5.26	-4.66
OVCAR-4	-4.96	-4.59	-4.21
OVCAR-5	-4.89	-4.59	-4.30
OVCAR-8	-5.25	-4.76	-4.36
SK-OV-3	-4.97	-4.62	-4.27
Renal Cancer			
786-0	-5.33	-4.80	-4.38
A498	-4.81	-4.51	-4.21
ACHN	-5.25	-4.73	-4.36
CAKI-1	-5.00	-4.64	-4.29
RXF 393	-4.98	-4.56	-4.14
SN12C	-5.12	-4.71	-4.35
TK-10	-4.84	-4.55	-4.25
UO-31	-4.92	-4.61	-4.30
Prostate Cancer			
PC-3	-5.18	-4.69	-4.31
DU-145	-5.17	-4.69	-4.31
Breast Cancer			
MCF7	-5.44	-4.79	-4.24
NCI/ADR-RES	-4.89	-4.50	-4.11
MDA-MB-231/ATCC	-5.26	-4.69	-4.27
HS 578T	-4.82	-4.21	> -4.00
MDA-MB-435	-5.59	-5.19	-4.57
MDA-N	-5.71	-5.45	-5.20
BT-549	-4.88	-4.56	-4.24
T-47D	-4.78	-4.38	> -4.00
MG_MID	-5.27	-4.79	-4.34
Delta	0.75	0.79	0.88
Range	1.24	1.37	1.22

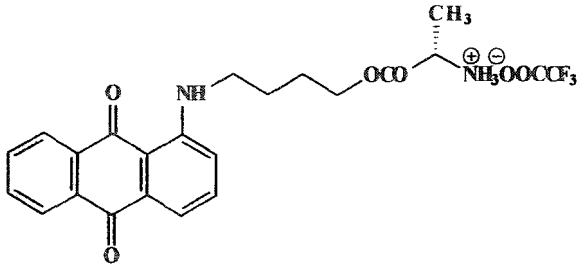


NU:UB 44 (254)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709768 -U / 1	**Experiment ID:** 9907MD59-23	**Test Type:** 08	**Units:** Molar
Report Date: August 6, 1999	**Test Date:** July 12, 1999	**QNS:**	**MC:**
COMI: NU:UB 73	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.300	0.738	0.718	0.750	0.695	0.584	0.055	95	103	90	65	-82	1.26E-05	2.77E-05	6.08E-05
HL-60(TB)	0.939	2.785	2.676	2.590	2.563	2.014	0.130	94	89	88	58	-86	1.14E-05	2.53E-05	5.62E-05
K-562	0.157	0.917	0.917	0.907	0.864	0.602	0.233	100	99	93	58	10	1.49E-05	>1.00E-04	>1.00E-04
MOLT-4	0.423	1.352	1.376	1.356	1.324	1.016	0.141	103	100	97	64	-67	1.28E-05	3.08E-05	7.45E-05
SR	1.084	2.814	2.674	2.697	2.761	1.976	0.214	92	93	97	52	-80	1.03E-05	2.46E-05	5.89E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.272	1.156	1.165	1.149	1.126	0.989	0.059	101	99	97	81	-78	1.57E-05	3.23E-05	6.64E-05
EKVX	0.307	0.788	0.796	0.794	0.759	0.623	0.076	102	101	94	66	-75	1.29E-05	2.92E-05	6.62E-05
HOP-62	0.362	0.887	0.915	0.901	0.894	0.822	0.130	105	103	101	88	-64	1.77E-05	3.78E-05	8.07E-05
HOP-92	0.506	0.777	0.778	0.760	0.794	0.720	0.150	100	94	106	79	-70	1.57E-05	3.38E-05	7.30E-05
NCI-H226	0.788	1.073	1.098	1.007	1.085	0.965	0.201	109	77	104	62	-75	1.22E-05	2.84E-05	6.61E-05
NCI-H23	0.291	0.931	0.985	0.986	0.980	0.894	0.026	108	108	108	94	-91	1.73E-05	3.22E-05	6.00E-05
NCI-H460	0.341	1.964	1.968	1.963	1.968	1.603	0.120	100	100	100	78	-65	1.56E-05	3.51E-05	7.86E-05
NCI-H522	0.267	0.830	0.855	0.831	0.816	0.694	0.082	104	100	98	76	-69	1.51E-05	3.33E-05	7.36E-05
Colon Cancer															
COLO 205	0.553	1.737	1.760	1.658	1.752	1.517	0.030	102	93	101	81	-95	1.51E-05	2.90E-05	5.58E-05
HCC-2998	0.333	0.868	0.867	0.849	0.897	0.828	0.037	100	96	105	93	-89	1.72E-05	3.23E-05	6.10E-05
HCT-116	0.119	1.002	0.982	0.963	1.006	0.765	0.004	98	96	101	73	-97	1.37E-05	2.70E-05	5.31E-05
HCT-15	0.084	0.536	0.519	0.524	0.541	0.378	-0.015	96	97	101	65	-100	1.23E-05	2.48E-05	4.98E-05
HT29	0.381	1.766	1.774	1.838	1.824	1.493	0.010	101	105	104	80	-97	1.48E-05	2.83E-05	5.41E-05
KM12	0.279	1.183	1.084	1.058	1.080	0.845	0.065	89	86	89	63	-77	1.23E-05	2.81E-05	6.42E-05
SW-620	0.081	0.488	0.476	0.439	0.462	0.362	0.055	97	88	94	69	-32	1.55E-05	4.82E-05	>1.00E-04
CNS Cancer															
SF-268	0.251	1.044	1.015	1.062	1.053	0.928	0.131	96	102	101	85	-48	1.84E-05	4.36E-05	>1.00E-04
SF-295	0.622	1.959	1.974	2.025	1.969	1.721	0.089	101	105	101	82	-86	1.55E-05	3.09E-05	6.13E-05
SNB-19	0.387	1.014	0.975	1.002	0.968	0.814	0.095	94	98	93	68	-75	1.34E-05	2.98E-05	6.65E-05
SNB-75	0.418	0.628	0.644	0.636	0.623	0.547	0.155	107	104	98	61	-63	1.23E-05	3.11E-05	7.87E-05
U251	0.268	1.358	1.293	1.276	1.271	1.006	0.038	94	92	92	68	-86	1.30E-05	2.76E-05	5.83E-05
Melanoma															
LOX IMVI	0.226	1.280	1.229	1.229	1.264	0.934	0.081	95	95	99	67	-64	1.35E-05	3.25E-05	7.80E-05
MALME-3M	0.319	0.803	0.808	0.797	0.791	0.768	0.111	101	99	98	93	-65	1.86E-05	3.86E-05	8.00E-05
M14	0.402	1.267	1.239	1.310	1.303	1.203	0.068	97	105	104	93	-83	1.75E-05	3.37E-05	6.48E-05
SK-MEL-28	0.220	0.679	0.677	0.686	0.679	0.600	0.067	100	101	100	83	-70	1.64E-05	3.49E-05	7.44E-05
SK-MEL-5	0.703	1.676	1.708	1.616	1.747	1.571	0.005	103	94	107	89	-99	1.61E-05	2.97E-05	5.48E-05
UACC-257	0.615	1.482	1.498	1.497	1.510	1.480	0.117	102	102	103	100	-81	1.89E-05	3.57E-05	6.74E-05
UACC-62	0.457	1.630	1.651	1.666	1.567	1.328	0.029	102	103	95	74	-94	1.39E-05	2.77E-05	5.50E-05
Ovarian Cancer															
IGROV1	0.279	1.021	1.029	0.992	1.005	0.790	0.068	101	96	98	69	-76	1.35E-05	2.99E-05	6.63E-05
OVCAR-3	0.534	1.026	1.065	1.046	1.024	0.840	0.111	108	104	100	62	-79	1.22E-05	2.75E-05	6.21E-05
OVCAR-4	0.510	1.249	1.252	1.269	1.178	0.941	0.131	100	103	90	58	-74	1.16E-05	2.75E-05	6.56E-05
OVCAR-5	0.596	1.335	1.338	1.407	1.406	1.254	0.062	100	110	110	89	-90	1.65E-05	3.15E-05	6.00E-05
OVCAR-8	0.230	0.832	0.821	0.795	0.792	0.662	0.077	98	94	93	72	-67	1.44E-05	3.30E-05	7.57E-05
SK-OV-3	0.356	0.852	0.853	0.882	0.840	0.743	0.119	100	106	97	78	-67	1.56E-05	3.46E-05	7.67E-05
Renal Cancer															
786-0	0.429	1.538	1.608	1.532	1.574	1.293	0.098	106	99	103	78	-77	1.51E-05	3.18E-05	6.68E-05
A498	0.991	1.682	1.738	1.669	1.641	1.446	0.014	108	98	94	66	-99	1.25E-05	2.51E-05	5.06E-05
ACHN	0.341	1.309	1.271	1.305	1.275	0.851	0.020	96	100	96	53	-94	1.04E-05	2.28E-05	5.00E-05
CAKI-1	0.413	1.217	1.171	1.183	1.106	0.870	0.133	94	96	86	57	-68	1.13E-05	2.85E-05	7.18E-05
RXF 393	0.583	1.220	1.180	1.139	1.139	0.927	0.226	94	87	87	54	-61	1.08E-05	2.94E-05	7.99E-05
SN12C	0.302	1.001	0.990	0.970	1.033	0.827	0.045	98	96	105	75	-85	1.43E-05	2.94E-05	6.04E-05
TK-10	0.520	1.310	1.328	1.389	1.405	1.141	0.080	102	110	112	79	-85	1.50E-05	3.03E-05	6.13E-05
Prostate Cancer															
PC-3	0.293	0.904	0.930	0.931	0.932	0.812	0.039	104	104	105	85	-87	1.60E-05	3.12E-05	6.11E-05
DU-145	0.265	0.833	0.833	0.766	0.808	0.610	0.082	100	88	96	61	-69	1.21E-05	2.93E-05	7.11E-05
Breast Cancer															
MCF7	0.316	1.580	1.599	1.481	1.538	1.470	0.054	102	92	97	91	-83	1.72E-05	3.34E-05	6.46E-05
NCI/ADR-RES	0.282	0.814	0.830	0.818	0.814	0.726	0.090	103	101	100	83	-68	1.66E-05	3.55E-05	7.60E-05
MDA-MB-231/ATCC	0.391	0.833	0.813	0.860	0.829	0.721	0.074	95	106	99	75	-81	1.44E-05	3.01E-05	6.31E-05
HS 578T	0.225	0.899	0.910	0.883	0.938	0.876	0.148	102	98	106	97	-34	2.27E-05	5.47E-05	>1.00E-04
MDA-MB-435	0.297	1.271	1.246	1.217	1.165	0.971	0.019	97	95	89	69	-94	1.31E-05	2.66E-05	5.40E-05
MDA-N	0.691	2.314	2.292	2.214	2.478	2.194	0.012	99	94	110	93	-98	1.67E-05	3.06E-05	5.59E-05
BT-549	0.304	0.746	0.745	0.714	0.767	0.698	0.050	100	93	105	89	-84	1.69E-05	3.28E-05	6.39E-05
T-47D	0.433	1.026	0.970	0.994	0.942	0.818	0.210	91	95	86	65	-52	1.34E-05	3.61E-05	9.69E-05



NU:UB 73

National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709768 -U / 1	Units: Molar	SSPL: 0B6Z	Exp. ID:9907MD59-23
Report Date: August 6, 1999		Test Date: July 12, 1999	

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-4.90	-4.56	-4.22
HL-60(TB)	-4.94	-4.60	-4.25
K-562	-4.83	> -4.00	> -4.00
MOLT-4	-4.89	-4.51	-4.13
SR	-4.99	-4.61	-4.23
Non-Small Cell Lung Cancer			
A549/ATCC	-4.80	-4.49	-4.18
EKVX	-4.89	-4.53	-4.18
HOP-62	-4.75	-4.42	-4.09
HOP-92	-4.80	-4.47	-4.14
NCI-H226	-4.91	-4.55	-4.18
NCI-H23	-4.76	-4.49	-4.22
NCI-H460	-4.81	-4.45	-4.10
NCI-H522	-4.82	-4.48	-4.13
Colon Cancer			
COLO 205	-4.82	-4.54	-4.25
HCC-2998	-4.76	-4.49	-4.21
HCT-116	-4.86	-4.57	-4.27
HCT-15	-4.91	-4.61	-4.30
HT29	-4.83	-4.55	-4.27
KM12	-4.91	-4.55	-4.19
SW-620	-4.81	-4.32	> -4.00
CNS Cancer			
SF-268	-4.74	-4.36	> -4.00
SF-295	-4.81	-4.51	-4.21
SNB-19	-4.87	-4.53	-4.18
SNB-75	-4.91	-4.51	-4.10
U251	-4.89	-4.56	-4.23
Melanoma			
LOX IMVI	-4.87	-4.49	-4.11
MALME-3M	-4.73	-4.41	-4.10
M14	-4.76	-4.47	-4.19
SK-MEL-28	-4.79	-4.46	-4.13
SK-MEL-5	-4.79	-4.53	-4.26
UACC-257	-4.72	-4.45	-4.17
UACC-62	-4.86	-4.56	-4.26
Ovarian Cancer			
IGROV1	-4.87	-4.52	-4.18
OVCAR-3	-4.91	-4.56	-4.21
OVCAR-4	-4.94	-4.56	-4.18
OVCAR-5	-4.78	-4.50	-4.22
OVCAR-8	-4.84	-4.48	-4.12
SK-OV-3	-4.81	-4.46	-4.12
Renal Cancer			
786-0	-4.82	-4.50	-4.18
A498	-4.90	-4.60	-4.30
ACHN	-4.98	-4.64	-4.30
CAKI-1	-4.95	-4.55	-4.14
RXF 393	-4.97	-4.53	-4.10
SN12C	-4.84	-4.53	-4.22
TK-10	-4.82	-4.52	-4.21
Prostate Cancer			
PC-3	-4.80	-4.51	-4.21
DU-145	-4.92	-4.53	-4.15
Breast Cancer			
MCF7	-4.76	-4.48	-4.19
NCI/ADR-RES	-4.78	-4.45	-4.12
MDA-MB-231/ATCC	-4.84	-4.52	-4.20
HS 578T	-4.64	-4.26	> -4.00
MDA-MB-435	-4.88	-4.58	-4.27
MDA-N	-4.78	-4.51	-4.25
BT-549	-4.77	-4.48	-4.19
T-47D	-4.87	-4.44	-4.01
MG_MID	-4.84	-4.50	-4.17
Delta	0.15	0.15	0.13
Range	0.34	0.64	0.30

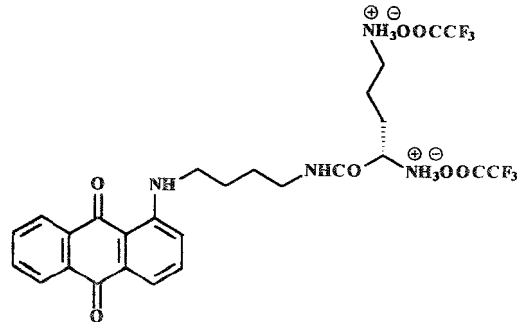
+3 +2 +1 0 -1 -2 -3

NU:UB 73 (194)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709772 -Y / 1	**Experiment ID:** 9907MD58-34	**Test Type:** 08	**Units:** Mola
Report Date: August 5, 1999	**Test Date:** July 6, 1999	**QNS:**	**MC:**
COMI: NU:UB 99	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration											
	Time		Mean Optical Densities					Percent Growth						
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.107	0.447	0.447	0.413	0.379	0.188	0.093	100	90	80	24	-14	3.41E-06	4.34E-05	>1.00E-0
HL-60(TB)	0.246	1.066	1.146	1.090	0.926	0.399	0.117	110	103	83	19	-53	3.25E-06	1.82E-05	9.18E-0
K-562	0.157	1.250	1.209	1.212	1.121	0.808	0.048	96	97	88	60	-70	1.18E-05	2.89E-05	7.03E-0
MOLT-4	0.149	0.635	0.636	0.613	0.592	0.400	0.055	100	95	91	52	-63	1.03E-05	2.81E-05	7.64E-0
Non-Small Cell Lung Cancer															
A549/ATCC	0.198	0.999	1.012	1.002	1.002	0.833	0.034	102	100	100	79	-83	1.52E-05	3.08E-05	6.27E-0
EKVX	0.630	1.370	1.393	1.379	1.385	1.338	0.090	103	101	102	96	-86	1.78E-05	3.37E-05	6.35E-0
HOP-62	0.199	0.534	0.545	0.503	0.524	0.449	0.059	103	91	97	75	-71	1.48E-05	3.26E-05	7.21E-0
HOP-92	0.394	0.919	0.941	0.945	0.965	0.903	0.166	104	105	109	97	-58	2.01E-05	4.23E-05	8.90E-0
NCI-H23	0.301	0.946	0.903	0.966	0.931	0.722	0.003	93	103	98	65	-99	1.24E-05	2.49E-05	5.03E-0
NCI-H322M	0.427	1.358	1.365	1.344	1.376	1.118	-0.005	101	99	102	74	-100	1.38E-05	2.67E-05	5.17E-0
NCI-H460	0.312	1.582	1.464	1.580	1.623	1.191	0.028	91	100	103	69	-91	1.32E-05	2.70E-05	5.55E-0
NCI-H522	0.262	0.690	0.724	0.718	0.711	0.584	0.026	108	106	105	75	-90	1.42E-05	2.84E-05	5.71E-0
Colon Cancer															
COLO 205	0.406	1.459	1.405	1.378	1.417	0.857	0.007	95	92	96	43	-98	7.33E-06	2.01E-05	4.54E-0
HCC-2998	0.360	0.667	0.648	0.707	0.721	0.478	0.008	94	113	118	38	-98	7.16E-06	1.92E-05	4.45E-0
HCT-116	0.254	1.538	1.550	1.520	1.487	1.234	0.041	101	99	96	76	-84	1.46E-05	2.99E-05	6.13E-0
HCT-15	0.169	1.142	1.155	1.154	1.131	0.965	0.009	101	101	99	82	-95	1.51E-05	2.91E-05	5.58E-0
HT29	0.162	0.999	1.035	0.966	0.994	0.853	0.002	104	96	99	83	-99	1.51E-05	2.85E-05	5.38E-0
KM12	0.286	1.218	1.214	1.166	1.213	1.079	0.091	100	94	100	85	-68	1.69E-05	3.59E-05	7.59E-0
SW-620	0.066	0.525	0.546	0.473	0.495	0.401	0.080	104	89	93	73	3	2.12E-05	>1.00E-04	>1.00E-0
CNS Cancer															
SF-268	0.250	0.940	0.954	0.975	0.931	0.818	0.042	102	105	99	82	-83	1.57E-05	3.14E-05	6.29E-0
SF-295	0.454	1.326	1.308	1.249	1.259	1.017	0.080	98	91	92	64	-82	1.25E-05	2.75E-05	6.01E-0
SF-539	0.568	1.040	1.005	1.004	1.075	0.971	0.011	93	92	107	85	-98	1.56E-05	2.92E-05	5.47E-0
SNB-19	0.335	1.023	1.072	1.052	0.997	0.841	0.060	107	104	96	74	-82	1.42E-05	2.97E-05	6.22E-0
SNB-75	0.265	0.600	0.610	0.570	0.597	0.586	0.026	103	91	99	96	-90	1.76E-05	3.27E-05	6.07E-0
U251	0.161	0.857	0.818	0.854	0.810	0.716	-0.003	94	100	93	80	-100	1.46E-05	2.78E-05	5.27E-0
Melanoma															
LOX IMVI	0.085	0.817	0.781	0.816	0.881	0.694	0.049	95	100	109	83	-43	1.83E-05	4.56E-05	>1.00E-0
MALME-3M	0.367	0.773	0.803	0.838	0.889	0.647	0.037	107	116	128	69	-90	1.32E-05	2.71E-05	5.60E-0
M14	0.295	1.065	1.059	1.052	1.065	0.735	0.025	99	98	100	57	-92	1.12E-05	2.42E-05	5.26E-0
SK-MEL-2	0.257	0.701	0.615	0.677	0.654	0.585	0.007	80	94	89	74	-97	1.38E-05	2.70E-05	5.29E-0
SK-MEL-28	0.267	1.070	1.045	1.020	1.090	1.099	0.126	97	94	103	104	-53	2.20E-05	4.60E-05	9.59E-0
SK-MEL-5	0.428	1.634	1.613	1.547	1.571	1.076	-0.014	98	93	95	54	-100	1.06E-05	2.24E-05	4.73E-0
UACC-257	0.554	1.129	1.041	1.138	1.134	1.100	0.048	85	102	101	95	-91	1.74E-05	3.23E-05	5.99E-0
UACC-62	0.700	1.699	1.649	1.613	1.682	1.592	0.025	95	91	98	89	-96	1.63E-05	3.03E-05	5.62E-0
Ovarian Cancer															
IGROV1	0.110	0.786	0.812	0.790	0.846	0.622	0.069	104	101	109	76	-38	1.68E-05	4.65E-05	>1.00E-0
OVCAR-3	0.446	0.828	0.834	0.816	0.839	0.769	0.137	102	97	103	84	-69	1.67E-05	3.54E-05	7.48E-0
OVCAR-4	0.265	0.816	0.803	0.822	0.803	0.740	0.102	98	101	98	86	-62	1.76E-05	3.83E-05	8.36E-0
OVCAR-5	0.774	1.725	1.760	1.701	1.748	1.778	0.036	104	97	102	106	-95	1.89E-05	3.35E-05	5.94E-0
OVCAR-8	0.121	0.875	0.857	0.885	0.869	0.634	0.065	98	101	99	68	-47	1.44E-05	3.92E-05	>1.00E-0
SK-OV-3	0.281	0.561	0.582	0.568	0.582	0.551	0.072	107	102	108	97	-74	1.87E-05	3.67E-05	7.20E-0
Renal Cancer															
786-0	0.226	0.877	0.859	0.853	0.850	0.749	0.027	97	96	96	80	-88	1.51E-05	3.00E-05	5.94E-0
A498	0.848	1.443	1.440	1.425	1.450	1.434	0.132	99	97	101	98	-84	1.84E-05	3.45E-05	6.48E-0
ACHN	0.237	0.853	0.860	0.835	0.861	0.720	.	101	97	101	78	-100	1.44E-05	2.75E-05	5.25E-0
CAKI-1	0.454	1.154	1.136	1.096	1.143	1.042	0.134	97	92	98	84	-70	1.66E-05	3.49E-05	7.37E-0
RXF 393	0.359	0.823	0.792	0.777	0.779	0.688	0.083	93	90	90	71	-77	1.38E-05	3.01E-05	6.57E-0
SN12C	0.377	0.972	0.991	0.935	0.944	0.798	0.051	103	94	95	71	-86	1.36E-05	2.82E-05	5.86E-0
TK-10	0.529	1.127	1.033	1.143	1.150	1.044	0.043	84	103	104	86	-92	1.59E-05	3.05E-05	5.82E-0
UO-31	0.242	0.661	0.663	0.615	0.551	0.603	0.031	100	89	74	86	-87	1.61E-05	3.13E-05	6.09E-0
Prostate Cancer															
PC-3	0.189	0.653	0.630	0.606	0.636	0.553	-0.006	95	90	96	78	-100	1.44E-05	2.75E-05	5.25E-0
DU-145	0.211	0.787	0.766	0.756	0.774	0.683	0.020	96	95	98	82	-91	1.53E-05	2.99E-05	5.82E-0
Breast Cancer															
MCF7	0.245	0.911	0.880	0.902	0.894	0.718	0.013	95	99	97	71	-95	1.34E-05	2.68E-05	5.36E-0
NCI/ADR-RES	0.299	0.889	0.866	0.916	0.898	0.774	0.126	96	105	102	80	-58	1.66E-05	3.81E-05	8.75E-0
MDA-MB-231/ATCC	0.322	0.871	0.799	0.823	0.806	0.753	0.010	87	91	88	79	-97	1.45E-05	2.80E-05	5.40E-0
HS 578T	0.106	0.465	0.476	0.452	0.469	0.401	0.057	103	96	101	82	-47	1.78E-05	4.34E-05	>1.00E-0
MDA-MB-435	0.279	1.011	1.004	0.973	0.997	0.813	0.052	99	95	98	73	-82	1.41E-05	2.97E-05	6.25E-0
MDA-N	0.352	1.370	1.357	1.349	1.366	0.921	0.032	99	98	100	56	-91	1.10E-05	2.40E-05	5.26E-0
BT-549	0.502	1.171	1.161	1.186	1.210	1.187	0.023	99	102	106	102	-96	1.84E-05	3.29E-05	5.89E-0
T-47D	0.300	0.725	0.711	0.678	0.674	0.575	0.121	97	89	88	65	-60	1.31E-05	3.31E-05	8.34E-0



NU:UB 99

(246)

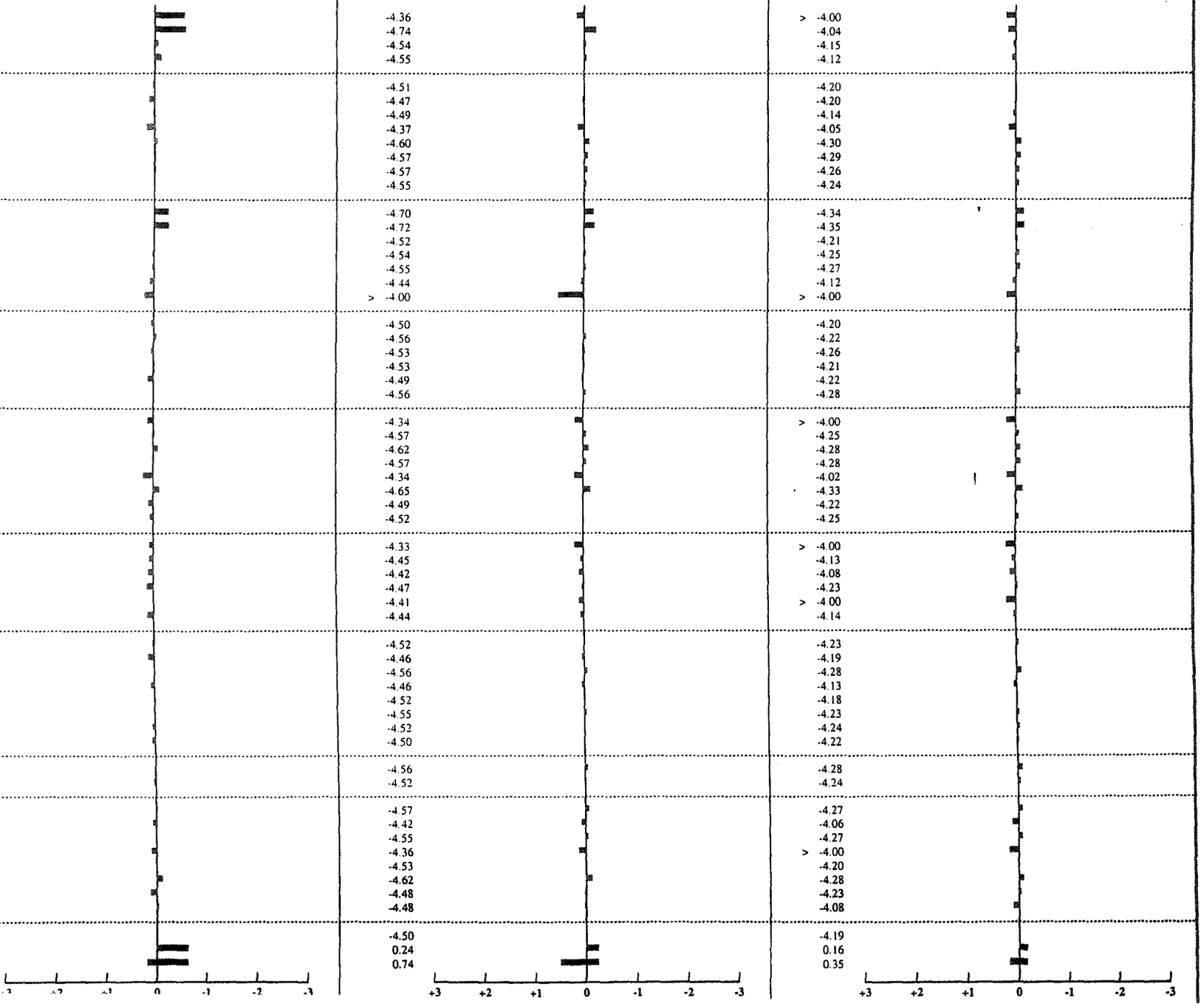
National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709772 -Y / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD58-34

Report Date: August 5, 1999 | Test Date: July 6, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-5.47	-4.36	> -4.00
HL-60(TB)	-5.49	-4.74	-4.04
K-562	-4.93	-4.54	-4.15
MOLT-4	-4.99	-4.55	-4.12
Non-Small Cell Lung Cancer			
A549/ATCC	-4.82	-4.51	-4.20
EKVX	-4.75	-4.47	-4.20
HOP-62	-4.83	-4.49	-4.14
HOP-92	-4.70	-4.37	-4.05
NCI-H23	-4.91	-4.60	-4.30
NCI-H322M	-4.86	-4.57	-4.29
NCI-H460	-4.88	-4.57	-4.26
NCI-H522	-4.85	-4.55	-4.24
Colon Cancer			
COLO 205	-5.13	-4.70	-4.34
HCC-2998	-5.15	-4.72	-4.35
HCT-116	-4.84	-4.52	-4.21
HCT-15	-4.82	-4.54	-4.25
HT29	-4.82	-4.55	-4.27
KM12	-4.77	-4.44	-4.12
SW-620	-4.67	> -4.00	> -4.00
CNS Cancer			
SF-268	-4.80	-4.50	-4.20
SF-295	-4.90	-4.56	-4.22
SF-539	-4.81	-4.53	-4.26
SNB-19	-4.85	-4.53	-4.21
SNB-75	-4.75	-4.49	-4.22
U251	-4.84	-4.56	-4.28
Melanoma			
LOX IMVI	-4.74	-4.34	> -4.00
MALME-3M	-4.88	-4.57	-4.25
M14	-4.95	-4.62	-4.28
SK-MEL-2	-4.86	-4.57	-4.28
SK-MEL-28	-4.66	-4.34	-4.02
SK-MEL-5	-4.97	-4.65	-4.33
UACC-257	-4.76	-4.49	-4.22
UACC-62	-4.79	-4.52	-4.25
Ovarian Cancer			
IGROV1	-4.77	-4.33	> -4.00
OVCAR-3	-4.78	-4.45	-4.13
OVCAR-4	-4.75	-4.42	-4.08
OVCAR-5	-4.72	-4.47	-4.23
OVCAR-8	-4.84	-4.41	> -4.00
SK-OV-3	-4.73	-4.44	-4.14
Renal Cancer			
786-0	-4.82	-4.52	-4.23
A498	-4.74	-4.46	-4.19
ACHN	-4.84	-4.56	-4.28
CAKI-1	-4.78	-4.46	-4.13
RXF 393	-4.86	-4.52	-4.18
SN12C	-4.87	-4.55	-4.23
TK-10	-4.80	-4.52	-4.24
UO-31	-4.79	-4.50	-4.22
Prostate Cancer			
PC-3	-4.84	-4.56	-4.28
DU-145	-4.82	-4.52	-4.24
Breast Cancer			
MCF7	-4.87	-4.57	-4.27
NCI/ADR-RES	-4.78	-4.42	-4.06
MDA-MB-231/ATCC	-4.84	-4.55	-4.27
HS 578T	-4.75	-4.36	> -4.00
MDA-MB-435	-4.85	-4.53	-4.20
MDA-N	-4.96	-4.62	-4.28
BT-549	-4.74	-4.48	-4.23
T-47D	-4.88	-4.48	-4.08
MG_MID	-4.85	-4.50	-4.19
Delta	0.63	0.24	0.16
Range	0.83	0.74	0.35

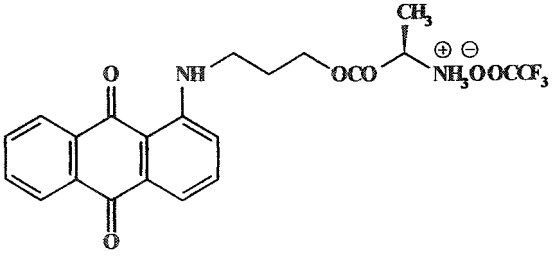


NU:UB 99 (246)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709773 -Z / 1	**Experiment ID:** 9907MD59-25	**Test Type:** 08	**Units:** Molar
Report Date: August 6, 1999	**Test Date:** July 12, 1999	**QNS:**	**MC:**
COMI: NU:UB 107	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.300	0.738	0.810	0.835	0.808	0.658	0.206	116	122	116	82	-32	1.90E-05	5.27E-05	>1.00E-04
HL-60(TB)	0.939	2.831	3.100	3.169	2.966	2.247	0.405	114	118	107	69	-57	1.42E-05	3.54E-05	8.82E-05
K-562	0.157	1.022	1.099	1.083	1.037	0.777	0.088	109	107	102	72	-44	1.54E-05	4.15E-05	>1.00E-04
MOLT-4	0.423	1.485	1.576	1.650	1.559	1.234	0.172	109	115	107	76	-59	1.56E-05	3.65E-05	8.52E-05
SR	1.084	3.005	3.109	3.193	3.106	2.766	0.463	105	110	105	88	-57	1.82E-05	4.02E-05	8.90E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.272	1.387	1.449	1.486	1.440	1.364	0.113	105	109	105	98	-59	2.02E-05	4.22E-05	8.81E-05
EKVX	0.307	0.749	0.791	0.814	0.770	0.517	0.190	110	115	105	48	-38	9.06E-06	3.59E-05	>1.00E-04
HOP-62	0.362	0.911	0.994	0.993	0.984	0.882	0.152	115	115	113	95	-58	1.96E-05	4.16E-05	8.84E-05
HOP-92	0.506	0.825	0.867	0.855	0.838	0.794	0.291	113	109	104	90	-43	2.01E-05	4.78E-05	>1.00E-04
NCI-H226	0.788	1.141	1.236	1.279	1.210	1.211	0.280	127	139	119	120	-65	2.39E-05	4.46E-05	8.34E-05
NCI-H23	0.291	0.920	0.997	1.023	0.980	0.927	0.002	112	116	109	101	-99	1.80E-05	3.19E-05	5.66E-05
NCI-H460	0.341	1.982	2.072	2.000	2.031	1.657	0.186	106	101	103	80	-46	1.74E-05	4.34E-05	>1.00E-04
NCI-H522	0.267	0.870	0.929	0.935	0.884	0.766	0.169	110	111	102	83	-37	1.88E-05	4.92E-05	>1.00E-04
Colon Cancer															
COLO 205	0.553	1.802	1.890	1.938	1.934	1.733	0.172	107	111	111	94	-69	1.87E-05	3.79E-05	7.66E-05
HCC-2998	0.333	0.863	0.896	0.901	0.910	0.827	0.083	106	107	109	93	-75	1.80E-05	3.58E-05	7.09E-05
HCT-116	0.119	1.067	0.986	0.988	1.016	0.864	0.041	92	92	95	79	-66	1.58E-05	3.51E-05	7.80E-05
HCT-15	0.084	0.603	0.635	0.612	0.613	0.482	0.008	106	102	102	77	-90	1.44E-05	2.87E-05	5.73E-05
HT29	0.381	1.815	1.812	1.863	1.812	1.614	0.082	100	103	100	86	-78	1.66E-05	3.33E-05	6.71E-05
KM12	0.279	1.185	1.191	1.152	1.151	0.929	0.104	101	96	96	72	-63	1.45E-05	3.41E-05	8.02E-05
SW-620	0.081	0.552	0.557	0.519	0.542	0.457	0.089	101	93	98	80	2	2.40E-05	>1.00E-04	>1.00E-04
CNS Cancer															
SF-268	0.251	1.061	1.158	1.142	1.154	1.012	0.296	112	110	112	94	6	3.14E-05	>1.00E-04	>1.00E-04
SF-295	0.622	1.863	1.949	1.950	1.938	1.795	0.059	107	107	106	95	-91	1.74E-05	3.24E-05	6.04E-05
SNB-19	0.387	1.120	1.200	1.173	1.233	1.073	0.338	111	107	115	94	-13	2.57E-05	7.58E-05	>1.00E-04
SNB-75	0.418	0.589	0.608	0.624	0.627	0.578	0.201	111	120	122	93	-52	1.99E-05	4.38E-05	9.68E-05
U251	0.268	1.297	1.272	1.241	1.262	1.184	0.097	97	94	97	89	-64	1.80E-05	3.82E-05	8.12E-05
Melanoma															
LOX IMVI	0.226	1.186	1.247	1.324	1.338	1.064	0.139	106	114	116	87	-39	1.98E-05	4.93E-05	>1.00E-04
MALME-3M	0.319	0.788	0.856	0.862	0.881	0.870	0.205	115	116	120	118	-36	2.75E-05	5.83E-05	>1.00E-04
M14	0.402	1.454	1.480	1.512	1.525	1.569	0.323	102	106	107	111	-20	2.92E-05	7.06E-05	>1.00E-04
SK-MEL-28	0.220	0.712	0.766	0.743	0.749	0.704	0.096	111	106	108	98	-56	2.06E-05	4.32E-05	9.10E-05
SK-MEL-5	0.703	1.697	1.854	1.950	1.903	1.779	0.002	116	125	121	108	-100	1.91E-05	3.31E-05	5.76E-05
UACC-257	0.615	1.459	1.507	1.484	1.543	1.503	0.212	106	103	110	105	-66	2.11E-05	4.13E-05	8.11E-05
UACC-62	0.457	1.646	1.799	1.726	1.710	1.529	0.028	113	107	105	90	-94	1.65E-05	3.09E-05	5.78E-05
Ovarian Cancer															
IGROV1	0.279	1.087	1.104	1.085	1.062	0.834	0.100	102	100	97	69	-64	1.38E-05	3.29E-05	7.82E-05
OVCAR-3	0.534	1.182	1.223	1.217	1.167	1.059	0.156	106	105	98	81	-71	1.60E-05	3.42E-05	7.30E-05
OVCAR-4	0.510	1.191	1.230	1.230	1.191	1.080	0.182	106	106	100	84	-64	1.69E-05	3.68E-05	8.00E-05
OVCAR-5	0.596	1.272	1.415	1.427	1.424	1.284	-0.005	121	123	123	102	-100	1.81E-05	3.19E-05	5.65E-05
OVCAR-8	0.230	0.838	0.887	0.877	0.856	0.761	0.225	108	106	103	87	-2	2.61E-05	9.46E-05	>1.00E-04
SK-OV-3	0.356	0.875	0.918	0.944	0.923	0.787	0.119	108	113	109	83	-67	1.66E-05	3.58E-05	7.73E-05
Renal Cancer															
786-0	0.429	1.357	1.454	1.369	1.438	1.310	0.004	111	101	109	95	-99	1.71E-05	3.09E-05	5.59E-05
A498	0.991	1.569	1.726	1.696	1.615	1.549	0.485	127	122	108	97	-51	2.07E-05	4.51E-05	9.83E-05
ACHN	0.341	1.323	1.344	1.347	1.303	1.002	0.033	102	102	98	67	-90	1.29E-05	2.67E-05	5.54E-05
CAKI-1	0.413	1.184	1.232	1.280	1.202	1.046	0.134	106	112	102	82	-68	1.64E-05	3.53E-05	7.62E-05
RXF 393	0.583	1.085	1.166	1.198	1.165	0.909	0.295	116	123	116	65	-49	1.35E-05	3.70E-05	>1.00E-04
SN12C	0.302	0.848	0.863	0.866	0.859	0.813	0.143	103	103	102	93	-53	1.98E-05	4.35E-05	9.57E-05
TK-10	0.520	1.261	1.397	1.276	1.300	1.100	0.025	118	102	105	78	-95	1.45E-05	2.82E-05	5.49E-05
UO-31	0.526	1.214	1.256	1.294	1.273	1.033	0.178	106	112	109	74	-66	1.48E-05	3.36E-05	7.66E-05
Prostate Cancer															
PC-3	0.293	0.980	0.936	0.958	0.924	0.891	0.039	94	97	92	87	-87	1.63E-05	3.17E-05	6.14E-05
DU-145	0.265	0.809	0.808	0.811	0.822	0.686	0.039	100	100	102	77	-85	1.47E-05	2.99E-05	6.07E-05
Breast Cancer															
MCF7	0.316	1.596	1.672	1.589	1.542	1.248	0.196	106	99	96	73	-38	1.61E-05	4.54E-05	>1.00E-04
NCI/ADR-RES	0.282	0.856	0.918	0.923	0.897	0.771	0.170	111	112	107	85	-40	1.91E-05	4.80E-05	>1.00E-04
MDA-MB-231/ATCC	0.391	1.031	1.038	1.099	1.029	0.944	0.091	101	111	100	86	-77	1.67E-05	3.39E-05	6.86E-05
HS 578T	0.225	0.936	1.067	1.029	1.003	0.940	0.297	118	113	109	100	10	3.62E-05	>1.00E-04	>1.00E-04
MDA-MB-435	0.297	1.166	1.238	1.242	1.211	1.100	0.140	108	109	105	92	-53	1.96E-05	4.33E-05	9.56E-05
MDA-N	0.691	2.522	2.654	2.499	2.665	2.529	0.353	107	99	108	100	-49	2.17E-05	4.70E-05	>1.00E-04
BT-549	0.304	0.739	0.797	0.789	0.772	0.772	0.059	113	111	108	107	-81	2.02E-05	3.72E-05	6.86E-05
T-47D	0.433	0.891	0.878	0.910	0.878	0.783	0.088	97	104	97	76	-80	1.47E-05	3.08E-05	6.45E-05



NU:UB 107

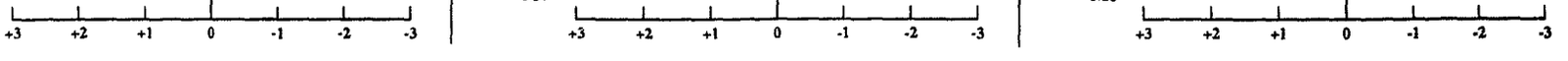
National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709773 -Z / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD59-25

Report Date: August 6, 1999 | Test Date: July 12, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-4.72	-4.28	> -4.00
HL-60(TB)	-4.85	-4.45	-4.05
K-562	-4.81	-4.38	> -4.00
MOLT-4	-4.81	-4.44	-4.07
SR	-4.74	-4.40	-4.05
Non-Small Cell Lung Cancer			
A549/ATCC	-4.69	-4.37	-4.06
EKVX	-5.04	-4.44	> -4.00
HOP-62	-4.71	-4.38	-4.05
HOP-92	-4.70	-4.32	> -4.00
NCI-H226	-4.62	-4.35	-4.08
NCI-H23	-4.74	-4.50	-4.25
NCI-H460	-4.76	-4.36	> -4.00
NCI-H522	-4.73	-4.31	> -4.00
Colon Cancer			
COLO 205	-4.73	-4.42	-4.12
HCC-2998	-4.74	-4.45	-4.15
HCT-116	-4.80	-4.45	-4.11
HCT-15	-4.84	-4.54	-4.24
HT29	-4.78	-4.48	-4.17
KM12	-4.84	-4.47	-4.10
SW-620	-4.62	> -4.00	> -4.00
CNS Cancer			
SF-268	-4.50	> -4.00	> -4.00
SF-295	-4.76	-4.49	-4.22
SNB-19	-4.59	-4.12	> -4.00
SNB-75	-4.70	-4.36	-4.01
U251	-4.74	-4.42	-4.09
Melanoma			
LOX IMVI	-4.70	-4.31	> -4.00
MALME-3M	-4.56	-4.23	> -4.00
M14	-4.53	-4.15	> -4.00
SK-MEL-28	-4.69	-4.36	-4.04
SK-MEL-5	-4.72	-4.48	-4.24
UACC-257	-4.68	-4.38	-4.09
UACC-62	-4.78	-4.51	-4.24
Ovarian Cancer			
IGROV1	-4.86	-4.48	-4.11
OVCAR-3	-4.80	-4.47	-4.14
OVCAR-4	-4.77	-4.43	-4.10
OVCAR-5	-4.74	-4.50	-4.25
OVCAR-8	-4.58	-4.02	> -4.00
SK-OV-3	-4.78	-4.45	-4.11
Renal Cancer			
786-0	-4.77	-4.51	-4.25
A498	-4.68	-4.35	-4.01
ACHN	-4.89	-4.57	-4.26
CAKI-1	-4.79	-4.45	-4.12
RXF 393	-4.87	-4.43	> -4.00
SN12C	-4.70	-4.36	-4.02
TK-10	-4.84	-4.55	-4.26
UO-31	-4.83	-4.47	-4.12
Prostate Cancer			
PC-3	-4.79	-4.50	-4.21
DU-145	-4.83	-4.52	-4.22
Breast Cancer			
MCF7	-4.79	-4.34	> -4.00
NCI/ADR-RES	-4.72	-4.32	> -4.00
MDA-MB-231/ATCC	-4.78	-4.47	-4.16
HS 578T	-4.44	> -4.00	> -4.00
MDA-MB-435	-4.71	-4.36	-4.02
MDA-N	-4.66	-4.33	> -4.00
BT-549	-4.69	-4.43	-4.16
T-47D	-4.83	-4.51	-4.19
MG_MID	-4.74	-4.38	-4.09
Delta	0.30	0.19	0.17
Range	0.60	0.57	0.26

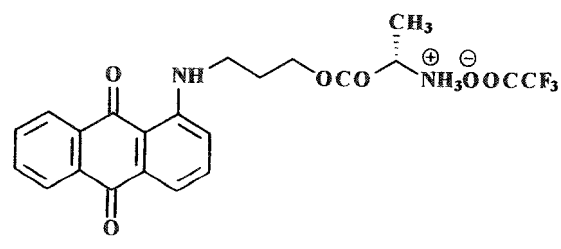


NU:UB 107 (192)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709774 -A / 1	**Experiment ID:** 9907MD59-26	**Test Type:** 08	**Units:** Molar
Report Date: August 6, 1999	**Test Date:** July 12, 1999	**QNS:**	**MC:**
COMI: NU:UB 108	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.300	0.738	0.751	0.878	0.829	0.729	0.221	103	132	121	98	-27	2.43E-05	6.12E-05	>1.00E-04
HL-60(TB)	0.939	2.831	3.167	3.224	3.097	2.635	0.289	118	121	114	90	-69	1.78E-05	3.67E-05	7.56E-05
K-562	0.157	1.022	1.047	1.022	0.972	0.865	0.096	103	100	94	82	-39	1.83E-05	4.76E-05	>1.00E-04
MOLT-4	0.423	1.485	1.569	1.541	1.521	1.241	0.184	108	105	103	77	-57	1.59E-05	3.77E-05	8.94E-05
SR	1.084	3.005	3.288	3.398	3.296	2.904	0.440	115	120	115	95	-59	1.95E-05	4.12E-05	8.68E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.272	1.387	1.459	1.436	1.464	1.318	0.084	106	104	107	94	-69	1.86E-05	3.76E-05	7.63E-05
EKVX	0.307	0.749	0.790	0.792	0.783	0.509	0.174	109	110	108	46	-43	8.53E-06	3.26E-05	>1.00E-04
HOP-62	0.362	0.911	1.024	0.965	0.974	0.918	0.175	120	110	111	101	-52	2.16E-05	4.59E-05	9.75E-05
HOP-92	0.506	0.825	0.849	0.846	0.855	0.783	0.273	108	107	109	87	-46	1.89E-05	4.50E-05	>1.00E-04
NCI-H226	0.788	1.141	1.144	1.207	1.208	1.089	0.352	101	119	119	85	-55	1.78E-05	4.04E-05	9.16E-05
NCI-H23	0.291	0.920	0.974	0.974	1.013	0.896	0.003	109	108	115	96	-99	1.72E-05	3.11E-05	5.60E-05
NCI-H460	0.341	1.982	1.985	2.313	2.066	1.674	0.144	100	120	105	81	-58	1.68E-05	3.84E-05	8.79E-05
NCI-H522	0.267	0.870	0.828	0.817	0.811	0.750	0.075	93	91	90	80	-72	1.57E-05	3.36E-05	7.16E-05
Colon Cancer															
COLO 205	0.553	1.802	1.757	1.845	1.794	1.588	0.009	96	103	99	83	-98	1.52E-05	2.86E-05	5.40E-05
HCC-2998	0.333	0.863	0.822	0.919	0.833	0.842	0.021	92	110	94	96	-94	1.75E-05	3.20E-05	5.88E-05
HCT-116	0.119	1.067	1.135	1.179	1.117	0.986	-0.006	107	112	105	92	-100	1.65E-05	3.00E-05	5.48E-05
HCT-15	0.084	0.603	0.592	0.628	0.600	0.483	-0.011	98	105	99	77	-100	1.42E-05	2.72E-05	5.22E-05
HT29	0.381	1.815	1.889	1.896	1.881	1.708	0.016	105	106	105	93	-96	1.68E-05	3.10E-05	5.71E-05
KM12	0.279	1.185	1.262	1.299	1.290	1.000	0.111	108	113	112	80	-60	1.63E-05	3.71E-05	8.45E-05
SW-620	0.081	0.552	0.573	0.575	0.549	0.484	0.072	104	105	99	85	-11	2.33E-05	7.67E-05	>1.00E-04
CNS Cancer															
SF-268	0.251	1.061	1.165	1.153	1.126	0.981	0.256	113	111	108	90	1	2.80E-05	>1.00E-04	>1.00E-04
SF-295	0.622	1.863	1.973	1.870	1.845	1.747	0.022	109	101	99	91	-96	1.65E-05	3.05E-05	5.64E-05
SNB-19	0.387	1.120	1.173	1.212	1.215	1.112	0.139	107	113	113	99	-64	2.00E-05	4.04E-05	8.18E-05
SNB-75	0.418	0.589	0.682	0.678	0.671	0.655	0.261	154	152	148	138	-38	3.18E-05	6.11E-05	>1.00E-04
U251	0.268	1.297	1.336	1.361	1.317	1.235	0.053	104	106	102	94	-80	1.79E-05	3.46E-05	6.69E-05
Melanoma															
LOX IMVI	0.226	1.186	1.237	1.233	1.232	1.007	0.065	105	105	105	81	-71	1.60E-05	3.41E-05	7.26E-05
MALME-3M	0.319	0.788	0.836	0.849	0.846	0.817	0.380	110	113	112	106	13	4.01E-05	>1.00E-04	>1.00E-04
M14	0.402	1.454	1.469	1.505	1.654	1.628	0.123	101	105	119	117	-70	2.28E-05	4.23E-05	7.85E-05
SK-MEL-28	0.220	0.712	0.768	0.766	0.775	0.735	0.084	111	111	113	105	-62	2.13E-05	4.25E-05	8.49E-05
SK-MEL-5	0.703	1.697	1.659	1.682	1.664	1.593	0.008	96	99	97	90	-99	1.62E-05	2.99E-05	5.50E-05
UACC-257	0.615	1.459	1.394	1.473	1.497	1.446	0.131	92	102	104	98	-79	1.88E-05	3.59E-05	6.88E-05
UACC-62	0.457	1.646	1.697	1.732	1.679	1.517	0.025	104	107	103	89	-95	1.63E-05	3.05E-05	5.72E-05
Ovarian Cancer															
IGROV1	0.279	1.087	1.125	1.100	1.066	0.842	0.168	105	102	97	70	-40	1.51E-05	4.32E-05	>1.00E-04
OVCAR-3	0.534	1.182	1.241	1.234	1.223	1.117	0.032	109	108	106	90	-94	1.65E-05	3.08E-05	5.76E-05
OVCAR-4	0.510	1.191	1.316	1.262	1.261	1.091	0.220	118	110	110	85	-57	1.77E-05	3.98E-05	8.93E-05
OVCAR-5	0.596	1.272	1.440	1.427	1.377	1.281	0.004	125	123	116	101	-99	1.80E-05	3.20E-05	5.68E-05
OVCAR-8	0.230	0.838	0.892	0.927	0.914	0.767	0.113	109	115	113	88	-51	1.88E-05	4.30E-05	9.82E-05
SK-OV-3	0.356	0.875	0.897	0.884	0.884	0.799	0.060	104	102	102	85	-83	1.62E-05	3.21E-05	6.36E-05
Renal Cancer															
786-0	0.429	1.357	1.475	1.596	1.513	1.383	0.008	113	126	117	103	-98	1.83E-05	3.25E-05	5.76E-05
A498	0.991	1.569	1.630	1.635	1.623	1.523	0.462	111	111	109	92	-53	1.94E-05	4.29E-05	9.48E-05
ACHN	0.341	1.323	1.308	1.349	1.288	1.000	0.049	98	103	96	67	-86	1.29E-05	2.75E-05	5.84E-05
CAKI-1	0.413	1.184	1.253	1.234	1.255	1.069	0.096	109	106	109	85	-77	1.65E-05	3.36E-05	6.83E-05
RXF 393	0.583	1.085	1.283	1.245	1.292	1.086	0.232	140	132	141	100	-60	2.06E-05	4.21E-05	8.63E-05
SN12C	0.302	0.848	0.936	0.963	0.929	0.867	0.091	116	121	115	103	-70	2.03E-05	3.95E-05	7.68E-05
TK-10	0.520	1.261	1.253	1.274	1.276	1.037	0.045	99	102	102	70	-91	1.32E-05	2.71E-05	5.54E-05
UO-31	0.526	1.214	1.260	1.300	1.334	1.037	0.130	107	113	117	74	-75	1.45E-05	3.14E-05	6.78E-05
Prostate Cancer															
PC-3	0.293	0.980	0.993	1.010	1.011	1.014	0.014	102	104	104	105	-95	1.88E-05	3.34E-05	5.94E-05
DU-145	0.265	0.809	0.892	0.858	0.848	0.756	0.105	115	109	107	90	-61	1.85E-05	3.97E-05	8.51E-05
Breast Cancer															
MCF7	0.316	1.596	1.520	1.561	1.542	1.330	0.149	94	97	96	79	-53	1.66E-05	3.98E-05	9.52E-05
NCI/ADR-RES	0.282	0.856	0.909	0.906	0.899	0.784	0.180	109	109	108	88	-36	2.01E-05	5.10E-05	>1.00E-04
MDA-MB-231/ATCC	0.391	1.031	1.077	1.050	1.044	0.962	0.100	107	103	102	89	-75	1.74E-05	3.51E-05	7.08E-05
HS 578T	0.225	0.936	0.996	0.958	0.944	0.882	0.328	108	103	101	92	14	3.49E-05	>1.00E-04	>1.00E-04
MDA-MB-435	0.297	1.166	1.245	1.221	1.290	1.166	0.099	109	106	114	100	-67	1.99E-05	3.98E-05	7.93E-05
MDA-N	0.691	2.522	2.708	2.783	2.738	2.642	0.041	110	114	112	107	-94	1.91E-05	3.40E-05	6.03E-05
BT-549	0.304	0.739	0.793	0.806	0.776	0.780	0.003	112	115	108	109	-99	1.93E-05	3.35E-05	5.82E-05
T-47D	0.433	0.891	1.036	0.927	0.964	0.918	0.308	131	108	116	106	-29	2.59E-05	6.10E-05	>1.00E-04



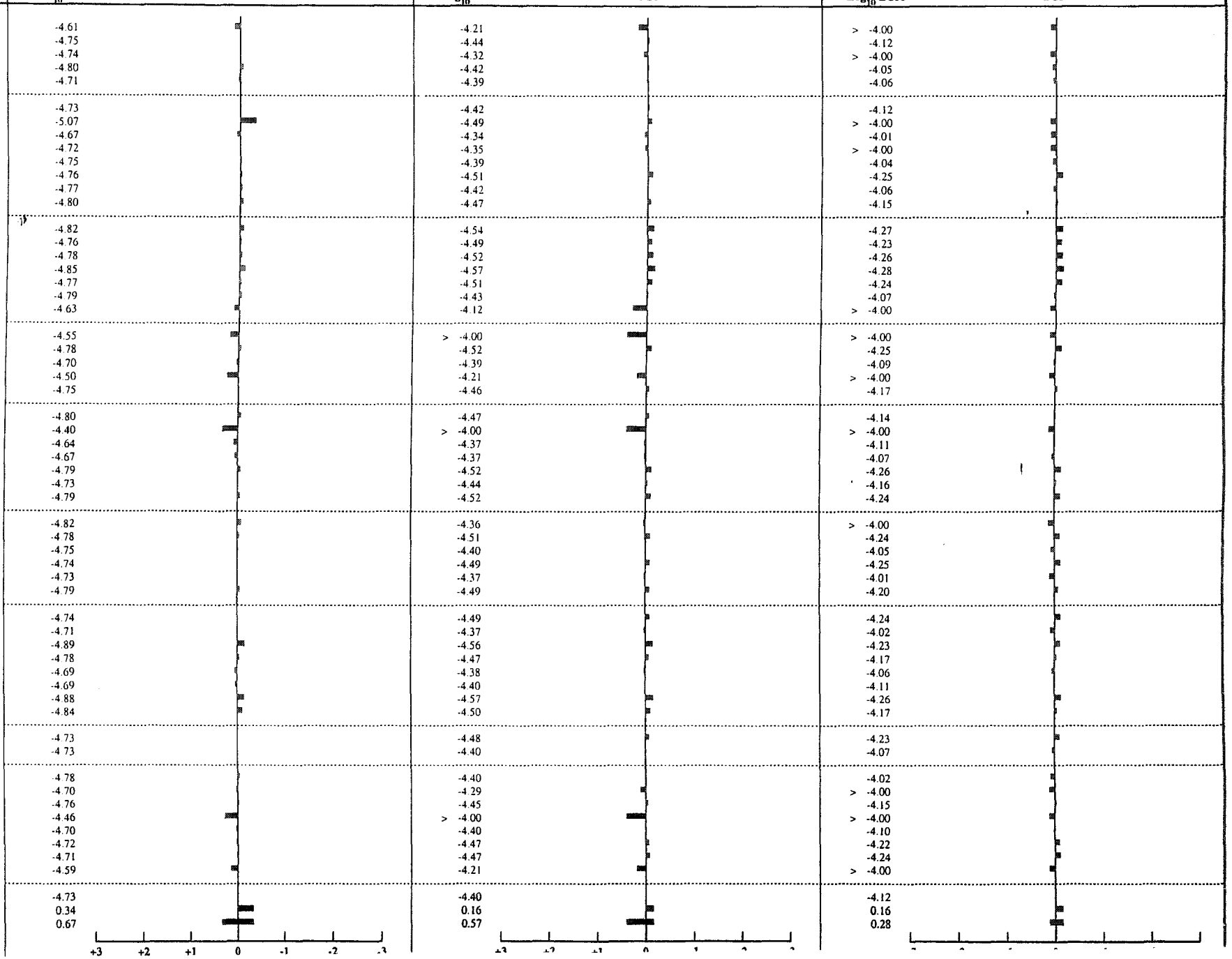
NU:UB 108

National Cancer Institute Developmental Therapeutics Program
Mean Graphs

NSC: D- 709774 -A / 1 | Units: Molar | SSPL: 0B6Z | Exp. ID:9907MD59-26

Report Date: August 6, 1999 | Test Date: July 12, 1999

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-4.61	-4.21	> -4.00
HL-60(TB)	-4.75	-4.44	-4.12
K-562	-4.74	-4.32	> -4.00
MOLT-4	-4.80	-4.42	-4.05
SR	-4.71	-4.39	-4.06
Non-Small Cell Lung Cancer			
A549/ATCC	-4.73	-4.42	-4.12
EKVX	-5.07	-4.49	> -4.00
HOP-62	-4.67	-4.34	-4.01
HOP-92	-4.72	-4.35	> -4.00
NCI-H226	-4.75	-4.39	-4.04
NCI-H23	-4.76	-4.51	-4.25
NCI-H460	-4.77	-4.42	-4.06
NCI-H522	-4.80	-4.47	-4.15
Colon Cancer			
COLO 205	-4.82	-4.54	-4.27
HCC-2998	-4.76	-4.49	-4.23
HCT-116	-4.78	-4.52	-4.26
HCT-15	-4.85	-4.57	-4.28
HT29	-4.77	-4.51	-4.24
KM12	-4.79	-4.43	-4.07
SW-620	-4.63	-4.12	> -4.00
CNS Cancer			
SF-268	-4.55	> -4.00	> -4.00
SF-295	-4.78	-4.52	-4.25
SNB-19	-4.70	-4.39	-4.09
SNB-75	-4.50	-4.21	> -4.00
U251	-4.75	-4.46	-4.17
Melanoma			
LOX IMVI	-4.80	-4.47	-4.14
MALME-3M	-4.40	> -4.00	> -4.00
M14	-4.64	-4.37	-4.11
SK-MEL-28	-4.67	-4.37	-4.07
SK-MEL-5	-4.79	-4.52	-4.26
UACC-257	-4.73	-4.44	-4.16
UACC-62	-4.79	-4.52	-4.24
Ovarian Cancer			
IGROV1	-4.82	-4.36	> -4.00
OVCAR-3	-4.78	-4.51	-4.24
OVCAR-4	-4.75	-4.40	-4.05
OVCAR-5	-4.74	-4.49	-4.25
OVCAR-8	-4.73	-4.37	-4.01
SK-OV-3	-4.79	-4.49	-4.20
Renal Cancer			
786-0	-4.74	-4.49	-4.24
A498	-4.71	-4.37	-4.02
ACHN	-4.89	-4.56	-4.23
CAKI-1	-4.78	-4.47	-4.17
RXF 393	-4.69	-4.38	-4.06
SN12C	-4.69	-4.40	-4.11
TK-10	-4.88	-4.57	-4.26
UO-31	-4.84	-4.50	-4.17
Prostate Cancer			
PC-3	-4.73	-4.48	-4.23
DU-145	-4.73	-4.40	-4.07
Breast Cancer			
MCF7	-4.78	-4.40	-4.02
NCI/ADR-RES	-4.70	-4.29	> -4.00
MDA-MB-231/ATCC	-4.76	-4.45	-4.15
HS 578T	-4.46	> -4.00	> -4.00
MDA-MB-435	-4.70	-4.40	-4.10
MDA-N	-4.72	-4.47	-4.22
BT-549	-4.71	-4.47	-4.24
T-47D	-4.59	-4.21	> -4.00
MG_MID	-4.73	-4.40	-4.12
Delta	0.34	0.16	0.16
Range	0.67	0.57	0.28

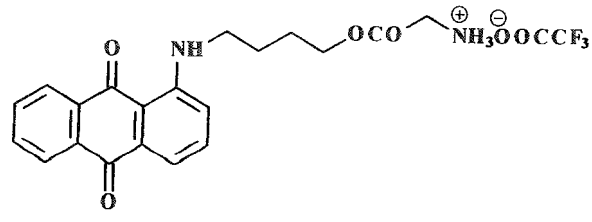


NU:UB 108 (190)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 709775 -C / 1	**Experiment ID:** 9907MD59-27	**Test Type:** 08	**Units:** Molar
Report Date: August 6, 1999	**Test Date:** July 12, 1999	**QNS:**	**MC:**
COMI: NU:UB 110	**Stain Reagent:** SRB Dual-P	**SSPL:** 0B6Z	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.300	0.757	0.751	0.801	0.769	0.565	0.137	99	110	103	58	-54	1.18E-05	3.28E-05	9.15E-05
HL-60(TB)	0.939	3.020	3.068	2.942	2.919	2.002	0.507	102	96	95	51	-46	1.03E-05	3.36E-05	>1.00E-04
K-562	0.157	0.938	0.972	0.957	0.952	0.534	0.021	104	102	102	48	-87	9.28E-06	2.27E-05	5.33E-05
MOLT-4	0.423	1.476	1.509	1.548	1.432	1.063	0.146	103	107	96	61	-66	1.22E-05	3.02E-05	7.52E-05
SR	1.084	3.075	3.141	3.142	3.026	2.307	0.444	103	103	98	61	-59	1.24E-05	3.23E-05	8.41E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.272	1.236	1.285	1.266	1.235	1.055	0.042	105	103	100	81	-85	1.54E-05	3.09E-05	6.18E-05
EKVX	0.307	0.730	0.735	0.734	0.286	0.599	0.100	101	101	-7	69	-68	.	.	7.43E-05
HOP-62	0.362	0.844	0.850	0.851	0.851	0.762	0.092	101	101	101	83	-75	1.62E-05	3.36E-05	6.98E-05
HOP-92	0.506	0.795	0.788	0.774	0.771	0.740	0.145	97	93	92	81	-71	1.60E-05	3.40E-05	7.24E-05
NCI-H226	0.788	1.013	0.997	1.074	1.044	0.938	0.292	93	127	114	66	-63	1.34E-05	3.26E-05	7.94E-05
NCI-H23	0.291	0.926	0.960	0.952	0.940	0.844	0.002	105	104	102	87	-99	1.58E-05	2.93E-05	5.43E-05
NCI-H460	0.341	2.010	1.968	1.949	1.909	1.528	0.192	97	96	94	71	-44	1.53E-05	4.16E-05	>1.00E-04
NCI-H522	0.267	0.842	0.876	0.875	0.846	0.699	0.083	106	106	101	75	-69	1.49E-05	3.32E-05	7.37E-05
Colon Cancer															
COLO 205	0.553	1.708	1.658	1.709	1.632	1.468	0.027	96	100	93	79	-95	1.47E-05	2.85E-05	5.51E-05
HCC-2998	0.333	0.923	0.913	0.963	0.885	0.840	0.118	98	107	94	86	-65	1.73E-05	3.72E-05	7.99E-05
HCT-116	0.119	1.110	1.123	1.138	1.113	0.754	0.018	101	103	100	64	-85	1.24E-05	2.68E-05	5.80E-05
HCT-15	0.084	0.565	0.566	0.577	0.573	0.366	-0.029	100	103	102	59	-100	1.13E-05	2.34E-05	4.84E-05
HT29	0.381	1.669	1.484	1.738	1.114	1.383	-0.001	86	105	57	78	-100	1.43E-05	2.74E-05	5.23E-05
KM12	0.279	1.227	1.148	1.144	1.114	0.885	0.040	92	91	88	64	-86	1.24E-05	2.67E-05	5.77E-05
SW-620	0.081	0.538	0.519	0.532	0.498	0.430	0.047	96	99	91	76	-43	1.66E-05	4.38E-05	>1.00E-04
CNS Cancer															
SF-268	0.251	1.103	1.112	1.105	1.068	0.940	0.140	101	100	96	81	-44	1.76E-05	4.42E-05	>1.00E-04
SF-295	0.622	1.887	1.823	1.871	1.856	1.629	0.071	95	99	98	80	-89	1.50E-05	2.97E-05	5.90E-05
SNB-19	0.387	1.028	1.034	0.997	1.066	0.887	0.070	101	95	106	78	-82	1.50E-05	3.07E-05	6.32E-05
SNB-75	0.418	0.646	0.655	0.656	0.661	0.632	0.161	104	105	106	94	-61	1.92E-05	4.02E-05	8.44E-05
U251	0.268	1.182	1.135	1.151	1.250	0.950	0.026	95	97	107	75	-90	1.41E-05	2.83E-05	5.69E-05
Melanoma															
LOX IMVI	0.226	1.208	1.220	1.226	1.215	0.948	0.016	101	102	101	74	-93	1.38E-05	2.77E-05	5.52E-05
MALME-3M	0.319	0.807	0.772	0.764	0.773	0.754	0.116	93	91	93	89	-64	1.80E-05	3.83E-05	8.12E-05
M14	0.402	1.426	1.525	1.518	1.537	1.374	-0.006	110	109	111	95	-100	1.70E-05	3.07E-05	5.54E-05
SK-MEL-28	0.220	0.710	0.707	0.718	0.676	0.616	0.137	99	102	93	81	-38	1.82E-05	4.81E-05	>1.00E-04
SK-MEL-5	0.703	1.591	1.567	1.537	1.515	1.403	0.023	97	94	91	79	-97	1.46E-05	2.81E-05	5.42E-05
UACC-257	0.615	1.532	1.537	1.484	1.506	1.533	0.175	101	95	97	100	-72	1.96E-05	3.83E-05	7.49E-05
UACC-62	0.457	1.703	1.678	1.605	1.601	1.277	0.098	98	92	92	66	-79	1.29E-05	2.86E-05	6.34E-05
Ovarian Cancer															
IGROV1	0.279	1.028	0.997	1.071	1.032	0.802	0.026	96	106	101	70	-91	1.33E-05	2.72E-05	5.57E-05
OVCAR-3	0.534	0.866	0.849	0.854	0.860	0.686	0.082	95	96	98	46	-85	8.24E-06	2.24E-05	5.42E-05
OVCAR-4	0.510	1.369	1.372	1.302	1.184	1.003	0.173	100	92	79	57	-66	1.15E-05	2.91E-05	7.40E-05
OVCAR-5	0.596	1.294	1.256	1.346	0.916	1.184	0.054	95	108	46	84	-91	.	3.03E-05	5.83E-05
OVCAR-8	0.230	0.886	0.873	0.852	0.868	0.699	0.026	98	95	97	71	-89	1.36E-05	2.79E-05	5.72E-05
SK-OV-3	0.356	0.776	0.738	0.758	0.766	0.642	0.096	91	96	98	68	-73	1.34E-05	3.04E-05	6.87E-05
Renal Cancer															
786-0	0.429	1.749	1.692	1.777	1.725	1.267	0.117	96	102	98	63	-73	1.26E-05	2.92E-05	6.81E-05
A498	0.991	1.620	1.565	1.514	1.520	1.392	0.266	91	83	84	64	-73	1.26E-05	2.92E-05	6.77E-05
ACHN	0.341	1.331	1.246	1.196	1.223	0.819	0.071	91	86	89	48	-79	9.09E-06	2.39E-05	5.90E-05
CAKI-1	0.413	1.228	1.171	1.220	1.165	0.902	0.100	93	99	92	60	-76	1.18E-05	2.76E-05	6.45E-05
RXF 393	0.583	1.013	0.985	1.006	1.002	0.807	0.252	93	98	97	52	-57	1.04E-05	3.01E-05	8.65E-05
SN12C	0.302	1.002	0.988	1.010	1.008	0.807	0.068	98	101	101	72	-77	1.40E-05	3.03E-05	6.55E-05
TK-10	0.520	1.281	1.236	1.271	1.283	1.068	0.213	94	99	100	72	-59	1.47E-05	3.54E-05	8.53E-05
UO-31	0.526	0.962	0.969	0.928	0.910	0.666	0.064	101	92	88	32	-88	4.77E-06	1.85E-05	4.83E-05
Prostate Cancer															
PC-3	0.293	0.897	0.867	0.840	0.890	0.800	0.003	95	91	99	84	-99	1.53E-05	2.88E-05	5.40E-05
DU-145	0.265	0.829	0.816	0.818	0.801	0.634	0.078	98	98	95	65	-71	1.30E-05	3.03E-05	7.06E-05
Breast Cancer															
MCF7	0.316	1.535	1.563	1.481	1.426	1.421	0.115	102	96	91	91	-64	1.83E-05	3.87E-05	8.16E-05
NCI/ADR-RES	0.282	0.830	0.828	0.840	0.820	0.694	0.083	100	102	98	75	-71	1.49E-05	3.27E-05	7.21E-05
MDA-MB-231/ATCC	0.391	0.917	0.886	0.906	0.978	0.839	0.103	94	98	112	85	-74	1.66E-05	3.44E-05	7.10E-05
HS 578T	0.225	0.976	0.985	0.969	0.996	0.891	0.197	101	99	103	89	-13	2.41E-05	7.50E-05	>1.00E-04
MDA-MB-435	0.297	1.249	1.236	1.247	1.180	1.052	0.039	99	100	93	79	-87	1.50E-05	3.00E-05	5.99E-05
MDA-N	0.691	2.549	2.648	2.786	2.864	2.413	0.056	105	113	117	93	-92	1.70E-05	3.18E-05	5.92E-05
BT-549	0.304	0.732	0.747	0.755	0.746	0.693	0.066	103	105	103	91	-78	1.74E-05	3.44E-05	6.79E-05
T-47D	0.433	0.836	0.792	0.821	0.813	0.705	0.224	89	96	94	67	-48	1.41E-05	3.83E-05	>1.00E-04



NU:UB 110

National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 709775 -C / 1 | Units: Molar | SSPL: 0B62 | Exp. ID:990/MD59-27

Report Date: August 6, 1999 | Test Date: July 12, 1999

Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
CCRF-CEM	-4.93		-4.48		-4.04	
HL-60(TB)	-4.99		-4.47		> -4.00	
K-562	-5.03		-4.64		-4.27	
MOLT-4	-4.91		-4.52		-4.12	
SR	-4.91		-4.49		-4.08	
Non-Small Cell Lung Cancer						
A549/ATCC	-4.81		-4.51		-4.21	
EKVX					-4.13	
HOP-62	-4.79		-4.47		-4.16	
HOP-92	-4.80		-4.47		-4.14	
NCI-H226	-4.87		-4.49		-4.10	
NCI-H23	-4.80		-4.53		-4.27	
NCI-H460	-4.82		-4.38		> -4.00	
NCI-H522	-4.83		-4.48		-4.13	
Colon Cancer						
COLO 205	-4.83		-4.55		-4.26	
HCC-2998	-4.76		-4.43		-4.10	
HCT-116	-4.91		-4.57		-4.24	
HCT-15	-4.95		-4.63		-4.32	
HT29	-4.84		-4.56		-4.28	
KM12	-4.91		-4.57		-4.24	
SW-620	-4.78		-4.36		> -4.00	
CNS Cancer						
SF-268	-4.75		-4.35		> -4.00	
SF-295	-4.82		-4.53		-4.23	
SNB-19	-4.82		-4.51		-4.20	
SNB-75	-4.72		-4.40		-4.07	
U251	-4.85		-4.55		-4.24	
Melanoma						
LOX IMVI	-4.86		-4.56		-4.26	
MALME-3M	-4.74		-4.42		-4.09	
M14	-4.77		-4.51		-4.26	
SK-MEL-28	-4.74		-4.32		> -4.00	
SK-MEL-5	-4.84		-4.55		-4.27	
UACC-257	-4.71		-4.42		-4.13	
UACC-62	-4.89		-4.54		-4.20	
Ovarian Cancer						
IGROV1	-4.88		-4.57		-4.25	
OVCAR-3	-5.08		-4.65		-4.27	
OVCAR-4	-4.94		-4.54		-4.13	
OVCAR-5			-4.52		-4.23	
OVCAR-8	-4.87		-4.55		-4.24	
SK-OV-3	-4.87		-4.52		-4.16	
Renal Cancer						
786-0	-4.90		-4.53		-4.17	
A498	-4.90		-4.53		-4.17	
ACHN	-5.04		-4.62		-4.23	
CAKI-1	-4.93		-4.56		-4.19	
RXF 393	-4.98		-4.52		-4.06	
SN12C	-4.85		-4.52		-4.18	
TK-10	-4.83		-4.45		-4.07	
UO-31	-5.32		-4.73		-4.32	
Prostate Cancer						
PC-3	-4.82		-4.54		-4.27	
DU-145	-4.89		-4.52		-4.15	
Breast Cancer						
MCF7	-4.74		-4.41		-4.09	
NCI/ADR-RES	-4.83		-4.49		-4.14	
MDA-MB-231/ATCC	-4.78		-4.46		-4.15	
HS 578T	-4.62		-4.12		> -4.00	
MDA-MB-435	-4.82		-4.52		-4.22	
MDA-N	-4.77		-4.50		-4.23	
BT-549	-4.76		-4.46		-4.17	
T-47D	-4.85		-4.42		> -4.00	
MG_MID	-4.86		-4.50		-4.16	
Delta	0.47		0.23		0.15	
Range	0.70		0.61		0.32	

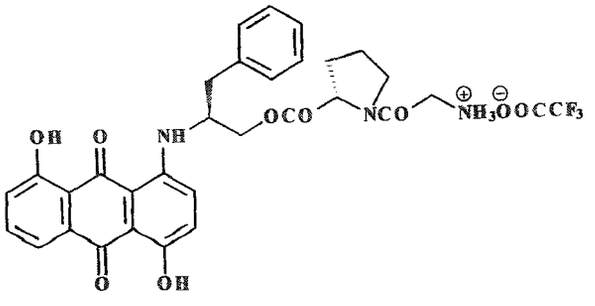
+3 +2 +1 0 -1 -2 -3

NU:UB 110 (172)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 725552 / 1	**Experiment ID:** 0210NS71-18	**Test Type:** 08	**Units:** Molar
Report Date: October 31, 2002	**Test Date:** October 7, 2002	**QNS:**	**MC:**
COMI: NU:UB 159 (16860)	**Stain Reagent:** SRB Dual-Pass	**SSPL:** 0JQS	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.875	2.091	2.072	2.063	1.749	0.836	0.161	98	98	72	-4	-82	1.93E-06	8.74E-06	3.89E-05
HL-60(TB)	0.743	2.544	2.691	2.581	2.308	0.988	0.220	108	102	87	14	-70	3.19E-06	1.45E-05	5.72E-05
K-562	0.563	1.245	1.231	1.351	1.195	0.417	0.099	98	116	93	-26	-83	2.29E-06	6.04E-06	2.66E-05
MOLT-4	0.717	0.958	1.178	1.266	1.050	0.580	0.107	192	228	138	-19	-85	3.64E-06	7.56E-06	2.94E-05
RPMI-8226	1.546	2.483	2.523	2.443	2.390	1.503	0.452	104	96	90	-3	-71	2.70E-06	9.33E-06	4.94E-05
Non-Small Cell Lung Cancer															
A549/ATCC	0.254	1.233	1.327	1.343	1.224	0.710	0.243	110	111	99	47	-4	8.60E-06	8.22E-05	>1.00E-04
EKVX	0.497	0.892	0.970	1.005	0.923	0.712	0.427	120	129	108	54	-14	1.16E-05	6.23E-05	>1.00E-04
HOP-62	0.624	1.332	1.326	1.294	1.234	0.917	0.231	99	95	86	41	-63	6.41E-06	2.49E-05	7.50E-05
HOP-92	0.388	0.954	0.973	0.959	0.938	0.746	0.405	103	101	97	63	3	1.66E-05	>1.00E-04	>1.00E-04
NCI-H226	0.556	1.120	1.098	1.061	0.962	0.672	0.465	96	89	72	20	-16	2.67E-06	3.59E-05	>1.00E-04
NCI-H23	0.710	1.282	1.206	1.242	1.171	0.877	0.225	87	93	81	29	-68	3.93E-06	1.99E-05	6.48E-05
NCI-H322M	0.451	0.733	0.790	0.788	0.733	0.567	0.230	120	120	100	41	-49	7.06E-06	2.86E-05	>1.00E-04
NCI-H460	0.277	1.800	1.867	1.842	1.651	0.680	0.168	104	103	90	26	-39	4.27E-06	2.52E-05	>1.00E-04
NCI-H522	0.229	0.949	1.014	1.068	0.937	0.657	0.208	109	117	98	59	-9	1.37E-05	7.31E-05	>1.00E-04
Colon Cancer															
COLO 205	0.288	1.129	1.150	1.239	1.174	0.496	0.159	102	113	105	25	-45	4.86E-06	2.26E-05	>1.00E-04
HCT-116	0.160	0.541	0.546	0.521	0.438	0.182	0.007	101	95	73	6	-96	2.20E-06	1.14E-05	3.55E-05
HCT-15	0.144	1.611	1.622	1.527	1.043	0.625	0.365	101	94	61	33	15	2.49E-06	>1.00E-04	>1.00E-04
HT29	0.168	1.107	1.179	1.195	1.061	0.514	0.118	108	109	95	37	-30	5.95E-06	3.58E-05	>1.00E-04
KM12	0.534	1.990	2.027	1.931	1.842	0.824	0.186	103	96	90	20	-65	3.71E-06	1.71E-05	6.63E-05
SW-620	0.197	0.754	0.821	0.797	0.817	0.356	0.161	112	108	111	28	-19	5.50E-06	4.03E-05	>1.00E-04
CNS Cancer															
SF-268	0.437	1.492	1.541	1.530	1.442	0.962	0.581	105	104	95	50	14	9.86E-06	>1.00E-04	>1.00E-04
SF-295	0.462	1.241	1.231	1.222	1.216	0.756	0.193	99	98	97	38	-58	6.20E-06	2.47E-05	8.19E-05
SF-539	0.540	1.011	1.091	1.093	1.101	0.719	0.128	117	117	119	38	-76	7.10E-06	2.15E-05	5.89E-05
SNB-19	0.304	0.927	0.854	0.954	0.888	0.486	0.252	88	104	94	29	-17	4.77E-06	4.25E-05	>1.00E-04
SNB-75	0.493	0.819	0.868	0.904	0.835	0.668	0.238	115	126	105	54	-52	1.08E-05	3.23E-05	9.63E-05
U251	0.248	0.939	0.936	0.967	0.791	0.415	0.155	100	104	79	24	-38	3.34E-06	2.45E-05	>1.00E-04
Melanoma															
LOX IMVI	0.325	1.331	1.440	1.419	1.325	0.550	0.115	111	109	99	22	-65	4.37E-06	1.81E-05	6.79E-05
MALME-3M	1.007	1.403	1.448	1.459	1.449	1.261	0.729	111	114	111	64	-28	1.42E-05	4.99E-05	>1.00E-04
SK-MEL-2	0.062	0.788	0.897	0.936	0.815	0.654	0.362	115	120	104	82	41	6.07E-05	>1.00E-04	>1.00E-04
SK-MEL-28	0.352	0.812	0.831	0.823	0.805	0.659	0.180	104	103	98	67	-49	1.40E-05	3.77E-05	>1.00E-04
SK-MEL-5	0.510	1.460	1.546	1.621	1.386	0.761	0.117	109	117	92	26	-77	4.37E-06	1.80E-05	5.47E-05
UACC-257	1.033	1.876	1.946	1.939	1.950	1.614	0.834	108	107	109	69	-19	1.64E-05	6.04E-05	>1.00E-04
UACC-62	0.617	1.306	1.442	1.355	1.244	0.733	0.217	120	107	91	17	-65	3.57E-06	1.61E-05	6.58E-05
Ovarian Cancer															
IGROV1	0.083	0.481	0.524	0.487	0.480	0.325	0.175	111	101	100	61	23	1.93E-05	>1.00E-04	>1.00E-04
OVCAR-3	0.612	1.200	1.205	1.190	1.132	0.733	0.243	101	98	88	21	-60	3.68E-06	1.79E-05	7.44E-05
OVCAR-4	0.665	1.537	1.563	1.580	1.509	0.875	0.487	103	105	97	24	-27	4.40E-06	2.98E-05	>1.00E-04
OVCAR-5	0.495	0.814	0.813	0.840	0.799	0.672	0.212	100	108	95	56	-57	1.12E-05	3.11E-05	8.64E-05
OVCAR-8	0.385	1.145	1.236	1.229	1.220	0.767	0.316	112	111	110	50	-18	1.01E-05	5.44E-05	>1.00E-04
SK-OV-3	0.368	0.959	0.926	0.963	0.985	0.348	0.201	94	101	104	-5	-45	3.13E-06	8.92E-06	>1.00E-04
Renal Cancer															
786-0	0.415	1.570	1.565	1.580	1.497	0.850	0.104	100	101	94	38	-75	6.02E-06	2.16E-05	6.00E-05
ACHN	0.358	1.185	1.180	1.186	1.120	0.610	0.148	99	100	92	30	-59	4.82E-06	2.20E-05	8.00E-05
CAKI-1	0.679	1.374	1.415	1.443	1.319	0.687	0.258	106	110	92	1	-62	2.91E-06	1.04E-05	6.44E-05
RXF 393	0.447	1.551	1.489	1.520	1.430	1.225	0.682	94	97	89	70	21	2.60E-05	>1.00E-04	>1.00E-04
SN12C	0.522	0.804	0.822	0.798	0.763	0.423	0.097	106	98	85	-19	-82	2.18E-06	6.58E-06	3.13E-05
TK-10	0.977	1.528	1.547	1.547	1.414	1.140	0.465	103	103	79	30	-52	3.87E-06	2.29E-05	9.33E-05
UO-31	0.127	1.278	1.402	1.365	1.242	0.970	0.605	111	108	97	73	42	5.41E-05	>1.00E-04	>1.00E-04
Prostate Cancer															
PC-3	0.157	0.553	0.511	0.524	0.500	0.291	0.064	89	92	86	34	-59	4.91E-06	2.30E-05	7.95E-05
DU-145	0.292	0.839	0.883	0.914	0.886	0.608	0.163	108	114	109	58	-44	1.19E-05	3.68E-05	>1.00E-04
Breast Cancer															
MCF7	0.142	1.379	1.398	1.360	1.295	0.745	0.319	102	98	93	49	14	9.38E-06	>1.00E-04	>1.00E-04
NCI/ADR-RES	0.708	1.010	1.027	1.001	0.975	0.557	0.246	106	97	88	-21	-65	2.24E-06	6.39E-06	4.48E-05
MDA-MB-231/ATCC	0.493	0.952	0.977	0.950	0.865	0.592	0.286	105	99	81	22	-42	3.32E-06	2.18E-05	>1.00E-04
HS 578T	0.799	1.209	1.411	1.423	1.300	0.956	0.514	149	152	122	38	-36	7.23E-06	3.29E-05	>1.00E-04
MDA-MB-435	0.644	1.683	1.762	1.747	1.616	0.686	0.313	108	106	94	4	-51	3.06E-06	1.18E-05	9.41E-05
BT-549	0.683	1.261	1.340	1.382	1.342	1.215	0.550	114	121	114	92	-20	2.38E-05	6.68E-05	>1.00E-04
T-47D	0.634	1.177	1.149	1.142	1.099	0.800	0.536	95	94	86	31	-16	4.43E-06	4.60E-05	>1.00E-04



NU:UB159

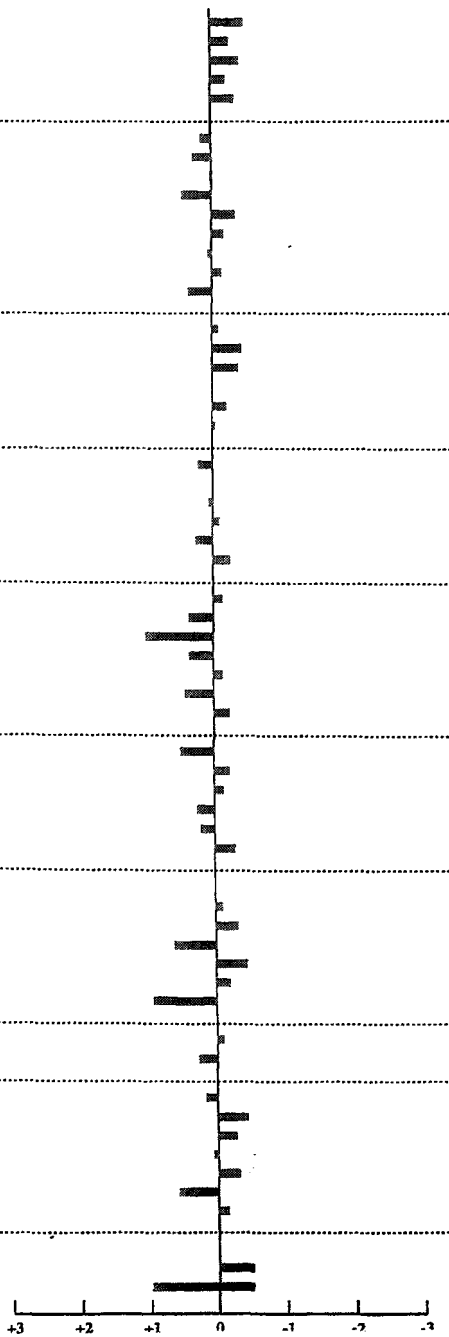
(273)

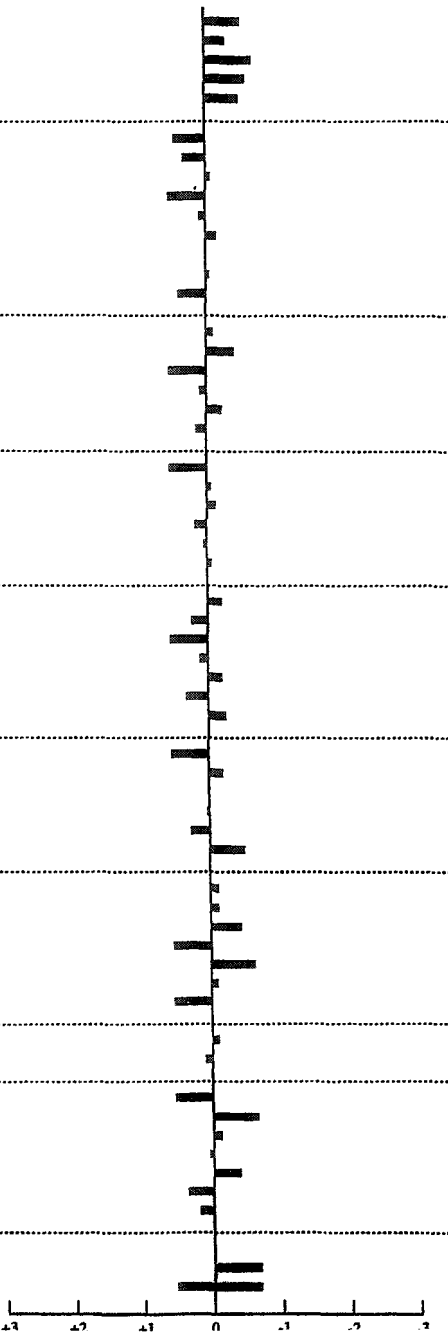
National Cancer Institute Developmental Therapeutics Program

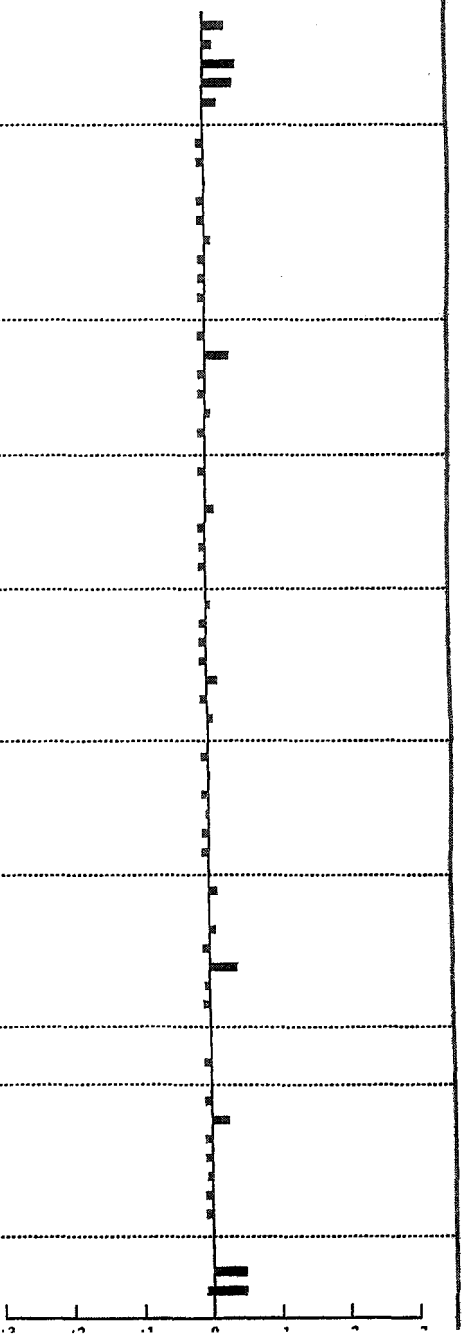
Mean Graphs

NSC: D- 725552 / 1	Units: Molar	SSPL: 0JQS	Exp. ID:0210NS71-18
Report Date: October 31, 2002		Test Date: October 7, 2002	

Panel/Cell Line	Log_{10} GI50	Log_{10} TGI	Log_{10} LC50
Leukemia			
CCRF-CEM	-5.71	-5.06	-4.41
HL-60(TB)	-5.50	-4.84	-4.24
K-562	-5.64	-5.22	-4.58
MOLT-4	-5.44	-5.12	-4.53
RPMI-8226	-5.57	-5.03	-4.31
Non-Small Cell Lung Cancer			
A549/ATCC	-5.07	-4.09	> -4.00
EKVX	-4.94	-4.21	> -4.00
HOP-62	-5.19	-4.60	-4.12
HOP-92	-4.78	> -4.00	> -4.00
NCI-H226	-5.57	-4.44	> -4.00
NCI-H23	-5.41	-4.70	-4.19
NCI-H322M	-5.15	-4.54	> -4.00
NCI-H460	-5.37	-4.60	> -4.00
NCI-H522	-4.86	-4.14	> -4.00
Colon Cancer			
COLO 205	-5.31	-4.65	> -4.00
HCT-116	-5.66	-4.94	-4.45
HCT-15	-5.60	> -4.00	> -4.00
HT29	-5.23	-4.45	> -4.00
KM12	-5.43	-4.77	-4.18
SW-620	-5.26	-4.39	> -4.00
CNS Cancer			
SF-268	-5.01	> -4.00	> -4.00
SF-295	-5.21	-4.61	-4.09
SF-539	-5.15	-4.67	-4.23
SNB-19	-5.32	-4.37	> -4.00
SNB-75	-4.97	-4.49	-4.02
U251	-5.48	-4.61	> -4.00
Melanoma			
LOX IMVI	-5.36	-4.74	-4.17
MALME-3M	-4.85	-4.30	> -4.00
SK-MEL-2	-4.22	> -4.00	> -4.00
SK-MEL-28	-4.85	-4.42	> -4.00
SK-MEL-5	-5.36	-4.74	-4.26
UACC-257	-4.79	-4.22	> -4.00
UACC-62	-5.45	-4.79	-4.18
Ovarian Cancer			
IGROV1	-4.71	> -4.00	> -4.00
OVCAR-3	-5.43	-4.75	-4.13
OVCAR-4	-5.36	-4.53	> -4.00
OVCAR-5	-4.95	-4.51	-4.06
OVCAR-8	-5.00	-4.26	> -4.00
SK-OV-3	-5.50	-5.05	> -4.00
Renal Cancer			
786-0	-5.22	-4.67	-4.22
ACHN	-5.32	-4.66	-4.10
CAKI-1	-5.54	-4.98	-4.19
RXF 393	-4.59	> -4.00	> -4.00
SN12C	-5.66	-5.18	-4.50
TK-10	-5.41	-4.64	-4.03
UO-31	-4.27	> -4.00	> -4.00
Prostate Cancer			
PC-3	-5.31	-4.64	-4.10
DU-145	-4.92	-4.43	> -4.00
Breast Cancer			
MCF7	-5.03	> -4.00	> -4.00
NCI/ADR-RES	-5.65	-5.19	-4.35
MDA-MB-231/ATCC	-5.48	-4.66	> -4.00
HS 578T	-5.14	-4.48	> -4.00
MDA-MB-435	-5.51	-4.93	-4.03
BT-549	-4.62	-4.18	> -4.00
T-47D	-5.35	-4.34	> -4.00
MG_MID	-5.21	-4.54	-4.10
Delta	0.50	0.68	0.47
Range	1.50	1.22	0.58





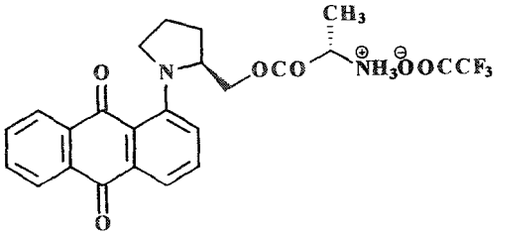


NU:UB 159 (273)

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC: D- 725553 / 1	**Experiment ID:** 0210NS71-34	**Test Type:** 08	**Units:** Molar
Report Date: October 31, 2002	**Test Date:** October 7, 2002	**QNS:**	**MC:**
COMI: NU:UB 171 (16861)	**Stain Reagent:** SRB Dual-Pass	**SSPL:** 0JQS	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.875	1.653	1.717	1.683	1.701	1.790	0.758	108	104	106	118	-13	3.28E-05	7.90E-05	>1.00E-04
HL-60(TB)	0.743	1.734	1.913	1.801	1.603	1.522	0.556	118	107	87	79	-25	1.88E-05	5.71E-05	>1.00E-04
K-562	0.563	0.882	0.957	1.022	1.091	0.979	0.305	123	144	165	130	-46	2.86E-05	5.49E-05	>1.00E-04
MOLT-4	0.717	0.899	0.953	0.877	0.949	0.996	0.403	130	88	127	154	-44	3.35E-05	5.99E-05	>1.00E-04
RPMI-8226	1.546	1.996	1.975	1.932	2.042	1.993	0.785	95	86	110	99	-49	2.15E-05	4.66E-05	>1.00E-04
Non-Small Cell Lung Cancer															
A549/ATCC	0.254	1.093	1.133	1.178	1.137	1.060	0.318	105	110	105	96	8	3.32E-05	>1.00E-04	>1.00E-04
EKVX	0.497	1.132	1.166	1.169	1.138	1.171	0.665	105	106	101	106	26	5.05E-05	>1.00E-04	>1.00E-04
HOP-92	0.388	1.005	0.891	0.845	0.870	0.849	0.503	82	74	78	75	19	2.75E-05	>1.00E-04	>1.00E-04
NCI-H23	0.710	1.179	1.085	1.081	1.098	1.048	0.633	80	79	83	72	-11	1.85E-05	7.39E-05	>1.00E-04
NCI-H322M	0.451	0.831	0.869	0.724	0.723	0.758	0.418	110	72	72	81	-7	2.24E-05	8.24E-05	>1.00E-04
NCI-H460	0.277	1.800	1.904	1.867	1.786	1.809	0.561	107	104	99	101	19	4.14E-05	>1.00E-04	>1.00E-04
NCI-H522	0.229	0.943	0.997	1.063	0.996	0.990	0.479	108	117	107	107	35	6.17E-05	>1.00E-04	>1.00E-04
Colon Cancer															
COLO 205	0.288	1.095	1.104	1.143	1.181	1.102	0.393	101	106	111	101	13	3.79E-05	>1.00E-04	>1.00E-04
HCT-15	0.144	1.316	1.117	1.227	1.214	1.123	0.309	83	92	91	84	14	3.04E-05	>1.00E-04	>1.00E-04
HT29	0.168	0.953	0.946	0.949	0.959	0.893	0.217	99	99	101	92	6	3.10E-05	>1.00E-04	>1.00E-04
KM12	0.534	2.023	1.914	1.893	1.773	1.506	0.278	93	91	83	65	-48	1.36E-05	3.77E-05	>1.00E-04
SW-620	0.197	0.762	0.776	0.734	0.780	0.780	0.189	102	95	103	103	-4	3.13E-05	9.17E-05	>1.00E-04
CNS Cancer															
SF-268	0.437	1.489	1.432	1.511	1.444	1.372	0.766	95	102	96	89	31	4.73E-05	>1.00E-04	>1.00E-04
SF-295	0.462	1.204	1.122	1.121	1.182	1.116	0.486	89	89	97	88	3	2.81E-05	>1.00E-04	>1.00E-04
SF-539	0.540	1.366	1.389	1.397	1.430	1.493	0.606	103	104	108	115	8	4.06E-05	>1.00E-04	>1.00E-04
SNB-19	0.304	0.832	0.849	0.896	0.872	0.733	0.447	103	112	108	81	27	3.78E-05	>1.00E-04	>1.00E-04
SNB-75	0.493	0.895	0.908	0.910	0.899	0.849	0.454	103	104	101	89	-8	2.51E-05	8.26E-05	>1.00E-04
U251	0.248	0.921	0.887	0.898	0.856	0.857	0.260	95	97	90	90	2	2.86E-05	>1.00E-04	>1.00E-04
Melanoma															
LOX IMVI	0.325	1.331	1.273	1.330	1.301	1.256	0.372	94	100	97	93	5	3.05E-05	>1.00E-04	>1.00E-04
MALME-3M	1.007	1.339	1.315	1.267	1.266	1.300	0.864	93	78	78	88	-14	2.36E-05	7.26E-05	>1.00E-04
SK-MEL-2	0.062	0.721	0.771	0.840	0.800	0.804	0.463	108	118	112	113	61	>1.00E-04	>1.00E-04	>1.00E-04
SK-MEL-28	0.352	0.967	0.922	0.960	0.951	0.937	0.490	93	99	97	95	22	4.17E-05	>1.00E-04	>1.00E-04
SK-MEL-5	0.510	1.797	1.843	1.829	1.828	1.879	0.518	104	102	102	106	1	3.41E-05	>1.00E-04	>1.00E-04
UACC-257	1.033	1.712	1.694	1.738	1.739	1.745	1.010	97	104	104	105	-2	3.25E-05	9.53E-05	>1.00E-04
UACC-62	0.617	1.429	1.445	1.500	1.337	1.319	0.608	102	109	89	86	-1	2.60E-05	9.63E-05	>1.00E-04
Ovarian Cancer															
IGROV1	0.083	0.740	0.728	0.759	0.751	0.735	0.285	98	103	102	99	31	5.23E-05	>1.00E-04	>1.00E-04
OVCAR-3	0.612	0.974	0.996	0.972	0.944	0.903	0.382	106	99	92	80	-38	1.80E-05	4.79E-05	>1.00E-04
OVCAR-4	0.665	1.522	1.567	1.600	1.596	1.563	0.946	105	109	109	105	33	5.76E-05	>1.00E-04	>1.00E-04
OVCAR-5	0.495	0.825	0.790	0.795	0.778	0.729	0.454	90	91	86	71	-8	1.83E-05	7.86E-05	>1.00E-04
OVCAR-8	0.385	1.072	1.038	1.040	1.070	1.052	0.323	95	95	100	97	-16	2.60E-05	7.19E-05	>1.00E-04
SK-OV-3	0.368	0.884	0.905	0.939	0.951	0.987	0.340	104	111	113	120	-8	3.54E-05	8.72E-05	>1.00E-04
Renal Cancer															
786-0	0.415	1.295	1.175	1.146	1.046	0.954	0.367	86	83	72	61	-12	1.43E-05	6.94E-05	>1.00E-04
ACHN	0.358	1.105	1.087	1.098	1.040	1.035	0.526	98	99	91	91	22	3.95E-05	>1.00E-04	>1.00E-04
CAKI-1	0.679	1.180	1.153	1.027	1.218	1.173	0.436	95	69	108	98	-36	2.30E-05	5.41E-05	>1.00E-04
RXF 393	0.447	1.712	1.685	1.605	1.675	1.545	1.075	98	91	97	87	50	9.78E-05	>1.00E-04	>1.00E-04
TK-10	0.977	1.702	1.713	1.600	1.557	1.595	0.757	101	86	80	85	-23	2.12E-05	6.18E-05	>1.00E-04
UO-31	0.127	1.161	1.167	1.190	1.186	1.143	0.501	101	103	102	98	36	5.98E-05	>1.00E-04	>1.00E-04
Prostate Cancer															
PC-3	0.157	0.566	0.497	0.469	0.490	0.448	0.149	83	76	81	71	-5	1.89E-05	8.50E-05	>1.00E-04
DU-145	0.292	1.051	1.064	1.113	1.120	1.140	0.616	102	108	109	112	43	7.84E-05	>1.00E-04	>1.00E-04
Breast Cancer															
MCF7	0.142	1.433	1.465	1.473	1.445	1.422	0.609	102	103	101	99	36	6.02E-05	>1.00E-04	>1.00E-04
MDA-MB-231/ATCC	0.493	0.844	0.821	0.771	0.706	0.699	0.395	94	79	61	59	-20	1.29E-05	5.59E-05	>1.00E-04
HS 578T	0.799	1.239	1.253	1.258	1.249	1.223	0.668	103	104	102	96	-16	2.57E-05	7.14E-05	>1.00E-04
MDA-MB-435	0.644	1.537	1.522	1.623	1.376	1.344	0.438	98	110	82	78	-32	1.81E-05	5.12E-05	>1.00E-04
BT-549	0.683	1.545	1.556	1.612	1.580	1.578	0.843	101	108	104	104	19	4.28E-05	>1.00E-04	>1.00E-04
T-47D	0.634	1.033	1.057	1.101	1.072	1.016	0.599	106	117	110	96	-6	2.83E-05	8.82E-05	>1.00E-04



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National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC: D- 725553 / 1	Units: Molar	SSPL: 0JQS	Exp. ID:0210NS71-34
Report Date: October 31, 2002		Test Date: October 7, 2002	

Panel/Cell Line	Log_{10} GI50	GI50	Log_{10} TGI	TGI	Log_{10} LC50	LC50
Leukemia						
CCRF-CEM	-4.48		-4.10		> -4.00	
HL-60(TB)	-4.73		-4.24		> -4.00	
K-562	-4.54		-4.26		> -4.00	
MOLT-4	-4.47		-4.22		> -4.00	
RPMI-8226	-4.67		-4.33		> -4.00	
Non-Small Cell Lung Cancer						
A549/ATCC	-4.48		> -4.00		> -4.00	
EKVX	-4.30		> -4.00		> -4.00	
HOP-92	-4.56		> -4.00		> -4.00	
NCI-H23	-4.73		-4.13		> -4.00	
NCI-H322M	-4.65		-4.08		> -4.00	
NCI-H460	-4.38		> -4.00		> -4.00	
NCI-H522	-4.21		> -4.00		> -4.00	
Colon Cancer						
COLO 205	-4.42		> -4.00		> -4.00	
HCT-15	-4.52		> -4.00		> -4.00	
HT29	-4.51		> -4.00		> -4.00	
KM12	-4.87		-4.42		> -4.00	
SW-620	-4.50		-4.04		> -4.00	
CNS Cancer						
SF-268	-4.33		> -4.00		> -4.00	
SF-295	-4.55		> -4.00		> -4.00	
SF-539	-4.39		> -4.00		> -4.00	
SNB-19	-4.42		> -4.00		> -4.00	
SNB-75	-4.60		-4.08		> -4.00	
U251	-4.54		> -4.00		> -4.00	
Melanoma						
LOX IMVI	-4.52		> -4.00		> -4.00	
MALME-3M	-4.63		-4.14		> -4.00	
SK-MEL-2	> -4.00		> -4.00		> -4.00	
SK-MEL-28	-4.38		> -4.00		> -4.00	
SK-MEL-5	-4.47		> -4.00		> -4.00	
UACC-257	-4.49		-4.02		> -4.00	
UACC-62	-4.59		-4.02		> -4.00	
Ovarian Cancer						
IGROV1	-4.28		> -4.00		> -4.00	
OVCAR-3	-4.74		-4.32		> -4.00	
OVCAR-4	-4.24		> -4.00		> -4.00	
OVCAR-5	-4.74		-4.10		> -4.00	
OVCAR-8	-4.59		-4.14		> -4.00	
SK-OV-3	-4.45		-4.06		> -4.00	
Renal Cancer						
786-0	-4.84		-4.16		> -4.00	
ACHN	-4.40		> -4.00		> -4.00	
CAKI-1	-4.64		-4.27		> -4.00	
RXF 393	-4.01		> -4.00		> -4.00	
TK-10	-4.67		-4.21		> -4.00	
UO-31	-4.22		> -4.00		> -4.00	
Prostate Cancer						
PC-3	-4.72		-4.07		> -4.00	
DU-145	-4.11		> -4.00		> -4.00	
Breast Cancer						
MCF7	-4.22		> -4.00		> -4.00	
MDA-MB-231/ATCC	-4.89		-4.25		> -4.00	
HS 578T	-4.59		-4.15		> -4.00	
MDA-MB-435	-4.74		-4.29		> -4.00	
BT-549	-4.37		> -4.00		> -4.00	
T-47D	-4.55		-4.05		> -4.00	
MG_MID	-4.50		-4.08		-4.00	
Delta	0.39		0.34		0.00	
Range	0.89		0.42		0.00	

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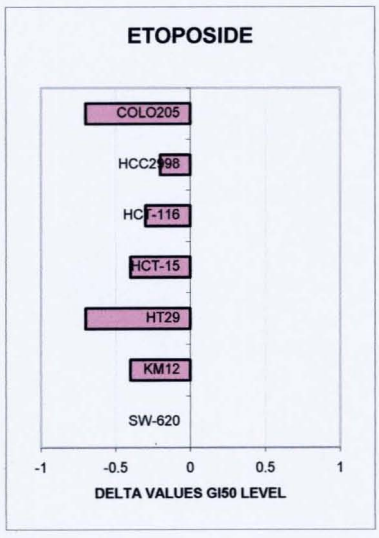
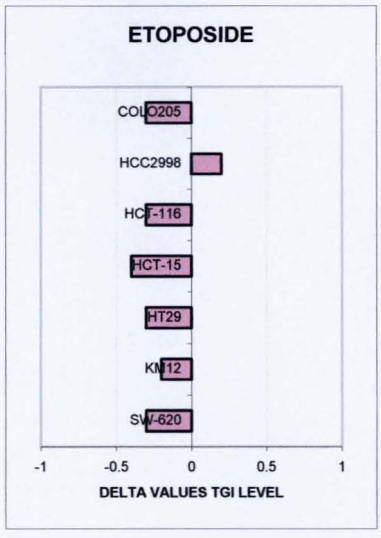
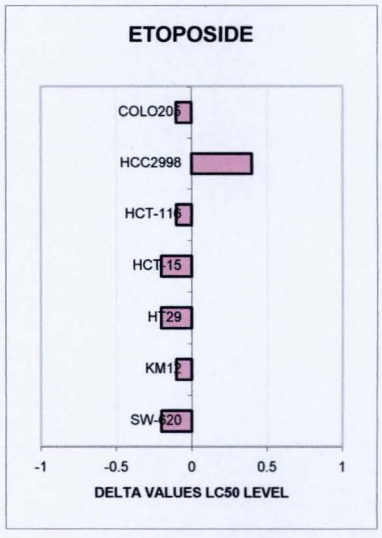
Appendix 3

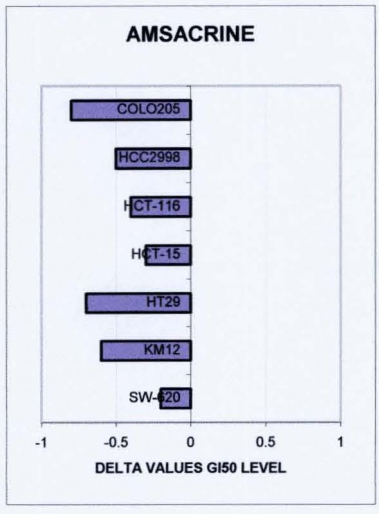
Comparison of Delta Values for the NCI Colon and Melanoma Cancer Sub-Panels

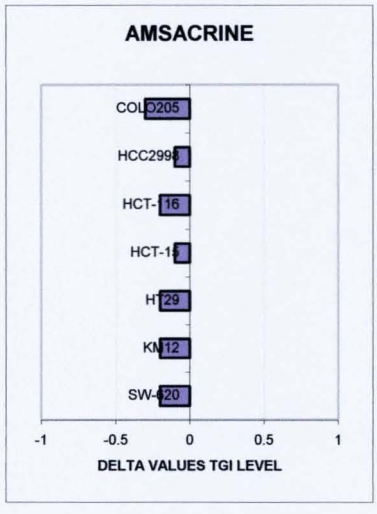
Comparison of Delta Values for the NCI Colon Cancer Sub-Panel (plotted in log mean graph format) for the topoisomerase II inhibitors, etoposide and mAMSA, and the dual topoisomerase I and II inhibitor intoplicine

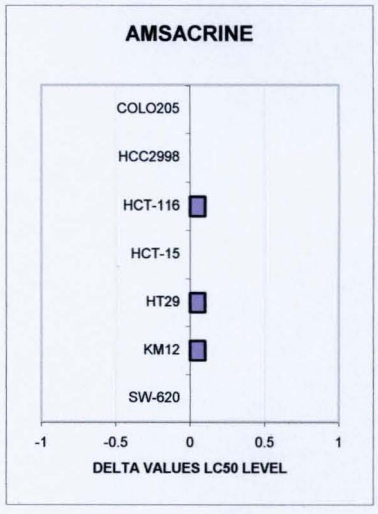


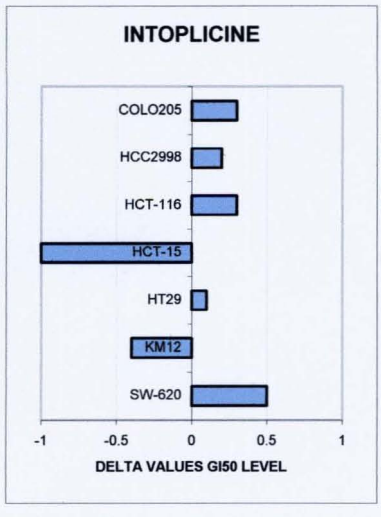


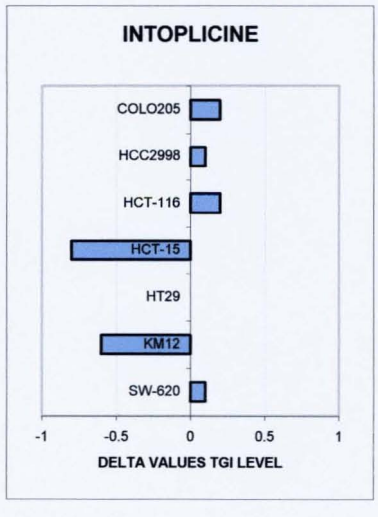


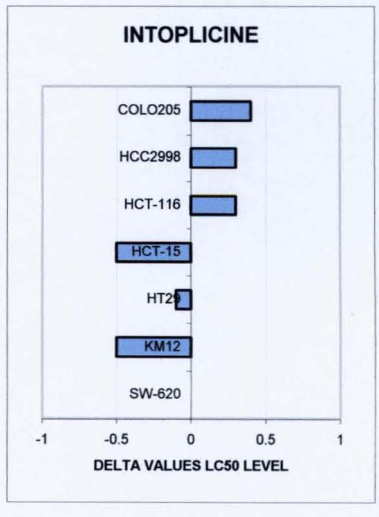












Comparison of Delta Values for the NCI Melanoma Cancer Sub-Panel (plotted in log mean graph format) for the topoisomerase II inhibitors, etoposide and mAMSA, and the dual topoisomerase I and II inhibitor intoplicine