Synthesis of agents targeting cancer cells while reducing MDR liability

Mohammad El-Dakdouki

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A Dissertation

Entitled

Synthesis of Agents Targeting Cancer Cells While Reducing MDR Liability

By

Mohammad El-Dakdouki

Submitted as partial fulfillment of the requirements for

the Doctor of Philosophy Degree in Medicinal Chemistry

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December 2009
An Abstract of

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The use of paclitaxel (PAC) for the treatment of certain types of cancers has been limited by its poor water solubility, toxicity to rapidly dividing normal cells, and weak activity against drug resistant tumors. Thus, there is a need to develop PAC conjugates that are selective toward cancer cells, while also having enhanced aqueous solubility and a reduced liability toward multidrug resistance (MDR). Previous studies in the Center for Drug Design and Development (CD3) have demonstrated that conjugating an acidic moiety on the Northern edge of PAC decreases the P-glycoprotein (Pgp) mediated MDR interactions. The acidic moiety not only reduces MDR liability, but also increases the aqueous solubility of these PAC conjugates. To further explore this structure-activity relationship (SAR) theme, several PAC analogues have been designed to examine the
effect on MDR liability of different functional groups, such as amino acids, aliphatic acids, and alcohols.

Selective targeting of cancer cells can be achieved by attaching a tumor recognizing agent or ‘address’ molecule to the anticancer drug wherein the address can then selectively bind to receptors over-expressed on cancer cells. γ-Linked glutamic acid residues (dipeptides and tripeptides), recognized by PSMA enzyme over-expressed on prostate cancer cells, were conjugated to PAC’s Northern edge to selectively target prostate cancer and its malignancy sites. In addition, a novel peptidomimetic analogue, **CD3-246**, was synthesized to target prostate cancer at the late stages of the disease. The latter inhibits the secondary processing enzyme *Peptidylglycine α-Amidating Monooxygenase* (PAM) that is believed to be crucial in activating growth hormones essential for hormone-independent tumor growth. Alternatively, several PAC-RGD conjugates have been designed to selectively target breast cancer cells. The RGD peptide is recognized by many of the integrin receptors, especially α₅β₃ which is over-expressed on blood vessels undergoing angiogenesis. In addition, the RGD address was utilized to develop a second generation contrast agent that may improve the accuracy of ultrasound imaging for breast cancer diagnosis. This was done by attaching a perfluorinated hydrocarbon chain as the ‘cargo’ for the RGD address system.

In another closely-related aspect of this overall ‘targeting’ program, several anthrapyrazole analogues were also designed in an attempt to overcome the side-effect toxicity of the anthracyclins when used to treat cancer. The proposed analogues were designed to have increased antitumor activity and reduced cardiotoxicity (being less prone to bioreduction).
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LIST OF ABBREVIATIONS

PAC        Paclitaxel
DAB        Deacetylpaclitaxel
Boc        t-Butoxycarbonyl
GTP        Guanosine triphosphate
GDP        Guanosine diphosphate
Pgp        Phosphoglycoprotein
MDR        Multidrug Resistance
ATP        Adenosine Triphosphate
EDA        Ethylene Diamine
DCM        Dichloromethane
MeOH       Methanol
psi        per square inch
AcOH       Acetic Acid
Cac        Choroacetyl
TES        Triethylsilyl
TFA        Trifluoroacetic Acid
DIC        Diisopropylcarbodiimide
DMAP       Dimethylaminopyridine
DIPEA      Diisopropylethylamine
PSMA       Prostate Specific Membrane Antigen
FOH I      Folate hydrolase I
NAALADase  $N$-acetylated $\alpha$-linked acidic dipeptidase
NAAG  $N$-acetylaspartylglutamate
SAR  Structure Activity Relationship
R6G  Rhodamine 6G
PyBOP  Benzotriazol-1-yl-oxytrpyrrolidinophosphorium hexafluorophosphate
HOBt  Hydroxybenzotriazole
DCC  Diicyclohexylcarbodiimide
PAM  Peptidylglycine $\alpha$-Amidating Monooxygenase
PHM  Peptidylglycine $\alpha$-hydroxylating monooxygenase
PAL  Peptidyl-$\alpha$-hydroxyglycine $\alpha$-amidating lyase
IC$_{50}$  Drug concentration that causes 50% inhibition of a biological process
GI$_{50}$  Drug concentration that causes 50% growth inhibition
DBU  1,8-Diazobicyclo(5.4.0)undec-7-ene
ECM  Extracellular matrix
Mtr  4-methoxy-2,3,6-trimethylbenzene-sulfonyl
Fmoc  Fluorenylmethyloxycarbonyl
TFA  Trifluoroacetic acid
NMR  Nuclear magnetic resonance
MS  Mass spectroscopy
PFOB  Perfluoroocytly bromide
MCPBA  $meta$-Chloroperbenzoic acid
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium on carbon</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>CD3</td>
<td>Center for Drug Design and Development</td>
</tr>
<tr>
<td>UT</td>
<td>University of Toledo</td>
</tr>
<tr>
<td>RES</td>
<td>Resistant</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
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Chapter 1.

Introduction

1.1. Discovery and Development of Paclitaxel

“One of few organic compounds, which like benzene and aspirin, is recognizable by name to the average citizen.”  


Much has been said about paclitaxel 1 (Taxol®; Fig. 1; PAC) where a simple search of “taxol or docetaxel or paclitaxel or taxotere” in SciFinder Scholar generates just over 45,000 hits. This anticancer ‘celebrity’ molecule was discovered by Drs. Wall and Wani at the Research Triangle Institute in North Carolina, where they isolated it from the stem-bark of the Pacific Yew tree (Taxus brevifolia).

![Paclitaxel (PAC) 1.](image_url)

*Figure 1. Paclitaxel (PAC) 1.*
The initial development of 1 was delayed by its low yield after a tedious isolation process from the natural source, coupled with moderate activity against slow growing cancers and the misconception that its anticancer properties stemmed from a common biological mechanism. The year 1979 constituted the breakthrough in PAC's research. It is the year when Horwitz et al.\textsuperscript{5} reported a unique mechanism of action for PAC derived from the over-stabilization of microtubules. The number of publications 'mushroomed'\textsuperscript{6} thereafter, and the rush to then move 1 into the clinic as rapidly as possible followed by the need to provide huge supplies to accommodate its immediate success, quickly led to considerable concern about the future supply of 1 because removal of a yew's bark leads to destruction of the tree.\textsuperscript{7} Amid extraordinary chemical efforts by several investigators to synthesize 1,\textsuperscript{4,8,9,10} it was eventually demonstrated by Potier et al. that 10-deacetyl-baccatin III (10-DAB in Scheme 1) which is obtained from the needles of both the Pacific and European yew in a manner that is not destructive to the source, constitutes a key intermediate for a practical semi-synthesis of what had by then become an extremely precious natural product.\textsuperscript{11} Although many notable chemists, as well as a modest effort in our labs (Scheme 2) using a Sharpless dihydroxylation procedure,\textsuperscript{12} contributed significantly to this chemistry arena, Holton's patented semi-synthesis that takes advantage of a lithium alkoxide\textsuperscript{13,14} eventually was adopted by Bristol Myers Squibb (BMS) for converting 10-DAB to 1 via reaction with the indicated β-lactam on a commercial scale according to Scheme 1.\textsuperscript{15}
Scheme 1. Semi-synthetic route ultimately used to prepare commercial quantities of paclitaxel shown as 1 from 10-deacetyl-baccatin III shown as 10-DAB.15 A similar semi-synthetic route can be used to prepare docetaxel (shown later) by deploying the t-butoxycarbonyl (BOC) version of the benzzyloxy β-lactam, as well as by utilizing other C-13 side-chain synthons. Conditions and yields: (a) Et3SiCl/Pyridine, 86%; (b) AcCl/Pyridine, 86%; (c) n-BuLi/THF then β-Lactam/THF, 98%; (d) HF/Pyridine, 98%.

Scheme 2. ‘Acyl migration’ route developed and confirmed in our labs to produce the C-13 side-chain via a Sharpless dihydroxylation procedure.11 (a) AD-a mixture, t-BuOH/H2O; (b) i. PhC(OCH3)3, cat. p-TsOH; ii. AcBr, -15°C; (c) i. NaN3, DMF; ii. H2, 10% Pd/C; Overall yield = 25%.

The extensive research that started in 1962 led to the approval of PAC by FDA for the treatment of ovarian cancer in 1992. Today, PAC is used for the treatment of several types of cancers including ovarian, breast, small-cell and large-cell lung cancers, and Karposi’s sarcoma. It is important to note that the tremendous success of 1 promoted a surge in basic research that led to the discovery of 1’s first clinically successful
synthetic analog derived from Analog Based Drug Design (ABDD), namely docetaxel 2 shown in Fig. 2. 

![Figure 2. Docetaxel 2.](image)

### 1.2 Mechanism of Action of PAC

Although PAC has been shown to have effects on several cell signaling pathways, its clinically relevant anticancer activity is attributed to its ability to bind to microtubules and promote tubulin polymerization and stabilization. This disruption of tubulin depolymerization dynamics leads to cell cycle arrest at the G2/M phase, and eventually cell death by apoptosis. 

Microtubules are cytoskeletal non-covalent polymers of the protein tubulin essential in all dividing eukaryotic cells and in most differentiated cell types. They are involved in a diverse range of cellular functions such as cell division, maintaining the cell shape and the intracellular movement of macromolecules and organelles. Microtubules are polar structures formed by the head-to-tail self-association of the α,β-tubulin heterodimers resulting in linear protofilaments, which in turn associate in a lateral manner to form 25 nm wide hollow cylindrical tubes. The in vivo number of protofilaments in microtubules is usually 13. Each monomer can be divided into three major structural domains: The N-terminal domain that is involved in the nucleotide
binding, the central domain that is involved in longitudinal and lateral interactions between the α,β-monomers and contains the hydrophobic pocket to which PAC binds, and the C-terminal which is negatively charged and believed to be involved in the binding of regulatory and motor proteins.\textsuperscript{19,20}

It is known that microtubules are highly dynamic, and characterized by a non-equilibrium behavior of tubulin called dynamic instability, a stochastic process based on nucleotide binding and hydrolysis. Dynamic instability is characterized by two processes: Catastrophe, or the transition from growth to shrinkage, and rescue, or transition from shrinkage to growth.\textsuperscript{21} Each tubulin monomer binds one GTP molecule. However, the nucleotide in the α-subunit is found in the non-exchangeable or N-site buried at the intradimer interface, while the nucleotide in the β-subunit is bound to the exchangeable or E-site that is partially exposed on the surface of the dimer allowing for GTP exchange in solution.\textsuperscript{21} Thus, upon the arrival of a new α,β-tubulin heterodimer, the α-subunit of the incoming dimer will interact with the nucleotide of the receiving β-subunit of the microtubule. As a consequence of such interaction, the GTP molecule in the receiving β-subunit will hydrolyze to GDP that will be buried in the interface and becomes non-exchangeable. As the GTP of the incoming dimer is not affected by polymerization, the plus end (the faster growing end where the β-chains of the tubulin heterodimer are exposed) should have a layer of GTP molecules forming what is called a GTP cap (GTP-cap model). This GTP-cap is crucial for stabilizing the microtubules that are themselves not stable.\textsuperscript{22} The loss of the GTP-cap will result in rapid depolymerization of microtubules with the protofilaments pealing outside. The resulting dimers will exchange GTP for GDP, and thus become primed for another cycle of polymerization. A different
scenario occurs at the minus end (the slower growing end where the α-chains are exposed) of the microtubule which is formed by the surface of α-tubulin containing the catalytic residue. The GTP bound to the E-site of the incoming dimer will be hydrolyzed following a new polymerization event. Thus, no GTP cap should exist at the minus end.

PAC binds to the microtubule polymer specifically and reversibly with a stiochiometry close to one (relative to the α,β-tubulin heterodimer). Microtubules that are formed in the presence of 1 exhibit unusual stability against conditions favoring depolymerization such as cold temperature, Ca\(^{2+}\) ions, and dilution. Of significant importance is the observation that 1 promotes the assembly of tubulin in the absence of GTP which, under normal conditions, is an absolute requirement for microtubule assembly. At a PAC concentration of 5 µM, the critical concentration of microtubule protein necessary for in vitro microtubule assembly decreases by a factor of 20 from 0.2 to less than 0.01 mg/ml. An in-depth account of how 1 is presently thought to bind with microtubules while inducing over-stabilization is discussed in the next section.

### 1.3 SAR and Receptor Binding

The accumulated studies directed toward total and semi-synthetic syntheses, plus the efforts directed toward improving aqueous solubility and establishing SAR, provide a reasonably detailed map of the pharmacophoric requirements associated with 1’s unique mechanism of action. Details reviews of the excellent work in this area can be found elsewhere such that only an overall summary pertaining to these structural aspects is provided herein. The latter is conveyed within Fig. 3.
Photoaffinity labeling strategies represent the first studies that were conducted to investigate the interaction of PAC with its microtubule target protein. Early studies using $[^3H]$-paclitaxel indicated that 1 preferentially binds to the β-subunits of microtubules, although the exact site for this interaction could not be ascertained due to insufficient photoincorporation of $[^3H]$-1.\textsuperscript{31} To more definitively map binding, analogs bearing photoaffinity groups at the C-2, C-7 and C-3\textsuperscript{'} positions of 1 have been employed (Fig. 4 below).\textsuperscript{32} Chemical and enzymatic digestion of the microtubules followed by N-terminal amino acid sequencing eventually led to identifying the specific residues that are in close proximity to the paclitaxel binding site. $[^3H]$-3\textsuperscript{'-}(p-azidobenzamido)-taxol\textsuperscript{33} and $[^3H]$-2-(m-azidobenzoyl)-taxol\textsuperscript{34} were found to crosslink to the amino acid residues 1-31 and 217-233 of β-tubulin, respectively, while $[^3H]$-7-(dihydrocinnamoyl-benzoyl)-taxol crosslinked with the Arg-282 residue in the β-subunit.\textsuperscript{35}
Based on the data obtained from photoaffinity studies and the atomic structure of α,β-tubulin junctions, it is generally accepted that 1 binds to a deep hydrophobic pocket near the surface of the β-tubulin while adopting a ‘T-shaped’ or ‘butterfly’ conformation. In this conformation, 1 ‘opens-up’ and allows for intermolecular hydrophobic association which is reflected by the irregularly stacked C-3’-benzamido, His-229 and C-2-benzoyl moieties. This arrangement is depicted in Fig. 5 where it can be seen that the C-3’-benzamido of 1 is in close proximity to Val-23 and the C-7-hydroxyl group is close to Thr-274. The C-2-benzoyl group fits into a pocket formed by the imidazole ring of His-227 and the side-chain of Asp-224, both of which are part of the H-7 helix that is in contact with the exchangeable nucleotide binding site of β-tubulin.
It is well-established that the hydrolysis of GTP to GDP in β-tubulin constitutes a key regulatory mechanism that affects microtubule polymerization/depolymerization.\textsuperscript{37,38} In this context, the H-7 helix could have a controlling effect on the overall tubulin molecule because its interaction with \textit{I} appears to induce a conformation that mimics the GTP-bound form of β-tubulin, thus promoting microtubule assembly.\textsuperscript{39,40} This binding site is close to the M-loop that participates in lateral interactions with the H-3 helix of the adjacent β-tubulin subunit. Therefore, the action of \textit{I} could be understood as a strengthening of the lateral contacts between protofilaments via a conformational change in the M-loop that leads to its increased stability.\textsuperscript{33,41,42,43} It is interesting to note that the position of \textit{I}'s binding site on the β-subunit is occupied by an eight amino acid loop associated with an α-subunit, namely the S-loop A-362 to A-369. It has been suggested that this loop acts as a microtubule stabilizing factor by promoting lateral contacts between protofilaments. This, in turn, has led to the hypothesis that \textit{I} could act by mimicking the stabilizing effect that the S-loop has on the M-loop.\textsuperscript{34,39-43} The dynamics
of this binding paradigm are shown in Figs. 5 and 6 with the latter also conveying the change in the binding surface’s hydrophobic character.

![Figure 6](image)

Figure 6. Solvent accessible surface of paclitaxel’s binding pocket on β-tubulin colored according to degree of hydrophobicity [taken directly from ref. 36]. Color gradient is on the left wherein the maximum hydrophobicity is indicated by red and the lowest by dark blue. (a) Empty binding pocket is burnt orange suggesting that it is highly hydrophobic. (b) Superposition of 1 in its ‘T-shaped’ conformation within the binding pocket. (c) Surface re-coloring which illustrates that the hydrophobic depression has been converted to a more hydrophilic surface upon paclitaxel’s binding.

The proposed ‘T-shaped’ bioactive conformation of 1 correlates strongly with the data obtained from SAR studies. As noted by others\textsuperscript{36}, these key features can be summarized according to the following six points.

1. The C-2’-hydroxyl group is a hydrogen-bond donor that is crucial for biological activity. In the proposed ‘T-shaped’ model, there is a hydrogen-bond from the C-2’-hydroxyl to the carbonyl of the ARG-369.

2. There is a notable biological insensitivity or ‘neutral SAR’\textsuperscript{44} of 1 to chemical modifications across the C-7 to C-10 region. This portion of 1 is not involved in binding to the β-subunit and is instead projected outward or away from the surface of the macromolecule.
Replacement of the phenyl rings with cyclohexyl rings in the C-2 and C-13 side-chain positions leads to sustained activity.\textsuperscript{45} In the proposed model, both of these phenyl rings are situated in a hydrophobic space.

Extension of the C-4 acetate with longer alkyl chains leads to sustained activity.\textsuperscript{46} In the proposed model, this group becomes positioned over a 10 residue hydrophobic basin.

Selected meta-substitutions on the C-2 benzoyl can enhance biological activity while para-substitutions cause a reduction in potency.\textsuperscript{47} The proposed model reveals that the hydrophobic subsite hosting the C-2 ring is reasonably tight on three sides but more open at one of the meta-locations.

The biological activity of the C-6 nor-paclitaxel analogs was found to be 10-20 times less than that of 1. In the proposed model, contraction of the six-membered C-ring of 1 to a five-membered ring pulls the oxetane D-ring away from Thr-276 which reduces the effectiveness of the O-21 to HN-Thr interactions.

\textbf{1.4 Clinical Limitations of Paclitaxel}

Despite its successful performance in treating several cancers, PAC’s clinical utility is hampered by several limitations such as poor water solubility, side-effect toxicity to rapidly dividing normal cells, and weak activity against drug resistant cancer cell lines.\textsuperscript{48} Due to its poor water solubility, PAC has to be formulated and administered with 50% Cremophor EL\textsuperscript{®} and 50% dehydrated ethanol, a formulation that can lead to severe allergic reactions due to histamine release and hypersensitivity reactions.\textsuperscript{49} Thus,
patients receiving 1 are often pretreated with histamine H1- and H2- receptor antagonists (e.g. dexamethasone) and corticosteroids to reduce the risk of developing hypersensitivity reactions.\textsuperscript{50} Like most agents that rely upon the rapid division of cancer cells to achieve selective toxicity relative to healthy cells, the therapeutic margin for 1 is not as large as would be desired. The systemic toxicity of PAC results in adverse effects, including bone marrow suppression, neurotoxicity and a variety of cardiac abnormalities.\textsuperscript{51} Most of these side-effects are the consequence of the action of the chemotherapeutic agent on normal cells, especially the rapidly dividing ones. Finally, a certain amount of chemotherapeutic failure can be related to the tumor being inherently resistant to the drug and/or to the acquisition of resistance during treatment. The mechanisms for resistance to PAC can occur directly on the microtubules, such as mutations, and at the level of regulatory proteins, with the latter typically having a predominant role in the drug resistance phenomena. Tumor cells that show resistance to different anticancer drugs, such as PAC, vinca alkaloids, and anthracyclines, are characterized by the over-expression of an energy-dependent drug transport protein, namely Pgp. Pgp is an efficient efflux system, where it is thought that it has at least two binding sites that can accommodate more than one ligand in a given efflux cycle.\textsuperscript{52} MDR will be addressed in more detail in Chapter II.

1.5 Towards Overcoming PAC’s Limitations

Avoiding the limitations of PAC can be achieved by designing an “ideal bullet”\textsuperscript{53} that will target the drug to cancer cells in the right amount and at the right time. Previous studies within the Center for Drug Design and Development (CD3) have demonstrated
that attaching an acidic group at position 10 of PAC can reduce MDR while retaining PAC’s biological activity towards over-stabilizing microtubules. The acidic group not only reduces MDR, but also increases the aqueous solubility and thus the bioavailability of these PAC analogues.

Selective targeting of the drug to cancer cells may be able to be achieved by attaching a tumor recognizing agent or ‘address’ molecule\(^5\) to the cytotoxic drug wherein the ‘address’ molecule will bind to receptors over-expressed on cancer cells. In combination with earlier results, an “ideal bullet” will contain the drug cargo attached to an address ligand bearing free acidic group(s) and having a selective binding affinity to cancer cells so as to lessen the toxicity to normal cells. This arrangement is shown in Fig. 7. Alternatively, if the acidic moieties can not be accommodated within the address system, they could be incorporated into the ‘linker’ moiety.

Figure 7. The ideal bullet: CD= Cancer drug (cargo) such as paclitaxel (PAC); Linker = Metabolically stable chemical connection; and, Triangle = Recognitional (address) system for proteins over-expressed on cancer cell. Note retention of an acidic moiety to attenuate MDR liability.
Chapter 2.

Addressing MDR

2.1 Pgp: A Promiscuous Transporter

The emergence of clinical resistance to PAC remains a major obstacle to improving the overall response and survival of cancer patients. Chemotherapeutic failure can be related to the tumor being inherently resistant to the drug and/or to the acquisition of resistance during treatment. Tumor cells can show resistance to structurally and mechanistically unrelated anticancer drugs, such as PAC, vinca alkaloids, and anthracyclines, a phenomenon referred to as multidrug resistance (MDR). MDR is a multifactorial process that often results from the over-expression of the multidrug transporter phosphoglycoprotein (Pgp), as well as from decreased sensitivity to death-inducing stimuli (e.g. decreased ceramide levels), altered drug metabolism, alterations in microtubule dynamics and activation of DNA repair machinery.\textsuperscript{55} The mechanisms for resistance to PAC can occur directly on the microtubules, such as mutations, and at the level of regulatory proteins, with the latter typically having a predominant role in the drug resistance phenomena. Tumor cells that show resistance to different anticancer drugs are characterized by the over-expression of the energy-dependent drug transport protein, namely Pgp.\textsuperscript{56} Pgp is an efficient, broad-spectrum efflux pump for hydrophobic bulky anticancer agents, thus preventing their accumulation in the cell at cytotoxic levels. Pgp belongs to the ATP-binding cassette (ABC) transporter family.\textsuperscript{57} It consists of 12
transmembrane regions that bind both neutral or positively charged hydrophobic substrates, and two ATP-binding sites.\textsuperscript{58} Two non-simultaneous ATP hydrolysis events occur in the process of transporting one drug molecule. Binding of the substrate to the transporter results in the hydrolysis of the first ATP molecule, causing conformational changes and the transport of the substrate. The hydrolysis of the second ATP molecule seems to be essential for the reconstitution of Pgp that is then made ready for another catalytic cycle.\textsuperscript{59} Photolabelling of Pgp with PAC analogues bearing photoreactive groups suggested that Pgp has at least two binding sites that can accommodate more than one ligand in a given efflux cycle.\textsuperscript{60} PAC analogues with benzophenone photoreactive groups at the C-3’ and C-7 positions photolabelled the peptide sequences 985-1088 and 683-760 of Pgp, respectively.\textsuperscript{61}

In addition to its negative impact upon chemotherapy, Pgp is now known to also be present in the epithelial lining of the human GI-tract where it can serve to limit the absorption of small molecule drugs that are prone to serve as substrates.\textsuperscript{7} The significance of these last two shortcomings is further exemplified by the plots displayed in Fig. 8 which conveys data generated from our laboratories. The narrow safety margin between paclitaxel’s effects on cancer cells versus its effects on rapidly dividing healthy cells is reflected by the very close proximity of the first two log dose-response curves for causing growth inhibition (GI) of cell cultures. Likewise, the profound effect that Pgp-derived resistance can have upon paclitaxel’s effects is illustrated by the third curve which has shifted to the right in such a manner that it now requires nearly three orders of magnitude higher drug concentration to cause 50\% inhibition of cell growth (GI\textsubscript{50} value). Although 1 and its related taxanes appear to be exceptional substrates for Pgp\textsuperscript{62}, the far from ideal
profile of activity conveyed in Fig. 8 is a common shortcoming that is displayed by many cancer chemotherapeutic agents in general.\(^7\)

![Log dose-response curves for the action of paclitaxel on three different human cell lines grown in cell culture. Control cultures did not receive drug. MCF7 (red curve with circled data points) is a non-resistant breast cancer. MCF12A (green curve with square data points) is a healthy breast epithelial cell line that is non-cancerous and non-resistant. NCI/ADR-RES (blue curve with triangular data points) is a drug resistant ovarian cancer. Pgp refers to P-glycoprotein which is either absent (-) or over-expressed (+) and ER refers to estrogen receptor responsiveness (+) or absence (-).](image)

**Figure 8.** Log dose-response curves for the action of paclitaxel on three different human cell lines grown in cell culture. Control cultures did not receive drug. MCF7 (red curve with circled data points) is a non-resistant breast cancer. MCF12A (green curve with square data points) is a healthy breast epithelial cell line that is non-cancerous and non-resistant. NCI/ADR-RES (blue curve with triangular data points) is a drug resistant ovarian cancer. Pgp refers to P-glycoprotein which is either absent (-) or over-expressed (+) and ER refers to estrogen receptor responsiveness (+) or absence (-).

### 2.2 Designing PAC Analogues to Reduce MDR Liability

In an attempt to explore chemical modifications that reduce MDR liability, we synthesized several PAC analogues bearing different chemical functionalities at C-7 or C-10 of paclitaxel, and evaluated the structure activity relationships (SAR) resulting from these studies. The goal was to provide general principles for design criteria potentially applicable to new anticancer drugs in order to avoid Pgp-mediated MDR interactions.\(^63\)

We studied the effect of a free basic (amino group), acidic (carboxyl group), and alcohol
(hydroxyl group) functionalities on the binding of 1 with the Pgp transporter. The chemistry and biology behind these studies are detailed in subsequent sections.

2.2.1 The 10-Asp-PAC Series

The first set of compounds involved attaching an aspartic acid (Asp) residue at position 10 of PAC, and was synthesized by Dr. W. Klis. The synthetic route that led to the synthesis of the different analogues is depicted in Schemes 3, 4 and 5.

Synthesis of the 10-PAC-Asp analogues started by removing the acyl-group normally present at position 10 of PAC by using a saturated solution of sodium bicarbonate (NaHCO$_3$) in 30% hydrogen peroxide (H$_2$O$_2$), to yield deacetylpaclitaxel 3 (DAP) as depicted in Scheme 3.$^{64}$ The 2’- and 7-hydroxyl groups were simultaneously protected using 2.5 equivalents of chloroacetic anhydride (ClCH$_2$CO)$_2$O so as to produce compound 4. Quenching the reaction mixture after 15-20 minutes is essential to avoid the further acylation of the 10-hydroxyl group.$^{65}$ The Boc-Asp-OBn residue was then attached to the free 10-hydroxyl group of PAC using DIC and catalytic amounts of DMAP to yield the key intermediate 5. Orthogonal deprotection procedures were then conducted on 5 to generate the different 10-Asp-PAC analogues as shown in Schemes 4 and 5.
Scheme 3. Synthesis of the PAC key intermediate 5: (a) Sat. NaHCO₃/30% H₂O₂, THF, RT, 6 h, 98%; (b) (ClCH₂CO)₂O (2.5 equivalents), DMAP, DCM, 15-20 min, 45%; (c) Boc-Asp-OBn, DIC, cat. DMAP, DCM, 18 h, 85%.

As revealed in Scheme 4, debenzylating 5 led to the production of intermediate 6, which upon treatment with EDA yielded target 7. Stirring of 7 in 50% formic acid in DCM afforded target 8. Alternatively, stirring of 6 in methanol for 18 hours led to the selective deprotection of the 2‘-hydroxyl group yielding target 9 in quantitative yields. However, the preparation of 10 from 9 was problematic and resulted in undesired and unidentified products.
Scheme 4. Synthesis of 10-Asp-PAC analogues for preliminary MDR studies: (a) 10% Pd/C, H₂, 25 psi, 2% AcOH, MeOH; (b) 0.1% EDA DCM, 90 min, 71.6% (from 5); (c) 50% Formic acid/DCM, 18 h, 72.5%; (d) MeOH, 18 h, 100%; (e) 1.7% Formic acid/DCM.

The other 10-Asp-PAC analogues were prepared as depicted in Scheme 5. Following the selective deprotection of the 2’-hydroxyl group of 6 to produce target 11, the latter was reacted with thiourea in ethanol to produce target 12. On the other hand, target 13 was obtained when 11 was treated with 50% formic acid in DCM. Treatment of 13 with EDA led to the production of target 14. The attempts to prepare 10 from 13 by debenzylation failed, where undesired results and unidentified products were obtained.
Scheme 5. Synthesis of 7-CAC-PAC-Asp analogues for preliminary MDR studies: (a) MeOH, 18 h, 100%; (b) Thiourea/ EtOH, 3 h, then sat. NaHCO₃, 93.8% (c) 50% Formic acid/ DCM, 73%; (d) 0.5% EDA in DCM, 50 min, 93%; (e) 10% Pd/C, H₂, 25 psi, MeOH.

A different strategy was proposed for the synthesis of the “neutral” Asp-PAC target 10. This strategy was based upon using an aspartic acid residue which has protecting groups that can be simultaneously removed in a single step. Two approaches were taken, namely using Cbz-Asp-OBn, in one route, and using Boc-Asp-OtBu, in another. Both of these approaches are shown in Scheme 6. Although the coupling steps (b) and (d) were successful, the deprotection steps (c) and (e) in either route proved to be problematic and did not produce the desired product. The most promising result was observed when we attempted to generate 10 through intermediate 16. The progress of the reaction was followed by mass spectroscopy. A procedure similar to that employed in the synthesis of 15 was used to produce intermediate 16 (Calculated M+Na 1259.14. Found M+Na 1257.30). The 2’-CAC protecting group was then selectively removed under methanolysis conditions to generate 7-CAC-10-Asp(Boc, OtBu)-DAP (16a: Calculated M+Na 1182.66. Found M+Na 1181.50). The latter was dissolved in 1:1 formic
acid:DCM mixture and stirred at -5°C for 18 hours. Only the Boc protecting group was removed as determined by mass spectroscopy [7-CAC-10-Asp(O'Bu)-DAP 16b: Calculated M+Na 1082.54. Found M+Na 1081.40]. Stirring the reaction at room temperature did not cause the removal of the tert-butoxy group. Thus, 10% TFA:DCM was added and the reaction was stirred for 3 hours. A mass spectroscopy spectrum taken 30 minutes following the addition of the TFA solution showed an intense peak that matched the molecular mass of target 10 (Calculated M+Na 1026.4. Found M+Na 1025.4). However, TLC indicated that only a very small fraction of 7-CAC-10-Asp(O'Bu)-DAP was converted to 10. Stirring was continued and undesired results were obtained. The peaks that corresponded to 7-CAC-10-Asp(O'Bu)-DAP 16b and 10 started disappearing gradually and unidentified spots on TLC and peaks in the mass spectrum were generated. The mass spectrums corresponding to the different intermediates will be displayed in the experimental section. Despite of the disappointment with the end result, it was the first time that we were able to trace the progress of the reaction and the formation of 10.
We then considered that the synthetic problems in the last steps (c) and (e) may be associated with the 7-chloroacetyl (CAC) group. Thus, a synthetic route was devised wherein a TES group replaced the 7-CAC group. This route is depicted in Scheme 7. Following protection of the 2'-hydroxyl group of DAP by a carbobenzyloxy (Cbz) group to afford 17, triethylsilyl chloride (TESCl) was used to protect the 7-hydroxyl group so as to yield 18. The key intermediate 19 was then obtained by coupling Cbz-Asp-OBn to position 10 of 18. The neutral analogue was approached from different fronts as depicted in Scheme 7. Attempts to debenzylate 19 were again disappointing although they did appear to be more promising than the case where a CAC was on the 7-position. The TES protecting group was then removed to generate 20. The latter was subjected to hydrogenation, but the neutral analogue was not obtained. The 7-hydroxyl group was protected with the relatively small acetyl group using acetic anhydride so as to produce
intermediate 21. Unfortunately, all attempts to generate the neutral analogue from 21 failed.

Scheme 7. Synthesis of 7-TES-Asp-DAP analogues. (a) CbzCl (1.2 equivalents), DIPEA, DCM, 18 h, -20°C, 92%; (b) TESCl (10 equivalent), DMAP, DCM, 18 hr, rt, 90%; (c) Cbz-Asp-OBn, , DIC, cat. DMAP, DCM, rt, 12 h, 80%; (d) 10% Pd/C, H₂, 25 psi, MeOH, 10 h; (e) HF/pyridine, pyridine, rt, 6 h, 85% (from 18); (f) Chloroacetic anhydride, DCM, rt, 2 h, 99%.

2.2.2 The 7-Glu-PAC Series

To further explore and potentially substantiate these findings, another set of 7-Glu-PAC analogues has been designed to study the effect of the additional methylene group in the Glu-side chain on the activity of the drug. We also predicted that the neutral 7-Glu-PAC analogue 29 would be synthetically more accessible. The different 7-Glu-PAC analogues were prepared as depicted in Schemes 8 and 9.

The synthetic route that can lead to the first set of analogues is shown in Scheme 8. The 2’-hydroxyl group of 1 was first selectively protected using 1.2 equivalents of
chloroacetyl chloride and compound 22 was afforded. A Boc-Glu-OBn residue was then coupled to the C-7 hydroxyl group of 22 using DIC as a coupling reagent to generate the fully protected intermediate 23. Stirring 23 in methanol for 18 hours produced target 24. Debenzylation of 24 by hydrogenation should afford target 25, while treating it with 50% formic acid in DCM can yield target 26.

The fully deprotected 7-Glu-PAC analogue 29 was synthesized as depicted in Scheme 9. Following the selective protection of the 2’-hydroxyl group of 1 with a Cbz group to generate intermediate 27, a Cbz-Glu-OBn residue was attached to the C-7 hydroxyl group so as to produce intermediate 28, which when subjected to hydrogenation afforded the fully deprotected target 29.

Scheme 8. Synthesis of the 7-Glu-PAC analogues. (a) ClCH₂COCl, DMAP, DCM, -20°C, 12 h, 74%. (b) Boc-Glu-OBn, DIC, DMAP, DCM, rt; (c) Methanol, 18 h, 100%; (d) 10% Pd/C, H₂, 25 psi, MeOH, 10 h; (e) 50% Formic acid/DCM.
2.2.3 The Aliphatic Acid-PAC Series

Several aliphatic acid-PAC analogues were designed and synthesized to confirm our finding that an acidic moiety reduces MDR liability. Thus, a succinic acid or glutaric acid moiety was attached to C-7 and C-10 of 1 as shown in Schemes 10 and 11. The synthesis of the 7-positon aliphatic acid series started by selectively protecting PAC at the 2'-position with a Cbz group to give 27, which was derivatized as an ester at the 7-position using succinic anhydride or glutaric anhydride to give 30 or 31, respectively. Compounds 30 and 31 were then subjected to hydrogenation conditions to selectively deprotect the 2'-hydroxyl group and yield targets 32 and 33, respectively.
Scheme 10. Synthesis of the 7-aliphatic acid-PAC analogues. (a) CbzCl, DIPEA, DCM, -20°C, 18 h, 98%. (b) Succinic anhydride, DMAP, DCM, rt, 80%, or glutaric anhydride, DMAP, toluene, reflux, 84%; (c) 10% Pd/C, H₂, 25 psi, MeOH, yields (32: 95%, 33: 95%).

The succinic acid series at the 10-position of PAC was synthesized by reacting compound 18 with succinic anhydride so as to produce intermediate 34, followed by hydrogenation to yield target 35. Selective deprotection of the 7-hydroxyl in 35 using HF/pyridine afforded target 36. These reactions are depicted in Scheme 11. Attempts to install a glutaric acid moiety at the 10-hydroxyl group of PAC proved to be laborious and yields were low.
Scheme 11. Synthesis of the 10-succinate-PAC series. (a) Succinic anhydride, DMAP, toluene, reflux, 12 h, 85%; (b) 10% Pd/C, H₂, 25 psi, MeOH, 5 h, 95%; (c) HF/pyridine, DCM, rt, 12 h, 75%.

2.2.4 Alcohols-PAC Conjugate Series

Noting that an acidic group can reduce MDR liability while a basic amine group appears to increase it (and suspecting that the latter result is likely observed when the amine is protonated and can only serve as an ionic partner or as a hydrogen-bond donor partner), it becomes beneficial to study the effect of coupling alcohols at position 10 of PAC. This study examines if the beneficial MDR result is due to the presence of an electron rich (but non-basic) group or a hydrogen-bond accepting group, rather than to a full-blown ionic effect from the carboxylate anion. In addition, such alcohol-PAC analogues are expected to have superior aqueous solubility as compared to that of 1. Along similar lines of coupling amino acid residues that offer different functional groups, a serine residue has been chosen to be attached to the C-7 hydroxyl group of 1. A similar approach to that used in Scheme 8 has been employed. This synthetic route is shown in
Scheme 12. Conjugate 37 was prepared by coupling a Boc-Ser-OBn residue to the C-7 hydroxyl group of 22. The selective removal of the 2’-chloroacetyl group yielded the key intermediate 38. Subjecting 38 to hydrogenation conditions resulted in 39 with a free hydroxyl group. The fully deprotected analogue 40 was obtained from 39 under acidic conditions. 39 was also prepared from 27 via intermediate 42. Treating 38 with 50% formic acid in DCM can produce 41 with a free amino group.

Scheme 12. Synthesis of the 7-Ser-PAC conjugates. (a) Boc-Ser-OBn, DIC, DMAP, DCM, rt, 12 h, 82%; (b) MeOH, rt, 12 h, 100%; (c) 10% Pd/C, H₂, 25 psi, MeOH, 18 h, 86%; (d) 50% Formic acid/DCM, 12 h, 90%; (e) Boc-Ser-OBn, DIC, DMAP, DCM, rt, 10 h, 85%.

2.3. Biological Evaluation

Biological studies were performed by Dr. J.G. Sarver. The MDR liability of the 10-Asp-PAC analogs was evaluated using an MCF7 cell line that does not express Pgp, and a NCI/ADR-RES cell line which over-expresses Pgp. The ideal result will be when
the PAC conjugates maintain or decrease the GI\textsubscript{50} within the MCF7 assay as compared to PAC, while decreasing dramatically the GI\textsubscript{50} within the NCI/ADR-RES assay. In addition, the selectivity between normal and cancerous cells was tested using the MCF12A cell line, a non-cancerous, rapidly dividing human breast epithelial cell line. The preliminary biological data is shown in Table 1 where it can be seen that a bulky, lipophilic group at position 10 of PAC (compounds 11 and 12) retains inherent anticancer activity (MCF7 potency) but exacerbates the MDR liability (MDR versus non-MDR ratio). Alternatively, the presence of a free acid group (7 and 9) reduced MDR liability although inherent potency was also diminished. Interestingly, a free amine (13 and 14) increased MDR liability while showing a smaller reduction in the inherent potency. Thus, these results suggested that the addition of a free acid group should be taken into consideration in designing paclitaxel analogues for the selective targeting of cancer cells.

Table 1. Biological data for the different 10-Asp-PAC analogues.

<table>
<thead>
<tr>
<th>Agent</th>
<th>N</th>
<th>MCF7 Non-MDR GI\textsubscript{50} (nM)\textsuperscript{a}</th>
<th>MDR vs Non-MDR GI\textsubscript{50} ratio\textsuperscript{b}</th>
<th>Normal vs Non-MDR GI\textsubscript{50} ratio\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel (PAC) 1</td>
<td>94</td>
<td>5.4 ± 0.3</td>
<td>1117 ± 47</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>10-D(Boc,OBn)-DAP 12</td>
<td>8</td>
<td>3.6 ± 0.4</td>
<td>4850 ± 713</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>10-D(Boc)-DAP 7</td>
<td>6</td>
<td>382 ± 30</td>
<td>421 ± 59</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>10-D(OBn)-DAP 14</td>
<td>6</td>
<td>7.1 ± 0.6</td>
<td>4353 ± 709</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>7-Cac-10-D(Boc, OBn)-DAP 11</td>
<td>6</td>
<td>44.7 ± 4.3</td>
<td>1951 ± 236</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>7-Cac-10-D(Boc)-DAP 9</td>
<td>6</td>
<td>596 ± 85</td>
<td>158 ± 10</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>7-Cac-10-D(OBn)-DAP 13</td>
<td>8</td>
<td>33.1 ± 8.3</td>
<td>2008 ± 620</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>10-D-DAP 8</td>
<td>6</td>
<td>11.0 ± 2.7</td>
<td>1212 ± 191</td>
<td>1.9 ± 0.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}GI\textsubscript{50}= Dose resulting in 50% growth inhibition; \textsuperscript{b} The smaller the ratio, the lower the MDR liability; \textsuperscript{c} The bigger the ratio, the better the selectivity.

The biological activity, including MDR liability and selectivity, of the different 7-Glu-PAC, 7- and 10- aliphatic acid-PAC, and the 7-Ser-PAC conjugates will be evaluated in the same type of assays conducted on the 10-Asp-PAC analogues. The conclusions that
will be drawn from these studies will answer several questions concerning the design criteria that should be considered when designing PAC analogues with reduced Pgp-mediated MDR.
Chapter 3.

Targeting Prostate Cancer Cells

3.1 The Protein-Specific Membrane Antigen (PSMA)

Prostate cancer is the most commonly diagnosed cancer in American men where it accounts for 29% of all new cancer cases each year.\textsuperscript{67} It is also one of the most fatal cancers, coming in second only to lung cancer and leading to over 30,000 fatalities each year.\textsuperscript{68} First discovered during the development of the LNCaP cell line\textsuperscript{69}, the Protein-Specific Membrane Antigen (PSMA) enzyme is expressed in normal and neoplastic prostate endothelial cells as well as in prostate tumor metastases. Although expressed in normal cells as well, PSMA expression levels are several hundred-fold higher in prostate cancer cells\textsuperscript{70}, with the expression level increasing as the stage and grade of the tumor progress.\textsuperscript{71} PSMA is also over-expressed in the neovasculature of non-prostatic solid tumors, but not in normal endothelial cells. This suggests a further role in angiogenesis.\textsuperscript{72}

PSMA, also known as folate hydrolase I (FOLH1), is a zinc metallopeptidase transmembrane protein localized on the apical plasma membrane in the secretory epithelial cells of the prostate gland. As a glutamate carboxypeptidase, its role is to sequentially cleave terminal $\gamma$-linked glutamated folates which are otherwise unable to be transported into cells in their poly-$\gamma$-glutamated form.\textsuperscript{73} Exhibiting activity similar to membrane-bound $N$-acetylated $\alpha$-linked acidic dipeptidase (NAALADase)\textsuperscript{74}, PSMA has the potential to hydrolyze the neuropeptide $N$-acetylaspartylglutamate (NAAG) as shown
in Fig. 12. This feature is commonly utilized in an *in vitro* radioenzymatic assay to monitor the PSMA activity.\textsuperscript{75} In addition, there is evidence that PSMA may be involved in multiple physiological functions within the cell, such as cell migration, signal transduction, receptor function, and nutrients uptake.\textsuperscript{76}

![Chemical structure](image.png)

*Figure 9.* PSMA-mediated hydrolysis of NAAG.

From this setting, it is not surprising that PSMA has been envisioned as a biomarker of prostate cancer, and has attracted significant attention as a target for immunotherapy,\textsuperscript{77} targeted chemotherapy,\textsuperscript{78} and targeted molecular imaging.\textsuperscript{79} The crystal structure of PSMA has been solved recently. This will be of tremendous assistance for designing even better PSMA substrates.\textsuperscript{80}

### 3.2 PAC Analogues for the Selective Targeting of Prostate Cancer Cells

Having already gained some knowledge from the PAC SAR studies, we started to synthesize composite target compounds to achieve selectivity toward cancer cells. Prostate cancer cells were targeted by taking advantage of the over-expression of PSMA on their surface. The latter recognizes γ-linked glutamate residues. Thus, we proposed
attaching the (Glu-γ-Glu) dipeptide and (Glu-γ-Glu-γ-Glu) tripeptide at the 7-position of PAC.

We were first interested in studying the binding affinity (or enzymatic activity) of PSMA to the γ-linked glutamate residues. Attaching the glutamate residues to a dye would make such an assay feasible and would assist in monitoring and evaluating the PSMA activity. Thus, we synthesized several glutamate residues attached to rhodamine 6G (R6G) dye in the desired γ-linkage as depicted in Schemes 13, 14 and 15. It is important to note that orthogonal protection/deprotection procedures were used to generate different analogues bearing different functional groups. Such analogues will help understand the structural requirements for the ultimate PSMA activity. The control conjugate D5 lacking the crucial γ-linkage required for the PSMA activity was synthesized as shown in Scheme 13. The first step in the synthetic route involved a transamidation reaction between R6G and EDA, the latter used as a linker connecting the γ-linked glutamate residues to the fluorescent dye, to afford intermediate D2. A Boc-Glu-OBn residue was then coupled to D2 using CDI as a coupling reagent generating D3 which when treated with a 1:1 TFA/DCM mixture produced intermediate D4. Debenzylation of D4 by hydrogenation yielded the control conjugate D5.

The synthesis of the R6G-Glu-γ-Glu analogues is shown in Scheme 14. Following the activation of Boc-Glu-OBn by PyBOP/HOBt, D4 was added and the fully protected conjugate D6 was produced. The Boc protecting group in D6 was then removed using 1:1 TFA/DCM mixture so as to yield D7, which when subjected to hydrogenation produced D8.
Scheme 13. Synthesis of R6G-Glu conjugate. (a) DCM, rt, 4 h; (b) Boc-Glu-OBn, CDI, DCM, 18 h, 90%; (c) 1:1 DCM/TFA, 12 h, 95%; (d) 10% Pd/C, H₂ (20 psi), MeOH, AcOH, 12 h, 75%.

Scheme 14. Synthesis of the R6G-Glu-γ-Glu conjugates. (a) Boc-Glu-OBn, PyBOP, HOBt, DCM/DMF, DIPEA, 13 hours, 80%; (b) 1:1 DCM/TFA, 12 h, 90%; (c) 10% Pd/C, H₂ (20 psi), MeOH, AcOH, 18h, 72%.
We then hypothesized that an extended Glu peptide with more exposed γ-linkages away from the bulky dye would have better PSMA binding affinity. Thus, the R6G- Glu-γ-Glu-γ-Glu conjugates were prepared as depicted in Scheme 15. D8 was coupled to another Boc-Glu-OBn yielding the fully protected R6G- Glu-γ-Glu-γ-Glu conjugate D9. The amino group in D9 was then deprotected and analogue D10 was produced. D11 was obtained by debenzylating D10.

Scheme 15. Synthesis of R6G- Glu-γ-Glu-γ-Glu conjugates. (a) Boc-Glu-OBn, PyBOP, HOBt, DCM/DMF, DIPEA, 14 h, 88%; (b) 1:1 DCM/TFA, 10 h, 92%; (c) 10% Pd/C, H₂ (20 psi), MeOH, AcOH, 10 h, 76%.

Having prepared the R6G-γ-linked glutamate residues, the next step in the study was to synthesis the glutamate dipeptides and tripeptides that are to be attached on the 7-
position of PAC. The dipeptides and tripeptides of interest were synthesized as depicted in Schemes 16, utilizing orthogonal protection/deprotection approaches to assure the formation of the desired γ-linkage. The Boc-protecting group in 43a was removed producing intermediate 44, which was then coupled to glutamic acid residue 43 to yield desired dipeptide 45. Tripeptide 46 was obtained from the reaction of 45 with another molecule of 44.

![Chemical structures](image)

Scheme 16. Synthesis of the γ-linked glutamic acid residues. (a) HCl/EtOAc, 1 h, 100%; (b) CDI, DCM, DIPEA, DCM, yields (45a: 82%; 45b: 80%); (c) CDI, DCM, DIPEA, DCM, yields 46a: 72%; 46b: 74%).

The γ-linked glutamate dipeptides and tripeptides were attached to the 7-position of PAC as depicted in Schemes 17, 18, and 19. Following the selective protection of the 2’-hydroxyl group of 1, the Cbz-Glu(OBn)-Glu-OBn 45b was coupled to the 7-hydroxyl group of 27 to generate 47. Shaking 47 under hydrogen resulted in the fully deprotected target 48. These reactions are shown in Scheme 17.
Target 50, with two free carboxyl groups and a protected amino group, was synthesized as depicted in Scheme 18. 45a was coupled to 27 at the 7-position yielding intermediate 49 which when subjected to hydrogenation afforded target 50.

Scheme 17. Synthesis of the neutral analogue 48. (a) Cbz-Cl, DCM, DIPEA, -20°C, 18 h, 98%; (b) Cbz-Glu(OBn)-Glu-OBn, DIC, DMAP, DCM, 11 h, 73%; (c) 10% Pd/C, H₂ (25 psi), AcOH, MeOH, 12 h, 93%.

Scheme 18. Synthesis of target 50. (a) Cbz-Cl, DCM, DIPEA, -20°C, 18 h, 98%; (b) Boc-Glu(OBn)-Glu-OBn, DIC, DMAP, DCM, 11 h, 70%; (c) 10% Pd/C, H₂ (25 psi), MeOH, 12 h, 91%.
A similar chemical approach was employed to generate PAC analogues having a Glu-γ-Glu-γ-Glu tripeptide hooked to the 7-position of PAC. These reactions are shown in Scheme 19. Conjugate 51 was obtained via the coupling of tripeptide 46 to 2’-Cbz-PAC 22. Targets 52a and 52b can be generated from 51a and 51b, respectively, under hydrogenation reaction.

Scheme 19. Synthesis of the 7-Glu-γ-Glu-γ-Glu-PAC analogues. (a) 46, DIC, DMAP, DCM, rt, 11 h, yield (51a: 71%). (b) 10% Pd/C, H2 (25 psi), MeOH.

3.3. Biological Evaluation

The biological activity of the different γ-linked glutamic acid-PAC analogs will be tested using two cell lines: LNCaP, which over-expresses PSMA; and PC3, which does not express PSMA. Two types of assays will be conducted wherein the uptake of the R6G-γ-linked glutamic acid and the cytotoxicity of the different γ-linked glutamic acid-PAC analogs are evaluated (Table 2).
Table 2. Prostate cancer cell lines chosen for biological testing.

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<td>Positive control for uptake of PSMA-dye</td>
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<tr>
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<td>PC3</td>
<td>Prostate cancer cell line PSMA -</td>
<td>Negative control for uptake of PSMA-dye</td>
</tr>
<tr>
<td>PMSA-targeted cytotoxicity</td>
<td>LNCaP</td>
<td>Prostate cancer cell line PSMA +</td>
<td>Experimental for cell death using PMSA-PAC</td>
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<tr>
<td>PMSA-targeted cytotoxicity</td>
<td>PC3</td>
<td>Prostate cancer cell line PSMA -</td>
<td>Negative control for cell death using PMSA-PAC</td>
</tr>
</tbody>
</table>

3.4 Designing CD3-246: A PAM Inhibitor

3.4.1. PAM’s discovery and mechanism of action

During early stages of the disease, prostate cancer cells rely on androgens to stimulate tumor growth.\(^{81}\) Therefore, hormone deprivation represents a main treatment approach as an initial therapy. However, as the disease progresses, the tumor develops the ability to grow even when its growth-promoting, androgenic hormone pathways are blocked or ablated. This has been attributed, in part, to specialized cells that overpopulate in the tumor where they produce their own hormones and growth factors that require posttranslational modifications, such as peptide amidation, to become biologically active. Such an activation process is triggered by secondary processing, the latter involving a bifunctional enzyme called *Peptidylglycine α-Amidating Monooxygenase* (PAM).\(^{82}\) PAM consists of two enzymes that act sequentially to convert peptidylglycine substrates into α-amidated products and glyoxylate: peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL). It is well established
that over half of all peptide hormones require amidation at their carboxy terminus to gain full biological activity.\textsuperscript{83} Such amides are not formed by a simple transamidation process. Instead, PAM catalyzes the conversion of peptidylglycine substrates into \( \alpha \)-amidated products by the oxidative cleavage of the glycine N-C\( \alpha \) bond.\textsuperscript{84} This conversion occurs in two steps as depicted in Fig. 10.A. The first enzymatic step is catalyzed by PHM and converts the substrate to an \( \alpha \)-hydroxylated intermediate, namely peptidyl \( \alpha \)-hydroxyglycine, in the presence of ascorbate, molecular oxygen and copper. The detailed enzymatic mechanism in which copper undergoes a reduction/oxidation cycle from Cu\( ^{2+} \) to Cu\( ^+ \) and back to Cu\( ^{2+} \) is given in Fig. 10.B. Both PHM-bound Cu\( ^{2+} \) ions are reduced independently by two ascorbate molecules in two one-electron reductions, and two semihydroascorbate radicals are formed. Molecular oxygen binds reversibly to the reduced CuB displacing the bound water molecule. Binding of the peptidylglycine substrate results in the formation of a ternary complex that appears to be crucial for the progress of the reaction. CuA and CuB transfer one electron each to molecular oxygen to form a hydroperoxide bound to CuB where the proton is believed to be provided by the carboxyl of the bound peptide substrate. The oxygen-oxygen bond cleaves and the hydroxyl radical abstracts the \( \text{pro S} \) hydrogen from the substrate glycine. The resulting glycyl radical combines with the Cu-bound oxo radical to form a Cu-bound alkoxide product. The latter then dissociate slowly affording peptidyl \( \alpha \)-hydroxyglycine and regenerating the enzyme that starts a new catalytic cycle. The second step is catalyzed by PAL where the \( \alpha \)-amidated peptide and glyoxylate are produced (Fig. 10.C).\textsuperscript{85}
Figure 10. The PAM reaction. (a) PAM’s overall enzymatic cycle; (b) PHM enzymatic cycle; (c) PAL enzymatic cycle.
3.4.2. Designing PAM inhibitors: Discovery and synthesis of CD3-246

From this background, it has been proposed that inhibiting PAM activity may prevent the activation of certain peptide hormones that contribute to tumor growth. Furthermore, co-treatment with hormone blocking drugs and PAM inhibitors during the early stages of the disease may circumvent the progression to hormone-independent cancer. Towards these possibilities, the CD3 launched a program to develop PAM inhibitors that have the potential to attenuate the growth of hormone-independent prostate cancer. One part of the program involved synthesizing a library of drug-like, peptidomimetic compounds as potential PAM inhibitors. From that effort, CD3-246 was identified as a lead compound (IC$_{50}$ = 2.5 µM), and gram quantities essential for the further in vitro and in vivo testings were required. Scheme 20 shows the synthetic route that was utilized to produce CD3-246. This route does not require a debenzylation step, a process that proved to be problematic in routes developed by other CD3 members.

The free amine form of 54 was coupled to Fmoc-protected methionine 53 using CDI as a coupling reagent to yield Fmoc-Met-Gly-O'Bu 55. The Fmoc- group of dipeptide 55 was removed using 10 equivalents of 1-octanethiol in the presence of a catalytic amount of DBU. Treating the reaction mixture with a saturated oxalic acid/EtOAc solution produced the oxalate salt 56. Tripeptide 57 was synthesized by coupling dipeptide 56 to (D)-Fmoc-Tyr-O'Bu using CDI as a coupling reagent. The Fmoc-protecting group on tripeptide 57 was removed, and Tyr-Met-Gly-OtBu oxalate salt 58 was obtained following the addition of a saturated oxalic acid /EtOAc solution. The free amine form of 58 was then coupled to N-4-(2-thienyl)butyric acid to give the
penultimate compound 59. **CD3-246** was obtained by treating compound 59 with a 1:1 DCM: TFA solution. The overall yield for this sequence is ca. 25%.

![Chemical reaction diagram]

Scheme 20. Synthesis of **CD3-246**. (a) CDI, DIPEA, DCM, rt, 3 h, 95%; (b) 10 eq. octanethiol, DBU, THF, rt, then oxalic acid/EtOAc, 6 h, 62% (c) CDI, DIPEA, DCM, rt, 6 h, 62%; (d) 10 eq. octanethiol, DBU, THF, rt, then oxalic acid/EtOAc, 6 h; (e) CDI, DIPEA, DCM, rt, 6 h, 75%; (f) 1:1 TFA:DCM, rt, 2 h, 90%.

### 3.4.3 Preliminary biological evaluation

Detailed biological evaluation studies have been conducted previously on **CD3-246**. These studies involved developing procedures to extract PAM from human prostate cancer cells, and establishing biochemical assays to evaluate PAM inhibitors. Whereas detailed biological data will be published elsewhere, only a brief summary is provided here. The *in vitro* growth inhibition assays led to the selection of **CD3-246** as a lead agent for further evaluation in *in vivo* and auxiliary tests. The data collected from an *in vivo* tumor xenograft study indicated that **CD3-246** attenuated the growth of implanted
hormone-independent CaP tumors. Auxiliary studies demonstrated that **CD3-246** is a non-substrate, competitive inhibitor of the first enzymatic step catalyzed by PHM.
Chapter 4.

Targeting Breast Cancer Cells

4.1. The Integrin Receptors

The ability of a given cell to interact with other cells or molecules in the extracellular matrix (ECM) is essential for maintaining that cell’s function and integrity. Receptors that are involved in these interactions belong to a class of cell surface α,β-heterodimers called integrins. Many of the integrin receptors recognize the hydrophilic amino acid triad Arg-Gly-Asp (RGD) which is found in several adhesion proteins including fibronectin, vitronectin, and fibrinogen. The main interactions occur between the positively charged arginine and the α-subunit, and between the anionic aspartic acid and the β-subunit, where the subunit selectivity is induced by the RGD conformation.\(^8^6\)

Although integrins are involved in normal processes such as platelet aggregation,\(^8^7\) bone resorption,\(^8^8\) immunological and inflammatory responses,\(^8^9\) they also play a critical role in the pathology and progression of several diseases such as osteoporosis, atherosclerosis, tumor metastasis, and tumor induced angiogenesis.\(^9^0\) These complex processes necessitate that the integrin receptors are able to bind several different ligands in the ECM.\(^9^1\) Such functional diversity stems from the ability of one α-subunit to associate with multiple β-subunits, and one β-subunit to complex with several α-subunits. There is also evidence that the integrin receptors can influence cell survival by generating cellular signals that can change gene expression.\(^9^2\)
In particular, the αv-subunit has gained special interest due to its ability to bind five different β-subunits: β1, β3, β5, β6, and β8. Such association resulted in heterodimers with distinct and diverse biochemical functions such as the ability to bind, internalize and degrade ligands, as well as to mediate cell migration. The integrin αvβ3 has received considerable attention due to its over-expression in a variety of cell types such as endothelial cells, melanoma, osteoclasts, and smooth muscle cells, as well as in breast cancer. It can bind at least four distinct adhesion proteins. αvβ3 is preferentially expressed on blood vessels undergoing angiogenesis and has been shown to have a role in neovascularization. It has been known for a long time that rapidly dividing cancer cells stimulate the formation of new blood vessels to ensure a sufficient supply of oxygen and nutrients, a process well-known as ‘angiogenesis’ and hypothesized to be of critical importance to tumor progression by Folkman over thirty years ago. However, the newly formed blood vessels are often abnormal in form and architecture leading to a leaky vasculature with enhanced permeability. Thus, the tumor blood vessels may represent potential targets for targeted antiangiogenic therapy. In fact, it is possible to differentiate between newly formed and mature tumor blood vessels using specific antibodies or medical imaging techniques. For example, it has been reported that mAb LM609 and small cyclic RGD-containing peptides induced rapid apoptosis in newly growing vessels without affecting the preexisting mature ones.

The fact that the over-expression of the αvβ3 integrin on breast cancer can signal the onset of widespread metastasis is supported by the evidence that nearly all breast cancers that metastasized to the bone expressed the αvβ3 integrin. In an attempt to study the contribution of the αv integrin to the adhesive and migratory behavior of breast cancer
cells, Smith et al. characterized the repertoire and function of $\alpha_v$ integrin in three well-established breast cancer cell lines.\textsuperscript{106} Interestingly, the authors reported substantial levels of $\alpha_v\beta_3$ in the highly metastatic MDA-MB-435 cell line, but not in the moderately metastasizing MDA-MB-231 or the inefficiently metastasizing MCF-7 breast cancer cell lines.

Collectively, these findings led to the conclusion that $\alpha_v\beta_3$ integrin can be a contributing factor in metastatic diseases making it an interesting target in developing integrin-specific RGD conjugates for targeted therapy. Several reports have been published utilizing RGD-containing moieties for selective targeting of tumor blood vessels,\textsuperscript{107} and for the \textit{in vitro} and \textit{in vivo} imaging of tumors.\textsuperscript{108}

Herein, we propose the synthesis of different 7-RGD-PAC analogues which will offer the following advantages in the treatment of breast cancer:

- The interaction of the RGD address with the integrin receptor $\alpha_v\beta_3$ will inhibit the latter’s interaction with the surrounding adhesion molecules bearing the RGD sequence. This can attenuate tumor growth and spreading;
- The over-expression of the integrin receptors, specifically $\alpha_v\beta_3$, on the malignant cells will result in localizing the RGD-drug analogues at the site of the tumor;
- The hydrophilic nature of the RGD tripeptide will increase the aqueous solubility of the 7-RGD-PAC analogues; and finally,
- As previously discussed, the presence of an acidic side chain on one of the amino acids may serve to decrease MDR.

In addition, the orthogonal procedures that will be used to deprotect the parent 7-PAC-RGD analogues will result in new conjugates that may afford a better understanding of
the RGD/integrin interaction SAR upon biological testing. It is worth noting that approaches that can stop the growth and spread of the tumor will be particularly beneficial in cases where long term treatment is required. For example, the long term treatment of breast cancer would result in the loss of the estrogen receptors that are expressed on breast cancer cells. This would render the treatment using selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene ineffective. It has been reported that adding PAC to the standard chemotherapy for women who have breast cancer that has spread to the lymph nodes lowers their chance of dying by 18%. Women whose cancer lacked estrogen receptors had the best results. Thus, the proposed 7-PAC-RGD analogues could be very important in such cases.

4.2. PAC Analogues for Targeting Breast Cancer Cells

4.2.1 Synthesis of the R6G-RGD conjugates

Although a cyclic constrained RGD moiety has the best binding affinity to the $\alpha_v\beta_3$ integrin receptor over-expressed on breast cancer cells, we hypothesized that a linear RGD tripeptide can answer preliminary questions concerning gross selectivity, binding affinity and initial feasibility for this approach to then be applied within the specific context of PAC. Likewise, rhodamine dye (R6G) conjugates were again envisioned to be useful probes to address initial questions pertaining to these types of issues. Thus, the R6G-RGD conjugates were first synthesized to study the binding affinity of the RGD tripeptide to the $\alpha_v\beta_3$ integrin. The synthetic route that led to these analogues is depicted in Scheme 21. The R6G-Asp intermediate D12 was synthesized by coupling a Boc-Asp-OBn to D2 that was made as described in Chapter 3. The Boc
protecting group of **D12** was removed by treating **D12** with a 1:1 TFA:DCM mixture to yield **D13**, which was then coupled to the preactivated Boc-Gly-OH residue affording R6G-Asp-Gly conjugate **D14**. Deblocking the amino group of **D14** produced **D15** which when reacted with a preactivated arginine residue gave R6G-Asp-Gly-Arg conjugate **D16a**. Subjecting **D16a** to hydrogenation resulted in targets **D17**.

![Chemical structure diagram](attachment:image.png)

**Scheme 21.** Synthesis of the Dye-RGD analogues. (a) DCM, rt, 95%; (b) Boc-Asp-OBn, CDI, DCM, rt, 12 h, 85%; (c) 1:1 DCM/TFA, rt, 10 h, 93%; (d) Boc-Gly-OH, PyBOP, HOBt, 13 h, rt, 60%; (e) 1:1 DCM/TFA, rt, 10 h, 90%; (f) Cbz-Arg(di-Cbz)-OH, PyBOP, HOBt, DCM, DMF, rt, 13 h, 70%; (g) 10% Pd/C, H\(_2\) (30 psi), AcOH, MeOH, 48 h, 80%.
The preparation of the PAC-RGD analogues was directed toward synthesizing 7-RGD-PAC analogues. The strategy was to synthesize the RGD address system separately before hooking it up on the 7-position of the cytotoxic drug. Thus, the Arg residue 60 was coupled to Gly 61 to yield dipeptide 62 whose carboxyl protecting group was deprotected under acidic conditions to produce 63. Asp 66 was prepared from Asp 64 in a separate reaction by protecting the α-carboxyl group with a benzyl moiety to generate 65, followed by the removal of the Fmoc protecting group and the oxalate salt 66 was produced. 66 was then coupled to dipeptide 63 so as to afford the fully protected tripeptide 67. The RGD address system 68 that is to be attached on PAC was obtained following the deprotection of the β-carboxyl group of 67. These reactions are shown in Scheme 22.

Scheme 22. Synthesis of the RGD tripeptide with a free carboxyl group. (a) CDI, DCM, DIPEA, yields (62a: 82%, 62b: 94%); (b) 1:1 TFA/DCM, yield (63a: 95%); (c) DCC, BnOH, DMAP, 95%; (d) 10 eq. octanethiol, DBU, then oxalic acid/EtOAc, 80%; (e) CDI, DCM, DIPEA, yields (67a: 60%, 67b: 70%); (f) 1:1 TFA/DCM, yield (68a: 92%).
The synthesis of the RGD tripeptide 75 that is to be connected through its N-terminal to PAC is depicted in Scheme 23. This analogue will assist in understanding the significance of linking the address system through its C- or N- terminals to PAC and the impact of such linkage on the binding to the integrin receptors. Dipeptide 71 was synthesized by coupling Gly 69 and Asp 70. The Boc protecting group was then removed and intermediate 72 was produced. The latter was reacted with a preactivated Arg 73 to generate the fully protected RGD 74. The tripeptide of interest 75 with a free amino group was obtained by subjecting 74 to acidic conditions.

Scheme 23. Synthesis of the RGD tripeptide with a free amino group. (a) CDI, DIPEA, DCM, rt, 7 h, 94%; (b) TFA/DCM, rt, 5 h; (c) CDI, DIPEA, DCM, rt, 12 h, 72% (from 71); (d) TFA/DCM, rt, 2 h.
The partially protected Dye-RGD analogue \textbf{D18} and the fully deprotected conjugate \textbf{D19} were synthesized as shown in Scheme 24. RGD tripeptide \textbf{68} was coupled to R6G \textbf{D2} to yield the fully protected intermediate \textbf{D16}. Shaking of \textbf{D16b} and \textbf{D16c} under hydrogen and in the presence of 10% Pd/C afforded the guanidine-protected \textbf{D18} and the fully deprotected \textbf{D19} conjugates, respectively.

\begin{center}
\textbf{Scheme 24.} Synthesis of Dye-RGD analogues. (a) CDI, DCM, rt, yields (\textbf{D16b}: 85%, \textbf{D16c}: 76%); (b) 10% Pd/C, H\textsubscript{2} (30 psi), 24 h, 85%; (c) 10% Pd/C, H\textsubscript{2} (30 psi), 24 h, 87%.
\end{center}

Scheme 25 shows the synthetic route that can be followed to generate the first 7-RGD-PAC target \textbf{77} where the RGD address system \textbf{68b} can be linked to 7-position of PAC through its C-terminal. Cbz-PAC \textbf{23} can be added following the activation of \textbf{68b} with DIC to afford conjugate \textbf{76}. Catalytic hydrogenation would remove all protecting groups and result in the fully deprotected target compound \textbf{77}. Initial attempts to
synthesize 76 resulted in its formation in very low yields (MS: M+Na 1607.50) and its isolation as a pure compound proved to be laborious.

Alternatively, target 79 where RGD is linked through its N-terminal to PAC was synthesized as depicted in Scheme 26. Cbz-PAC 23 was first functionalized with a succinic acid moiety to produce 26 that can accommodate the incoming RGD 75. 26 was

Scheme 25. C-terminal coupling of the RGD 68b to PAC. (a) DIC, DMAP, rt; (b) 10% Pd/C, H₂ (30 psi), AcOH, MeOH.
activated using DIC after which 75 was added to generate 78. 79 can generated from 78 under catalytic hydrogenation conditions.

Scheme 26. N-terminal coupling of the RGD 75 to PAC. (a) succinic anhydride, DMAP, DCM, rt; (b) 75, DIC, DMAP, rt, 52%; (b) 10% Pd/C, H₂ (30 psi), AcOH, MeOH.

4.3. Biological Evaluation

The uptake and cytotoxicity of the R6G-RGD and RGD-PAC analogs will be tested using an MCF7 cell line (a human breast cancer cell line that does not express αvβ3
integrin), and an MDA-MB-435 cell line (a metastatic human breast cancer cell line that over-expresses $\alpha_\beta_3$).

**Table 3.** Breast cancer cell lines chosen for biological testing.

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</table>

### 4.4 Targeted Ultrasound Imaging in Breast Cancer

Advances in the field of medical imaging over the past decade have led to the development of “state-of-the-art” modalities that can detect/diagnose events at the molecular level. While each technique has its own advantages and disadvantages, an ideal imaging modality should offer high sensitivity, specificity, rapid execution of imaging protocols, and relatively low cost. These features can all be found in the non-invasive, ultrasound imaging technique. Ultrasound or ultrasonography is a medical imaging technique that involves exposing a part of the body to high frequency sound waves (1 to
10 MHz) to produce real time pictures of the tissue interface within the body. It has been used to detect the changes in the appearance and function of organs, tissues, or abnormal masses such as tumors. The ultrasound waves emitted by an ultrasound scanner travel into the body and hit a boundary between tissues having different densities (e.g. between fluid and soft tissue). Part of the sound waves become reflected back to a probe, while the rest travel further till they reach a different boundary and become reflected. The reflected waves are relayed to a machine that calculates the distance from the probe to the tissue (boundaries) using the speed of the sound in the tissue and the time of each echo’s return. The machine displays the distance and the intensities of echoes on a screen forming a two dimensional image. Examples of these images are provided below in Fig. 14.

![Ultrasound of a kidney and gallbladder](image.png)

**Figure 11.** Ultrasound of a kidney and gallbladder.

Due to the small difference in densities and compressibility of neighboring soft tissues, ultrasound contrast agents were developed to enhance the accuracy and improve the sensitivity of this technique in such settings. The ultrasound contrast agents are usually microbubbles that consist of a shell encapsulating the contrast agent that can be
air, perfluoro gases (e.g. C₃F₈) or sulfur hexafluoride. The choice of the shell material determines how easily the microbubble is taken up by the immune system. Microbubble shells can consist of albumin, lipid, or polymer. Microbubbles are usually 1-4 µm in diameter making them smaller than red blood cells and allowing them to flow easily through vessels and microvessels. It is important to note that microbubbles are intravascular tracers, meaning that following their intravenous injection, they would not leave the vascular tissue in an intact form. Thus, microbubbles are designed to image molecular targets only in the blood stream or on the endothelium surface.

Several strategies have been designed to target microbubbles to their molecular destination. One strategy relies on the chemical or electrostatic interactions between the albumin or lipid components of the microbubble with the receptors over-expressed on the targeted cells. Another strategy involves attaching a ligand, such as a peptide or an antibody, to the microbubble shell. These ligands bind preferentially to receptors over-expressed on the host cell. A ligand-directed strategy involves administrating a biotinylated monoclonal antibody followed by the injection of streptavidin to which the biotinylated emulsion contrast agent binds (Fig.12.A). Ligands are attached to microbubbles via two general strategies. In the first approach, the ligand is attached to the shell-forming molecule (e.g. lipid), and the resulting conjugate is mixed with the bulk material suited for shell preparation. The second method involves attaching the ligands, covalently or noncovalently, to a preformed microbubble (Fig.12.B).

Several studies have been reported discussing targeted contrast agents that can be used to image diseases such as inflammation, thrombus, and angiogenesis. For example, Lindner et al. designed a microbubble targeted to αvβ3 integrin receptor to
detect early tumor angiogenesis. They attached echistatin, an RGD-containing snake
venom peptide, to the microbubble surface. The authors reported that the $\alpha_v\beta_3$-targeted,
but not the control microbubbles, were retained preferentially within the tumor
microcirculation. The greatest ultrasound signal was at the periphery of tumors where the
$\alpha_v$-integrin expression levels are high.

A. Ligand-directed strategy.

B. Covalent attachment of a ligand to a preformed microbubble.

Figure 12. Strategies towards targeting microtubules to their molecular destination. A. Design of
biotinylated microbubbles and attachment of biotinylated ligand via a streptavidin and PEG spacer arm; B. Coupling of amino-containing ligand to carboxyl carrying microbubble.

Products producing air bubbles and perfluorocarbon gases have been used to
enhance ultrasound imaging. However, these types of ultrasound contrast agents have
been limited by the short lifetime and size instability of the bubbles.\textsuperscript{122} Thus, a novel contrast agent has been developed utilizing perfluourocarbon emulsions due to their dramatic differences in compressibility and elasticity. In this strategy, the RGD tripeptide will serve as the address component that is recognized by the integrin receptors over-expressed on breast cancer. The dye or perfluorinated compound will serve as the monomer from which bubble-like systems will form and thus serve as the biological marker or probe, the latter in particular to serve as an ultrasound contrast agent. If the perfluorinated cargo accumulates at the site of cancer cells, this could result in an enhanced diagnosis of the cancer mass. The proposed analogues may offer one or more of the following advantages:

- The selective binding of the RGD-perfluorinated analogues to integrin receptors over-expressed on cancer cells would result in “painting” the cancer cells’ membranes with perfluorinated tags;

- The RGD-perfluorinated conjugates may form micelles in solution where the RGD hydrophilic portion will be exposed on the surface of the micelle, while the hydrophobic perfluorinated chain becomes buried inside the micelle. This formation would constitute fluoroglobs that could preferentially associate with cancer cell membranes over-expressing integrins;

- The RGD-perfluorinated micelles may additionally be able to be used by entrapping perfluorocarbon gases which are commercially available ultrasound contrast agents. Based on the fact that “like” prefers to interact with “like,” these micelles will bear stronger hydrophobic stability than the simple hydrocarbon tails
now present in the commercially available products. The latter should thus improve their stability and half-life within the body; and,

- The RGD-perfluorinated conjugates can be useful in monitoring the proliferative growth of vascular tissues around cancer cells since vascular cells express the integrin on their surface as well.

### 4.4.1. RGD-Perfluorinated Conjugates for Enhancing Ultrasound Accuracy

The synthesis of this perfluorinated cargo is depicted in Scheme 27. The perfluorinated iodide 80 was refluxed with benzylamine to afford 81, which when subjected to catalytic hydrogenation, gave perfluorinated amine 82. The RGD tripeptide, whose synthesis is depicted in Scheme 23, was then attached to the perfluorinated amine 82 to yield the perfluorinated-RGD adduct 83. Catalytic hydrogenation resulted in the desired product 84. It is worth noting that controlling the amount of DIPEA used to free 82 is crucial for the success of the reaction. The best result was obtained when 1.2 equivalents of DIPEA were used. Utilizing two or more equivalents of DIPEA caused the formation of the cyclized product 85. The structure of 85 was confirmed by NMR and mass spectroscopy. The $^1$H NMR demonstrated the presence of a multiplet in the aromatic region corresponding to five protons and another at 5.0 ppm corresponding to one benzylic methylene group in a CBZ group. Cyclization was also verified by mass spectroscopy which showed M+H and M+Na peaks that are 108 units (OBn group) lower than what is expected for the uncyclized product. 85a was isolated and subjected to hydrogenation where 86 was formed as the acetate salt. 86 will be utilized as part of the overall SAR investigation where it will help to elucidate the importance of the carboxyl
group of the Asp unit in the binding of the RGD tripeptide address system to integrin receptors.

Scheme 27. Synthesis of the RGD-perfluorinated analogues: (a) Benzylamine, EtOAc, reflux, 24 h; (b) 10% Pd/C, H₂, 30 psi, MeOH, 18 h, 55% (from 80); (c) 68b, CDI, 1.2 eq. DIPEA, DCM, 18 h, 73%; (d) 10% Pd/C, H₂, 30 psi, MeOH, 48 h, 85%; (e) 68, CDI, > 2 eq. DIPEA, DCM, yields (85a: 81%, 85b: 78%); (f) 10% Pd/C, H₂, 25 psi, AcOH, MeOH, 12 h, 95%.

4.4.2. Biological Evaluation: Developing an in vitro Model

The feasibility and utility of the RGD-perfluorinated analogs as contrast agents will be examined by Dr. J.G Sarver, Dr. J. Trendell and N. Bearss based on the model developed for the control experiments. This model is described as follows: the MDA-MB-435 cell line was placed in wells crafted within a gel “plate” prepared from 10% gelatin containing 0.1% sodium azide to provide a uniform low-echo-emitting surrounding environment. The RGD-perfluorinated analogs were then be applied and the ultrasound echoes were measured with a General Electric Logiq Book XP portable ultrasound device with 8L-KS linear probe purchased by the CD3. This imaging procedure is illustrated as a cartoon in Fig. 13.
The utility and practicality of the prepared gel plate as a medium that can maximize the image signal-to-noise ratio was assessed by taking images using perfluorooctyl bromide (PFOB) as control contrast agent. The obtained images are shown in Fig. 14 and the collected data is given in Table 4. Brightness luminosity was determined by Photoshop software. The first image in Fig. 14 represents two cell-free wells where PFOB was applied in one (on the left side) while the second (on the right side) contained the gel material only and served as a control. As can be noticed visually and as determined by the Photoshop software measurements, there was no considerable difference in brightness luminosity between the two wells (3.4 in the well without PFOB and 5.8 for the well with PFOB). The second image in Fig. 14 shows two wells in the gel plate where the first (on the left side) was crafted with NCI/ADR-RES breast cancer cells while the second (on the right side) contained NCI/ADR-RES breast cancer cells and PFOB. A significant difference in brightness luminosity was observed between the wells. The ratio of the brightness luminosity of the second well to that of the background (no cells, the first image) was more than thirty [(72.7/2.4) = 30.2]. A similar result was seen in the third image where the wells were crafted with MCF7 breast cancer cells, with only
the second well incorporating PFOB. These observations suggest that the prepared gel plate is feasible for running in vitro ultrasound imaging tests. Further tests will be performed using our RGD targeted ultrasound contrast agents on MDA-MB-435 cells over-expressing integrin $\alpha_v\beta_3$ which binds the RGD peptide motif. Should the results be encouraging, the contrast agent will be attached to anticancer chemotherapeutic drugs where the conjugates will be evaluated for cytotoxicity and acoustic properties.

![Gel images](image)

**Figure 14.** Ultrasound images collected for validating the in vitro model.

| Table 4. Brightness Luminosity as determined by Photoshop software. |
|---|---|---|---|---|
| | No PFOB | With PFOB | Comparison |
| | avg. n=2 | avg. n=2 | Difference | x Background |
| No cells | 3.4 | 5.8 | 2.4 | 1.0 |
| NCI/ADR-RES | 5.7 | 78.4 | 72.7 | 30.2 |
| MCF7 | 7.5 | 87.9 | 80.3 | 33.4 |
5.1. Introduction

It has been known for many years that human solid tumors grow within a unique microenvironment characterized by significantly lower oxygen levels than that found in normal tissues, a phenomenon known as hypoxia. The median oxygen tension in human tumors ranges from 1.3-3.9% (10-30 mm Hg), where 82% of the measurements taken in solid tumors were less than 0.33% (2.5 mm Hg). On the other hand, higher values were recorded in normal cells with oxygen tension values in the range of 3.1-8.7% (24-66 mm Hg). This can be attributed in part to the vasculature in solid tumors that is abnormal in form and architecture, leading to insufficient supply of oxygen and nutrients. There is a plethora of experimental data demonstrating that the low oxygen tension in solid tumors is associated with low extracellular pH, low glucose concentrations, and high lactate concentrations because glycolysis, the oxygen-independent metabolic pathway, becomes the primary mechanism of generating ATP. In addition, there is convincing evidence that hypoxia promotes metastasis and angiogenesis which, in turn, leads to the selection of cells with a more malignant phenotype. These morphological and physiological differences between hypoxic tumor cells and normal cells can be exploited as markers to selectively target tumor cells. However, targeting hypoxic cells is limited by systemic toxicity, inefficient delivery of metabolites (e.g.
cytotoxic drugs) to cells that are distant from blood vessels, and reduced uptake of metabolites by nondividing severely hypoxic cells. Such features rendered hypoxic tumor cells intrinsically more resistant to conventional treatments by ionizing radiation\textsuperscript{132} and chemotherapeutic drugs.\textsuperscript{133}

The anthracyclin antitumor and antibiotic agents daunorubicin and doxorubicin (Fig. 15) have been used for the treatment of several types of cancers such as breast and ovarian cancers, acute non-lymphocytic leukemia in adults, and acute lymphocytic leukemia in adults and children.\textsuperscript{134} Anthracyclins impose their cell-killing ability by intercalating and wedging between the DNA bases, thus blocking DNA synthesis and transcription. They also inhibit the activity of topoisomerase II, leading to breaks in genomic DNA.\textsuperscript{135} However, these anticancer agents possess serious side-effect toxicity, especially cardiotoxicity.

Although mitoxantrone (Fig.15), an anticancer drug structurally similar to doxorubicin, proved to have an improved tolerance profile compared to daunorubicin and doxorubicin, it is still not devoid of serious side-effects that are associated with myelosuppression and cardiotoxicity. Moreover, cell histotypes that developed resistance to doxorubicin due to the over-expression of Pgp also showed resistance to mitoxantrone.\textsuperscript{136} The biochemical basis for cardiotoxicity is not fully understood, but it is thought that the \textit{in vivo} reduction of the quinone moiety plays an important role. The addition of an electron to the quinone leads to the formation of a semi-quinone free radical which, in turn, transfers an electron to molecular oxygen to generate superoxide radical anions. The so-formed radical anions lead to hydroxyl radicals which can damage cardiac tissue.
Several studies have been published discussing the structural modification of mitoxantrone in an attempt to reduce/eliminate its adverse side effects. These studies led to the discovery of the 9-aza-anthrapyrazole analogues whose general structure is shown in Fig. 16.

These anthrapyrazole analogues were designed to have increased antitumor activity and reduced cardiotoxicity, since they were expected to be less prone to undergo bioreduction which might be responsible for their cardiotoxic potential. The presence of two amine side chains in the proposed anthrapyrazoles would enhance their DNA binding affinity and cytotoxicity, which is believed to be achieved through topoisomerase inhibition.
However, the selective targeting of the drugs to cancer cells will be an important issue since these analogues have the capability to intercalate with the DNA of normal cells, as well as with cancer cells.\textsuperscript{138}

One approach to achieve the selective targeting is to mask the anthrapyrazole analogues and then demask them at the cancer site.\textsuperscript{136,139} In line with such a proposal, we have synthesized several 9-aza-anthrapyrazole-N-oxide analogues as “bio-reductive-dependent agents and as potential hypoxia-selective cytotoxins for cancer therapy.”\textsuperscript{15} The lower pH and oxygen-deprived (reductive) nature of hypoxic cancer cells may be able to reduce these inactive prodrugs to their active amine forms. Cellular reduction of the N-oxide functionalities will then result in “bis-armed” anthrapyrazole amines \textsuperscript{93} that regain DNA binding affinity and cytotoxicity. Such anthrapyrazole moieties are unique DNA – complexing agents that exhibit reduced or no cardiotoxicity due to their diminished tendency to form semiquinone free radicals.\textsuperscript{140}

\textbf{5.2. Chemistry Results and Discussion}

The synthesis of the anthrapyrazole bis-N-oxide analogues started by preparing the substituted hydrazine \textsuperscript{89} that is essential for constructing the pyrazole ring via the reaction of hydrazine monohydrate \textsuperscript{87} and dimethylaminoethyl chloride \textsuperscript{88} under basic conditions (Scheme 28).\textsuperscript{141} The regioselective construction of the pyrazole ring with an N-substituent can be achieved by treating anthraquinone \textsuperscript{90} with hydrazine \textsuperscript{89}.\textsuperscript{137} Two aspects should be highlighted in this respect. First, based on previous SAR studies where regioisomers bearing nitrogen atoms at positions 7, 8, 9, or 10 of the anthrapyrazole chromophore have been prepared, the most active 9-aza-regioisomer has been selected
for this synthesis. Second, the regioselective construction of the pyrazole ring was induced by choosing the fluorine and chlorine atoms as substituents at C-6 and C-9 of anthraquinone 90, respectively, due to the difference in their electrophilicity during S\textsubscript{N}Ar displacements.

Scheme 28. Synthesis of the 9-aza-anthrapyrazole-N-oxide analogues. (a) K\textsubscript{2}CO\textsubscript{3}, reflux, then NaOH, 62%; (b) DIPEA, THF, rt, 75%; (c) [2-(dimethylamino)-ethyl]amine, anhydrous pyridine, 90°C, 60%; (d) 2.5 eq. 94, CH\textsubscript{2}Cl\textsubscript{2}:MeOH (3:1), 0-5°C, then HCl (g), 87%; (e) 1 eq. 94, CH\textsubscript{2}Cl\textsubscript{2}:MeOH (3:1), 0-5°C, 49%.
Thus, the most nucleophilic nitrogen of the hydrazine adduct selectively displaced the C-9 fluorine atom yielding intermediate 91 which under the reaction conditions underwent spontaneous cyclization to yield anthrapyrazole 92. Refluxing 92 with N,N-dimethylamino-ethylamine in anhydrous pyridine under a nitrogen atmosphere displaced the C-5 chlorine atom and resulted in the bis-armed anthrapyrazole diamine 93. Oxidation of anthra-pyrazole 93 using 2.5 equivalents of the mild oxidizing oxaziridine 94 resulted in anthrapyrazole bis-N-oxide 95. Oxidizing 93 with 1 equivalent of oxaziridine 94 afforded the anthrapyrazole mono-N-oxide adducts in a 1:1 mixture of 96 and 97 as determined by NMR where eight distinct peaks corresponding to the different eight methylene groups in 96 and 97 were revealed, as compared to only four peaks in anthrapyrazole bis-N-oxide analogue 95.

The oxaziridine mild oxidizing agent 94 was synthesized as summarized in Scheme 29. Condensing sulfonamide 98 with diethyl aldehyde diacetal 99 resulted in imine 100, with the release of ethanol, which when oxidized using MCPBA yielded the corresponding oxaziridine 94 (Scheme 30).

![Scheme 29](image)

Scheme 29. Synthesis of oxaziridine 94. Reagents and conditions: (a) 150-160°C; 99%; (b) NaHCO₃, 0.11 eq. benzyltriethyl-ammonium chloride (BTEAC), 0-5°C, then MCPBA, 90%.

5.3. Biology Results and Discussion

The biological data that is presented and discussed below was conducted by Dr. M. Hacker and corresponds to the free amine form 93, which is referred to as CD3-1, and
the bis-N-oxide analogue 95 that is referred to as CD3-1N. The first piece of data was collected from topoisomerase II inhibition experiments designed to study the ability of CD3-1 and CD3-1N to inhibit the activity of topoisomerase II. The results are summarized in the Southern plot given in Fig. 19. Portion 1 of the gel corresponds to the DNA which gives one band in the Southern plot. Addition of topoisomerase II sequenced DNA resulted in multiple bands on the gel (Portion 2). Different concentrations of CD3-1 were mixed with DNA and topoisomerase II and the mixtures were applied on the gel as shown in portions 3, 4, and 5. One band was observed at all three concentrations indicating that no sequencing of the DNA by topoisomerase II occurred, whose activity was apparently inhibited by CD3-1.

Figure 17. Southern plot obtained from the topoisomerase II inhibition assay.
Alternatively, mixing different concentration of CD3-1N with DNA and topoisomerase II resulted in multiple bands on the gel as given is portions 6, 7, and 8. This observation led to the conclusion that CD3-1N did not inhibit the activity of topoisomerase II that was able to sequence DNA. Thus, the topoisomerase II inhibition assay gave evidence that the prodrug form anthrapyrazole bis-N-oxide CD3-1N did not inhibit topoisomerase II while the free-amine (drug) version CD3-1 did.

The interaction between DNA and CD3-1 and CD3-1N was studied using an ethidium bromide displacement assay. In this assay, the ability of CD3-1 and CD3-1N to displace ethidium bromide, which is known to interact with DNA, is determined. The results are summarized in Table 5. DNA was mixed with ethidium bromide and the relative fluorescence was measured. CD3-1 or CD3-1N was then added, and the drug concentration that caused 50% reduction in fluorescence was determined. Mitoxantrone, which was used as a control, caused 50% reduction in fluorescence at a concentration of 4.5 µM. Interestingly, 50% reduction in fluorescence was obtained at CD3-1 concentration of 6.8 µM (Fig. 20), whereas no reduction in fluorescence was observed when CD3-1N was added. Such data provided evidence that CD3-1 was able to interact with DNA as highlighted in its ability to displace ethidium bromide while CD3-1N did not, a profile that is again typical to the designed prodrug approach.
Table 5. Summary of the results collected from the Ethidium bromide displacement assay.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone</td>
<td>4.5</td>
</tr>
<tr>
<td>CD3-1</td>
<td>6.8</td>
</tr>
<tr>
<td>CD3-1N</td>
<td>No displacement</td>
</tr>
</tbody>
</table>

The cytotoxicity of CD3-1 and CD3-1N was measured using an MTT assay. The IC$_{50}$ values were measured under oxic (oxygen rich) and hypoxic (oxygen deficient) conditions using three different cancer cell lines: LoVo that is a colon cancer cell line, MCF-7/S which is a breast cancer cell line sensitive to doxorubicin, and MCF-7/Dox which is a breast cancer cell line that is resistant to doxorubicin. Mitoxatrone was used as a control. Cells were incubated in 96 well plates (5,000 cells/ well) in a CO$_2$ incubator (37°C) for 96 hours with varying concentrations of the test drug. Cytotoxicity was determined by MTT assay where viable cells converted colorless solution to blue. The more dense the blue color, the greater the number of viable cells. The anoxic medium was created by incubating the cells in an anaerobic chamber flushed with 95% N$_2$ and 5%
CO₂ at 37°C for 96 hours, and cytotoxicity was determined by MTT assay. In the LoVo and MCF-7/S cell lines, **CD3-1** showed comparable activity under oxic and hypoxic conditions as shown in Table 6. However, **CD3-1N** showed profound activity under hypoxic conditions as compared to the oxic conditions (e.g. MCF-7/S: IC₅₀ (oxic) > 25 µM; IC₅₀ (hypoxic) = 5.0 µM). A very promising result was obtained when **CD3-1** and **CD3-1N** were tested against the doxorubicin-resistant cell line MCF-7/Dox. Both compounds were more cytotoxic than Mitoxantrone under hypoxic conditions indicating that such agents were able to avoid interaction with the Pgp transporter system which is believed to be the main cause of resistance.

Table 6. IC₅₀ values collected from the MTT cytotoxicity assay.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>IC₅₀ (µM) (Oxic)</th>
<th>IC₅₀ (µM) (Anoxic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo</td>
<td>Mitoxantrone</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>CD3-1</td>
<td>1.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>CD3-1N</td>
<td>&gt;25</td>
<td>1.5</td>
</tr>
<tr>
<td>MCF-7/S</td>
<td>Mitoxantrone</td>
<td>0.08</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>CD3-1</td>
<td>0.95</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CD3-1N</td>
<td>&gt;25</td>
<td>5.0</td>
</tr>
<tr>
<td>MCF-7/Dox</td>
<td>Mitoxantrone</td>
<td>4.7</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>CD3-1</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>CD3-1N</td>
<td>&gt;25</td>
<td>1.3</td>
</tr>
</tbody>
</table>
The ability of the cytochrome P450 system (CYP) to reduce under hypoxic conditions was also examined. The test agent was incubated with the S-9 fraction (drug metabolizing fraction) of a rat liver in normal air or in 1% O\textsubscript{2} for 2 hours at 37°C. Following the precipitation of the protein by ethanol, the samples were centrifuged and the supernatant was collected and tested for DNA binding, topoisomerase II activity, and cytotoxicity. The results summarized in Table 7 showed that the liver metabolism can activate CD3-1N under hypoxic conditions.

<table>
<thead>
<tr>
<th>Activity</th>
<th>+ O\textsubscript{2}</th>
<th>- O\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Binding</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>Topo II</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>None</td>
<td>Yes</td>
</tr>
</tbody>
</table>

In summary, anthrapyrazole N-oxide analogues 95, 96, and 97 have been synthesized. The bis-N-oxide adduct was prepared using 2.5 equivalents of the oxidizing agent, while the mono-N-oxide moiety was afforded using 1 equivalent of the oxidizing agent. Cytotoxicity assays indicated that CD3-1N was highly active under hypoxic conditions, but not under normal conditions. While DNA reactivity assays proved that no interaction occurred between the anthrapyrazole N-oxide CD3-1N and DNA, considerable interaction was observed between the demasked tertiary amine CD3-1 and DNA. Finally, the ability of the CYP system to cause reductive metabolism of CD3-1N under hypoxic conditions has been validated.
Chapter 6

Summary and Future Work

Towards identifying general criteria that makes anticancer drugs less susceptible to MDR, several PAC analogs bearing different functional groups at the 7- or 10-positions were synthesized, and the effect of such chemical modifications on the interaction between PAC and P-gp was evaluated. Our hypothesis that acidic groups reduce MDR liability tested positive in MDR assays, although yet to be confirmed through the biological evaluation of several PAC analogs modified with aliphatic acid or alcohol moieties. The preliminary data collected from the MDR liability assays directed us towards designing PAC analogues for the selective targeting of cancer cells, namely prostate and breast cancer cells. Address systems incorporating acidic functionalities in their structure were utilized as the drug-directing moieties. This formulation has a dual advantage where the interaction between the address system [$\gamma$-linked glutamic acid (prostate cancer) and RGD peptides (breast cancer)] and the receptor over-expressed on cancer cells [PSMA (prostate cancer) and integrins (breast cancer)] will localize the drug at the tumor site accompanied with a reduction in MDR liability. The RGD address system was also exploited in designing targeted ultrasound contrast agents for breast cancer diagnosis. The theme of selective targeting of cancer cells was employed in synthesizing the lead compound CD3-246 as a PAM inhibitor, and the anthrapyrazoleN-oxide conjugates as prodrugs for hypoxia cancer cells.
Future work involves assessing the biological activity of the different synthesized conjugates as summarized below:

1. Evaluate the 7-aliphatic acid-PAC, 10-aliphatic acid-PAC, and 7-Ser-PAC analogues in cytotoxicity, MDR liability, and selectivity assays;
2. Study the binding affinity of the PSMA to the $\gamma$-linked glutamic acid residues utilizing the R6G-$\gamma$-linked glutamic acid conjugates;
3. Measure the cytotoxicity of the $\gamma$-linked glutamic acid-PAC analogues in growth inhibition assays;
4. Study the binding affinity of the RGD tripeptide to the $\alpha_v\beta_3$ integrin receptor using the R6G-RGD conjugates; and,
5. Evaluate the utility of the RGD-perfluorocarbon adducts as ultrasound contrast agents.
Chapter 7

Experimental Section

Amino acid building blocks were purchased from EMD Biosciences. All other reagents and solvents were obtained from Sigma-Aldrich Chemical Co. and from Fisher Scientific, respectively. Thin-layer chromatography (TLC) was conducted on 250 µ fluorescent plates acquired from VWR and visualized by using UV light, iodine vapor or ninhydrin stain. Normal phase flash column chromatography was performed using silica gel (200-425 mesh 60 Å pore size) and ACS grade solvents. Hydrogen gas was used as received from AirGas, Co. $^1$H-NMR, $^{13}$C-NMR, and $^{19}$F-NMR spectra were recorded on a Varian 200-MHz, 400-MHz, or 600 MHz Fourier transform spectrometer in D$_2$O, CDCl$_3$, CD$_2$Cl$_2$, CD$_3$OD, CD$_3$COCD$_3$, or DMSO (Cambridge Isotope Laboratories, Inc.). Chemical shifts are reported in ppm (δ) and spin multiplicities are described as s (singlet), d (doublet), dd (doublet of doublet), dq (doublet of quartet), t (triplet), br (broad), and m (multiplet). Melting points were determined in open capillaries using an Electro thermal digital melting point apparatus and are uncorrected. Drying of samples was accomplished on high vacuum pumps (Welch) or a lyophilization instrument (Virtis, Inc). Mass spectra were collected on an Esquire-LC instrument equipped with an Electron Spray Ionization (ESI) source. Elemental analyses were performed by Atlantic MicroLab., Norcross, GA. The ultrasound images were taken by a General Electric Logiq Book XP portable ultrasound device with 8L-KS linear probe purchased by the CD3.
10-Deacetylpaclitaxel (DAP) (3)

PAC 1 (150 mg, 0.175 mmol) was dissolved in THF (20 ml) and 30% hydrogen peroxide (10 ml) was added. After stirring the reaction mixture for 30 min, a saturated NaHCO₃/H₂O₂ solution (6 ml) was added. The reaction mixture was allowed to stir at room temperature for 6 hours after which it was diluted by brine solution (100 ml). The product was extracted using DCM (6 x 50 ml). The organic layer was dried over anhydrous sodium sulfate, and DCM was then evaporated under vacuum. The resulting product was purified by column chromatography using DCM: MeOH (20: 1). 0.12 g of 3 were collected as a white solid (85%). The unreacted 1 was also recovered. Mp: 197-199°C. ¹H NMR (CD₂Cl₂, 100 MHz) δ: 8.13 (d, 2H), 7.75 (d, 2H), 7.63(t, 1H), 7.45 (m, 11H), 7.14 (d, 1H), 6.19 (t, 1H), 5.77 (d, 1H), 5.64 (d, 1H), 5.18 (s, 1H), 4.93 (d, 1H), 4.79 (s, 1H), 4.23 (d, 1H), 4.21 (m, 3H), 3.88 (d, 1H), 3.76 (d, 1H), 2.55 (m, 1H), 2.35 (s, 3H), 2.26 (m, 2H), 1.88 (s, 2H), 1.81 (m, 4H), 1.78 (m, 1H), 1.18 (s, 3H), 1.09 (s, 3H).

¹³C NMR (CD₂Cl₂, 100 MHz) δ: 9.93, 14.37, 20.72, 22.72, 26.61, 36.10, 37.05, 43.25, 46.75, 55.50, 57.87, 72.14, 72.51, 73.62, 74.69, 75.09, 76.73, 78.86, 81.29, 84.35, 127.27, 127.29, 128.33, 128.83, 128.92, 129.05, 129.61, 130.34, 132.08, 133.87, 134.06, 138.43, 138.54, 166.97, 167.49, 170.82, 172.87, 211.44. Anal. Cald for C₄₅H₄₉NO₁₃. 0.75 H₂O: C, 65.48, H, 6.17, N, 1.70. Found C, 65.29, H, 6.16, N, 1.82.

2’,7-Dichloroacetyl-10-[N-α-carbobenzyloxy-L-Aspartic Acid, α-Benzyl Ester] paclitaxel (15)

To a solution of 3 (0.391 g, 0.484 mmol) dissolved in DMF (2 ml) and DCM (20 ml) was added DMAP (20 mg, 0.16 mmol). 2.3 equivalents of chloroacetic anhydride (0.19 g,
1.113 mmol) were added in one portion. The reaction mixture was stirred at room temperature for 15-20 minutes after which it was diluted by 50 ml of DCM. The reaction mixture was washed successively by a saturated NaHCO₃ aqueous solution (20 ml) and water (20 ml). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was then evaporated under vacuum and an oily residue was obtained. The latter was purified by column chromatography (DCM:MeOH, 20:1) and the desired product 4 was collected as a white solid.

Cbz-Asp-OBn (22 mg, 0.062 mmol) was dissolved in DCM (5 ml) and DIC (12 mg, 0.093 mmol) was added. The reaction mixture was stirred at room temperature for 30 minutes. 4 (30 mg, 0.031 mmol) and DMAP (5mg, 0.041 mmol) were then added and the reaction stirred at room temperature for 18 hours. DCM (20 ml) was added and the reaction mixture was washed with 2% aqueous HCl solution (10 ml) and brine (10 ml). The organic layer was collected and dried over anhydrous sodium sulfate, and the volatiles were evaporated under vacuum. The residue purified on a silica column (DCM:MeOH, 50:1) and 29.5 mg of 15 were collected (73%). \(^1\)H NMR (CDCl₃, 400 MHz) δ: 8.11 (d, 2H), 7.74 (d, 2H), 7.63 (t, 1H), 7.35 (m, 21H), 7.01 (d, 1H), 6.20 (m, 2H), 6.02 (d, 1H), 5.78 (d, 1H), 5.65 (d, 1H), 5.57 (m, 1H), 5.24 (d, 1H), 5.12 (s, 1H), 4.95 (d, 1H), 4.81 (s, 1H), 4.71 (m, 1H), 4.32 (d, 1H), 4.17 (s, 1H), 3.89 (d, 1H), 3.85 (m, 2H), 3.27 (dd, 1H), 2.96 (dd, 1H), 2.59 (m, 1H), 2.39 (s, 3H), 2.32 (d, 1H), 1.83 (s, 3H), 1.80 (s, 3H), 1.25 (s, 3H), 1.10 (s, 3H). MS: Cald M+Na 1327.17. Found M+Na 1325.2.
2'-Carbobenzyloxydeacetylpaclitaxel (17)

To solution of DAP (100 mg, 0.123 mmol) in DCM was added DIPEA (32 mg, 0.246 mmol) and the mixture was cooled to -20°C. A solution of benzyl chloroformate (25.2 mg, 0.148 mmol) in DCM (2 ml) was then added dropwise, and the reaction mixture was stirred at -20°C for 18 hours. The reaction was first diluted by DCM (25 ml) and then quenched by washing with a saturated aqueous NaHCO₃ solution (50 ml). The organic layer was collected and washed with brine (25 ml). Following drying over anhydrous sodium sulfate, DCM was evaporated, and the resulting residue was purified by column chromatography (DCM: MeOH, 50:1) and 107 mg of 17 were collected (92%) yield. Mp: 153-155°C. ¹H NMR (CDCl₃, 400 MHz) δ: 8.13 (d, 2H), 7.72 (d, 2H), 7.63 (m, 1H), 7.32 (m, 15H), 6.92 (d, 1H), 6.23 (t, 1H), 5.97 (d, 1H), 5.70 (d, 1H), 5.45 (s, 1H), 5.14 (m, 3H), 4.96 (d, 1H), 4.69 (d, 1H), 4.32 (d, 1H), 4.21 (m, 3H), 3.95 (d, 1H), 3.47 (d, 1H), 2.58 (m, 1H), 2.45 (s, 3H), 2.36 (m, 1H), 2.16 (m, 1H), 1.94 (s, 3H), 1.85 (m, 1H), 1.76 (s, 3H), 1.22 (s, 3H), 1.12 (s, 3H). Anal. Cald for C₅₃H₆₅NO₁₅·0.25H₂O: C, 66.97, H, 5.89, N, 1.47. Found C, 66.79, H, 5.90, N, 1.50.

2'-Carbobenzyloxy-7-triethylsilyl-deacetylpaclitaxel (18)

2'-Cbz-DAP (50 mg, 0.053 mmol) was dissolved in DCM (10 ml) to which was added DMAP (6.5 mg, 0.053 mmol) and 10 equivalents of triethylsilyl chloride (TESCl) (79.88 mg, 0.53 mmol). The reaction mixture was stirred over overnight at room temperature after which it was washed with a saturated ammonium chloride (NH₄Cl) solution (20 ml) and water (20 ml). The organic layer was collected and dried over anhydrous sodium sulfate. The volatiles were evaporated and the resulting residue was dissolved in DCM (1
ml) to which hexane (20 ml) was added. The resulting white solid was filtered and purified by column chromatography using DCM: MeOH (50:1) as an eluent. 51 mg of the desired product 18 were collected as a white solid (90%). Mp: 145-147°C. 1H NMR (CDCl₃, 600 MHz) δ: 8.11 (d, 2H), 7.71 (d, 2H), 7.58 (t, 1H), 7.46 (m, 3H), 7.36 (m, 16H), 6.88 (d, 1H), 6.26 (t, 1H), 5.97 (d, 1H), 5.65 (d, 1H), 5.44 (d, 1H), 5.45 (d, 1H), 5.10 (m, 4H), 4.91 (d, 1H), 4.36 (t, 1H), 4.31 (d, 1H), 4.25 (s, 1H), 4.18 (m, 2H), 3.86 (d, 1H), 2.43 (s, 3H), 2.34 (m, 1H), 2.14 (m, 1H), 1.94 (s, 3H), 1.73 (s, 3H), 1.59 (s, 1H), 1.19 (s, 3H), 1.09 (s, 3H), 0.92 (t, 9H), 0.53 (m, 6H). Anal. Cald for C₅₉H₆₉NO₁₅Si. 0.5 H₂O. 0.1 CH₂Cl₂: C, 65.80, H, 6.50, N, 1.29. Found C, 65.66, H, 6.16, N, 1.31.

2'-Carbobenzyloxy-10-[N-α-carbobenzyloxy-L-Aspartic Acid, α-Benzyl Ester] paclitaxel (20)

Cbz-Asp-OBn (232.3 mg, 0.651 mmol) was dissolved in DCM (5 ml) and 3 equivalents of DIC (246 mg, 1.953 mmol) were added. The reaction stirred at room temperature for 1 hour. The resulting mixture and catalytic amount of DMAP (5 mg, 0.041 mmol) were then added to 18 (230 mg, 217 mmol). The reaction mixture was stirred at room temperature for 12 hours after which it was washed successively with 5% aqueous HCl solution (5 ml), saturated sodium bicarbonate solution (5 ml) and brine (5 ml). The organic layer was collected, dried over anhydrous sodium sulfate, and the volatiles were then evaporated. The resulting residue was purified by column chromatography (DCM:MeOH, 50:1) and compound 19 was collected as a white solid. 19 was then dissolved in anhydrous pyridine (5 ml) and 12 drops of HF/pyridine were added. The reaction was stirred for 6 hours after which DCM (10 ml) was added and the reaction
mixture was washed with 0.2% aqueous HCl solution (10 ml), saturated ammonium chloride solution (5 ml) and water (5 ml), successively. The organic layer was collected and dried over anhydrous sodium sulfate. The volatiles were evaporated under vacuum and the resulting residue was purified by column chromatography (DCM:MeOH, 50:1) as an eluent. 237 mg of the desired product 20 were obtained as a white solid (85%). Mp: 118-120°C. 1H NMR (CDCl₃, 600 MHz) δ: 8.12 (d, 2H), 7.71 (d, 2H), 7.57 (t, 1H), 7.48 (m, 3H), 7.35 (m, 22H), 6.86 (d, 1H), 6.31 (s, 1H), 6.24 (t, 1H), 6.10 (d, 1H), 5.96 (d, 1H), 5.62 (d, 1H), 5.42 (d, 1H), 5.15 (m, 6H), 4.94 (d, 1H), 4.71 (m, 1H), 4.39 (m, 1H), 4.31 (d, 1H), 4.18 (d, 1H), 4.10 (q, 1H), 3.47 (d, 2H), 3.29 (dd, 1H), 3.08 (dd, 1H), 2.52 (m, 1H), 2.43 (s, 3H), 2.34 (m, 1H), 2.26 (d, 1H), 2.16 (m, 1H), 2.03 (s, 2H), 1.88 (s, 3H), 1.67 (s, 3H), 1.24 (t, 3H), 1.16 (s, 3H), 1.07 (s, 3H). Anal. Cald for C₇₂H₇₂N₂O₂₀ . 3.5 H₂O: C, 64.14, H, 5.91, N, 2.08. Found C, 64.11, H, 5.97, N, 1.92. MS: Cald M+Na 1308.34. Found M+Na 1307.7.

2’-Carbobenzyloxy-7-acetyl-10-[N-α-carbobenzyloxy-L-Aspartic Acid, α-Benzyl Ester] paclitaxel (21)

2’-Cbz-10-Asp(Cbz, OBn)-DAP 20 (100 mg, 0.078 mmol), dissolved in anhydrous DCM (5 ml), was reacted with 5 equivalents of acetic anhydride (39.7 mg, 0.39 mmol) and 5 equivalents of DMAP (48 mg, 0.39 mmol). The reaction mixture was stirred at room temperature for 2 hours after which it was diluted with DCM (10 ml) and washed with saturated aqueous sodium bicarbonate solution (10 ml) and brine (10 ml). The organic layer was collected and dried over anhydrous sodium sulfate. The volatiles were evaporated. The resulting residue was purified by column chromatography
(DCM:MeOH, 50:1) and 102 mg of 21 were obtained (99%). Mp: 130-132°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 8.11 (d, 2H), 7.71 (d, 2H), 7.60 (t, 1H), 7.50 (m, 3H), 7.38 (m, 22H), 6.87 (d, 1H), 6.31 (s, 1H), 6.21 (t, 1H), 6.05 (d, 1H), 5.96 (d, 1H), 5.66 (d, 1H), 5.56 (d, 1H), 5.43 (s, 1H), 5.16 (m, 6H), 4.93 (d, 1H), 4.69 (m, 1H), 4.32 (d, 1H), 4.17 (d, 1H), 3.90 (d, 1H), 3.28 (dd, 1H), 2.95 (dd, 1H), 2.57 (m, 1H), 2.43 (s, 3H), 2.36 (m, 1H), 2.19 (m, 1H), 1.96 (s, 3H), 1.88 (s, 3H), 1.81 (s, 3H), 1.67 (s, 1H), 1.24 (s, 1H), 1.15 (s, 3H), 1.10 (s, 3H). Anal. Cald for C$_{74}$H$_{74}$N$_2$O$_{21}$. 0.75 H$_2$O: C, 66.29, H, 5.68, N, 2.09. Found C, 66.29, H, 5.53, N, 2.14. MS: Cald M+Na 1350.38. Found M+Na 1350.0.

**2’-Chloroacetylpaclitaxel (22)**

PAC 1 (100 mg, 0.117 mmol) and DMAP (29 mg, 0.234 mmol) were dissolved in DCM (20 ml) and the mixture was cooled to -20°C. Chloroacetyl anhydride (13.2 mg, 0.117 mmol) was added, and the reaction mixture was stirred at -20°C for 12 hours. The reaction mixture was then quenched with a saturated aqueous sodium bicarbonate solution (25 ml) and the organic layer was separated and dried over anhydrous sodium sulfate. DCM was evaporated under vacuum, and the resulting residue was purified by column chromatography (DCM:MeOH, 100:1) affording 81 mg of 22 (74%) while recovering the unreacted PAC. Mp: 163-165°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 8.12 (d, 1H), 7.71 (d, 1H), 7.36 (m, 15H), 6.83 (d, 1H), 6.28 (m, 2H), 5.99 (d, 1H), 5.67 (d, 1H), 5.53 (m, 1H), 4.96 (d, 1H), 4.44 (m, 1H), 4.30 (d, 1H), 4.18 (m, 3H), 3.79 (d, 1H), 2.55 (m, 1H), 2.49 (d, 1H), 2.44 (s, 3H), 2.36 (m, 1H), 2.22 (s, 3H), 2.18 (m, 1H), 1.92 (s, 3H), 1.85 (m, 1H), 1.67 (s, 3H), 1.24 (s, 3H), 1.12 (s, 3H). Anal. Cald for C$_{49}$H$_{52}$NClO$_{15}$: C,
2'-Carbobenzyloxypaclitaxel (27)

To a solution of PAC (150 mg, 0.176 mmol) and DIPEA (45.5 mg, 0.352 mmol) in DCM (15 ml) at -20°C was added benzyl chlorofomate (36 mg, 0.211 mmol). The reaction mixture was stirred at -20°C for 18 hours after which it was diluted by DCM (50 ml) and quenched by a saturated aqueous sodium bicarbonate solution (25 ml). The organic layer was collected and dried over anhydrous sodium bicarbonate, and the volatiles were evaporated under vacuum. The resulting residue was purified by column chromatography (DCM: MeOH 25:1) and 170 mg of the desired product 27 were collected as a white solid (98%). Mp: 142-144°C. 1H NMR (CDCl₃, 600MHz): δ 1.13 (s, 3H), 1.23 (s, 3H), 1.67 (s, 3H), 1.91 (s, 3H), 2.18 (m, 1H), 2.22 (s, 3H), 2.37 (m, 1H), 2.43 (s, 3H), 2.54 (m, 1H), 3.79 (d, 1H), 4.18 (d, 1H), 4.30 (d, 1H), 4.43 (m, 1H), 4.96 (d, 1H), 5.12 (m, 2H), 5.43 (m, 1H), 5.67 (d, 1H), 5.96 (d, 1H), 6.28 (m, 2H), 6.89 (d, 1H), 7.36 (m, 20H), 7.71 (d, 1H), 8.12 (d, 1H). Anal. Cald C₅₅H₇₅NO₁₆: C, 66.86, H, 5.81, N, 1.42. Found C, 66.62, H, 5.89, N, 1.41. MS: Calc M+Na 1011.4. Found M+Na 1009.80.

2'-Carbobenzyloxy-7-[N-α-carbobenzyloxy-L-glutamic Acid, α-Benzyl Ester] paclitaxel (28)

To a solution of Cbz-Glu-OBn (45 mg, 0.121 mmol) in DCM (5 ml) was added DIC (18.4 mg, 0.146 mmol) and the solution was stirred for 1 hour. Cbz-PAC 27 (60 mg, 0.0607 mmol) and DMAP (18 mg, 0.146 mmol) were then added and the solution was
stirred at room temperature for 18 hours. The reaction mixture was diluted with DCM (25 ml) and washed with 2% aqueous HCl solution (15 ml) and brine (15 ml). The DCM layer was collected and dried over anhydrous sodium sulfate. DCM was evaporated and the residue was purified on a silica column (EtOAc:hexane, 3:2) and 28 was collected. 28 was dissolved in DCM (1 ml) and precipitated using hexane (30 ml). The precipitate was filtered and dried under vacuum. 68 mg of 28 were collected as a white solid (83%). Mp: 120-123°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 8.10 (d, 1H), 7.71 (d, 2H), 7.60 (t, 1H), 7.48 (m, 3H), 7.36 (m, 24H), 6.88 (d, 1H), 6.23 (t, 1H), 6.13 (s, 1H), 5.66 (d, 1H), 5.57 (m, 1H), 5.43 (d, 1H), 5.13 (m, 1H), 4.93 (d, 1H), 4.31 (m, 2H), 4.15 (d, 1H), 3.91 (d, 1H), 2.43 (s, 3H), 2.36 (m, 8H), 2.00 (m, 3H), 1.95 (s, 3H), 1.78 (s, 3H), 1.24 (s, 1H), 1.19 (s, 3H), 1.13 (s, 3H). MS: Cald M+Na 1363.49. Found M+Na 1363.60.

7-[$\gamma$-L-Glutamic Acid]paclitaxel Acetate (29)

2'-Cbz-7-Glu(Cbz, OBn)-paclitaxel 28 (40 mg, 0.03 mmol) was dissolved in methanol (25 ml) and three drops of acetic acid were added. The solution was cooled to -20°C and 10% Pd/C (10 mg) were added. The mixture was shaken under hydrogen gas (25 psi) for 10 hours. Methanol was evaporated and the residue was washed with hexane (2 x 10 ml) to remove the acetic acid. The residue was dissolved in DCM and 29 was precipitated out of the solution using hexane (50 ml). The white solid was filtered and dried under vacuum, and 28 mg of 29 were collected (92%). Mp: 183-186°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 8.08 (d, 1H), 7.83 (d, 2H), 7.60 (m, 1H), 7.46 (m, 10H), 7.27 (t, 1H), 6.21 (s, 1H), 6.13 (t, 1H), 5.62 (d, 1H), 4.97 (d, 1H), 4.73 (d, 1H), 4.18 (m, 2H), 3.89 (d, 1H), 3.55 (br, s, 1H), 2.47 (s, 4H), 2.35 (s, 3H), 2.19 (m, 2H), 2.15 (m, 3H), 1.99 (m, 2H), 1.86 (s,
2’-Carbobenzyloxy-7-succinylpaclitaxel (30)

Cbz-PAC 27 (45 mg, 0.046 mmol), dissolved in DCM (5 ml), was reacted with succinic anhydride (13.7 mg, 0.137 mmol) in the presence of DMAP (16.7 mg, 0.137 mmol) at room temperature for 10 hours. The reaction mixture was washed with 2% aqueous HCl solution (25 ml). The organic layer was collected and dried over anhydrous sodium sulfate. After removal of solvent, the residue was purified on a silica column (EtOAc:hexane, 2:1) to provide 40 mg of product 30 (80%). Mp: 157-160°C. 1H NMR (CDCl3, 600 MHz) δ: 8.11 (d, 2H). 7.71 (d, 1H); 7.60 (m, 1H); 7.51 (m,3H), 7.37 (m, 12H); 6.91 (d, 1H); 6.21 (m, 2H); 5.95 (d, 1H); 5.67 (d, 1H), 5.60 (m, 1H); 5.43 (s, 1H); 5.11 (m, 2H), 4.94 (d, 1H); 4.31 (d, 1H); 4.17 (d, 1H); 3.92 (d, 1H); 2.60 (m, 5H); 2.43 (s, 3H), 2.36 (m, 1H); 2.14 (s, 3H); 1.97 (s, 3H); 1.79 (s, 3H); 1.20 (s, 1H); 1.14 (s, 3H).

Anal. Cald for C59H61NO19·0.5 H2O: C, 64.59, H, 5.70, N, 1.28. Found C, 64.40, H, 5.72, N, 1.32. MS: Cald M+Na 1111.11. Found M+Na 1110.50.

2’-Carbobenzyloxy-7-glutarylpaclitaxel (31)

2’-Cbz-PAC 27 (50 mg, 0.051 mmol) was dissolved in anhydrous toluene (10 ml), and DMAP (12.2 mg, 0.10 mmol) and glutaric anhydride (23.3 mg, 0.20 mmol) were added. The reaction mixture was heated to 80-90°C and allowed to stirred for 8 hours. Toluene was then evaporated and the residue was dissolved in DCM. The reaction mixture was
washed with saturated NaHCO₃ (15 ml), 3% aqueous HCl solution (15 ml), and brine (20 ml). The organic layer was dried over anhydrous sodium sulfate, and DCM was then evaporated. The resulting residue was purified by column chromatography using DCM:MeOH (25:1) as an eluent. 47 mg of 31 were collected as a white solid (84%). Mp: 118-121°C. ¹H NMR (CDCl₃, 600 MHz) δ: 8.11 (d, 2H), 7.71 (d, 1H), 7.60 (t, 1H), 7.48 (m, 3H), 7.37 (m, 20H), 6.90 (d, 1H), 6.23 (m, 3H), 5.94 (d, 1H), 5.69 (d, 1H), 5.60 (t, 1H), 5.43 (d, 1H), 5.16 (q, 2H), 5.11 (s, 1H), 4.94 (d, 1H), 4.32 (d, 1H), 4.17 (d, 1H), 3.94 (d, 1H), 2.75 (t, 1H), 2.60 (m, 2H), 2.40 (m, 10H), 2.32 (m, 2H), 2.16 (m, 2H), 2.14 (s, 3H), 1.94 (s, 3H), 1.85 (t, 1H), 1.76 (s, 3H), 1.20 (s, 3H), 1.18 (s, 1H), 1.15 (s, 3H). Anal. Cald for C₆₀H₆₃NO₁₉·0.5 H₂O: C, 64.86, H, 5.81, N, 1.26. Found C, 64.69, H, 5.76, N, 1.28. MS: Cald M+Na 1125.14. Found M+Na 1124.40.

7-Succinylpaclitaxel (32)

To a solution of 2’-Cbz-7-succinate-PAC 30 (40 mg, 0.037 mmol) in methanol (50 ml) cooled to ca. -20°C was added 10% Pd/C (4 mg). The mixture was shaken under hydrogen gas for 5 hours after which it was filtered over a celite bed. Methanol was evaporated under vacuum and the resulting residue was dissolved in minimal amount of DCM (1 ml). The addition of hexane (30 ml) to the solution precipitated the product as a white solid. The latter was filtered and 33.5 mg of 32 were collected (95%). Mp: 201-203°C. ¹H NMR (CDCl₃, 600 MHz) δ: 8.10 (d, 2H), 7.75 (d, 1H), 7.60 (m, 1H), 7.37 (m, 10H), 7.05 (m, 1H), 6.15 (m, 2H), 5.78(d, 1H), 5.64 (d, 1H), 5.56 (m, 1H), 4.92 (d, 1H), 4.78 (s, 1H), 4.30 (m, 1H), 4.16 (m, 1H), 3.89 (d, 1H), 2.64 (m, 5H), 2.37 (m, 4H), 2.14
(s, 3H), 1.79 (m, 6H), 1.19 (s, 1H), 1.14 (s, 3H). MS: Cald M+Na 976.98. Found M+Na 976.60.

7-Glutarylpaclitaxel (33)

To a solution of 2’-Cbz-7-glutaryl-PAC 31 (30 mg, 0.027 mmol) in methanol (50ml) at -20°C was added 10% Pd/C (3 mg), and the mixture was shaken under hydrogen (20 psi) for 5 hours after which the TLC showed the disappearance of the starting material. After filtration, 25 mg of 33 were obtained (95%). Mp: 153-155°C. 1H NMR (CDCl₃, 600 MHz) δ: 8.11 (d, 2H), 7.75 (d, 1H), 7.60 (t, 1H), 7.60 (m, 1H), 7.37 (m, 12H), 7.05 (d, 1H), 6.20 (s, 1H), 6.15 (t, 1H), 5.78 (d, 1H), 5.63 (d, 1H), 5.55 (m, 1H), 4.94 (d, 1H), 4.78 (m, 1H), 4.32 (d, 1H), 4.17 (d, 1H), 3.85 (d, 1H), 2.58 (m, 2H), 2.35 (m, 11H), 2.14 (s, 3H), 1.80 (s, 3H), 1.76 (s, 3H), 1.20 (s, 3H), 1.18 (s, 1H), 1.14 (s, 3H). Anal. Cald for C₅₂H₇₅NO₁₇. 0.3 CH₂Cl₂: C, 63.23, H, 5.84, N, 1.41. Found C, 63.01, H, 5.98, N, 1.63.

2’-Carbobenzyloxy-7-triethylsilyl-10-succinyldeacetylpaclitaxel (34)

A mixture of 18 (30 mg, 0.028 mmol), succinic anhydride (11 mg, 0.11 mmol), and DMAP (13 mg, 0.11 mmol) in anhydrous toluene (10 ml) was refluxed for 12 hour. Toluene was evaporated under vacuum, and the residue was dissolved in DCM (25 ml). The solution was washed with 2% HCl aqueous solution (5 ml) and the organic layer was dried over anhydrous sodium sulfate. DCM was evaporated and 28 mg of 34 were collected (85%) after purification by column chromatography (EtOAc:Hexanes 2:1). Mp: 123-126°C. 1H NMR (CDCl₃, 600 MHz) δ: 8.11 (d, 2H), 7.70 (d, 2H), 7.58 (t, 1H), 7.46 (m, 3H), 7.36 (m, 15H), 6.93 (d, 1H), 6.45 (s, 1H), 6.25 (t, 1H), 5.96 (d, 1H), 5.68 (d,
1H), 5.45 (d, 1H), 5.10 (m, 4H), 4.92 (d, 1H), 4.44 (m, 1H), 4.29 (d, 1H), 4.17 (d, 1H), 3.80 (d, 1H), 2.65 (m, 4H), 2.51 (m, 1H), 2.44 (s, 3H), 2.37 (m, 1H), 2.19 (m, 1H), 2.03 (s, 3H), 1.67 (s, 3H), 1.23 (s, 3H), 1.14 (s, 3H), 0.90 (t, 9H), 0.55 (m, 6H). MS: Cald M+Na 1183.34. Found M+Na 1182.60.

7-Triethylsilyl-10-succinyldeacetylpaclitaxel (35)

2’-Cbz-7-TES-10-succinyl-DAP 34 (50 mg, 0.043 mmol) was dissolved in methanol (50 ml) and the solution was cooled to ca. -20°C. 10% Pd/C (5mg) was added, and the mixture was subjected to hydrogenation (20 psi) for 5 hours. The mixture was filtered over a celite bed and the filtrate was collected. Methanol was evaporated under vacuum and the residue was dissolved in DCM (1 ml). Hexane (50 ml) was added causing the precipitation of the desired product 35 as a white solid, and 42 mg of the latter were collected by filtration (95%). Mp: 131-134°C. 1H NMR (CDCl₃, 600 MHz) δ: 8.10 (d, 2H), 7.72 (d, 2H), 7.58 (m, 1H), 7.35 (m, 10H), 7.03 (d, 1H), 7.02 (s, 1H), 6.16 (t, 1H), 5.78 (m, 1H), 5.67 (d, 1H), 4.89 (d, 1H), 4.78 (s, 1H), 4.40 (m, 1H), 4.28 (d, 1H), 4.16 (d, 1H), 3.77 (d, 1H), 3.48 (m, 1H), 2.68 (m, 4H), 2.49 (m, 1H), 2.36(s, 3H), 2.29 (m, 1H), 1.89 (s, 3H), 1.67 (s, 3H), 1.19 (s, 3H), 1.15 (s, 3H), 0.88 (t, 9H), 0.55 (m, 6H). Anal. Cald for C₅₅H₇₇NO₁₆Si. 0.75 H₂O: Cald C, 63.54, H, 6.64, N, 1.35. Found C, 63.33, H, 6.51, N, 1.36. MS: Cald M+Na 1049.2. Found M+Na 1048.2.

10-Succinyl-deacetylpaclitaxel (36)

7-TES-10-succinyl-DAP 35 (15 mg, 0.015 mmol) was dissolved in pydrine (4 ml) and the solution was cooled to 0°C. HF/pyridine (6 drops) was then added and the reaction
mixture was stirred at room temperature for 12 hours. Pyridine was evaporated and the residue was dissolved in DCM. The resulting solution was washed with 5% aqueous HCl solution (2 x 10 ml) and the organic layer was collected and dried over anhydrous sodium sulfate. The volatiles were evaporated and the residue was purified by column chromatography (DCM:MeOH, 25:1) where 10 mg of the desired product 36 were collected (75%). $^1$H NMR (CDCl$_3$, 600 MHz) δ: 8.10 (d, 2H), 7.72 (d, 2H), 7.60 (m, 1H), 7.35 (m, 10H), 7.06 (d, 1H), 6.25 (s, 1H), 6.20 (m, 1H), 5.75 (d, 1H), 5.64 (d, 1H), 4.91 (d, 1H), 4.78 (s, 1H), 4.38 (m, 1H), 4.28 (d, 1H), 4.17 (d, 1H), 3.76 (d, 1H), 2.74 (m, 4H), 2.51 (m, 2H), 2.36 (s, 3H), 2.29 (m, 1H), 1.78 (s, 3H), 1.65 (s, 3H), 1.21 (s, 3H), 1.11 (s, 3H). MS: Calc. M+Na 934.94, Found M+Na 934.50.

2’-Chloroacetyl-7-[N-$\alpha$-tert-butoxy-L-serine, O-Benzyl Ether]paclitaxel (37)

Boc-Ser(OBn)-OH (32 mg, 0.11 mmol) was reacted with DIC (21 mg, 0.17 mmol) in DCM (5 ml) for 1 hour. 2’-CAC-PAC 22 (50 mg, 0.054 mmol) and catalytic amount of DMAP (5 mg, 0.041 mmol) were added, and the mixture was stirred at room temperature for 12 hours. The reaction mixture was treated 2% HCl aqueous solution (20 ml) and brine (20 ml) successively, and the organic layer was dried over anhydrous sodium sulfate. DCM was evaporated, and the resulting residue was purified by column chromatography (EtOAc: hexane, 2:1) to produce 54 mg of the desired product 37 (82%). Mp: 129-131°C. $^1$H NMR (CDCl$_3$, 600 MHz) δ: 8.11 (d, 1H), 7.71 (d, 1H), 7.36 (m, 20H), 6.90 (d, 1H), 6.28 (s, 1H), 6.21 (d, 1H), 5.94 (d, 1H), 5.69 (d, 1H), 5.58 (m, 1H), 5.43 (d, 1H), 5.15 (m, 2H), 4.92 (d, 1H), 4.31 (d, 1H), 4.17 (d, 2H), 3.93 (d, 1H), 3.74 (m, 1H), 2.62 (m, 1H), 2.44 (s, 3H), 2.38 (m, 1H), 2.20 (m, 1H), 2.07 (s, 3H), 1.99 (s,
7-\[N-\alpha\text{-}\text{tert-Butoxy-L-serine, O-Benzyl Ether]\text{paclitaxel (38)}

2’-Chloroacetyl-7-Boc-Ser-OBn-paclitaxel 37 (50 mg, 0.041 mmol) was dissolved in methanol (50 ml) and the mixture was stirred at room temperature for 12 hours. Methanol was then evaporated under vacuum and the residue was dissolved in minimal amount of DCM. The addition of n-hexanes precipitated 38 out of the solution in quantitative yields (47 mg, 100%) where no additional purifications were required. Mp: 141-144°C. \(1^H\) NMR (CDCl₃, 600 MHz) \(\delta\) : 8.11 (d, 1H), 7.72 (d, 1H), 7.52 (t, 1H), 7.36 (m, 21H), 6.88 (d, 1H), 6.27 (s, 1H), 6.21 (m, 1H), 5.98 (dd, 1H), 5.67 (d, 1H), 5.60 (m ,1H), 5.56 (d, 1H), 5.25 (m, 1H), 4.92 (d, 1H), 4.72 (m ,1H), 4.32 (d, 1H), 4.18 (d, 1H), 3.92 (d, 1H), 3.74 (d, 1H), 2.58 (m, 1H), 2.43 (s, 3H), 2.34 (m ,1H), 2.18 (m, 1H), 2.06 (s, 2H), 1.98 (s, 3H), 1.78 (s, 3H), 1.64 (s, 3H), 1.45 (s, 9H), 1.21 (s, 3H), 1.13 (s, 3H). Anal. Cald for C₆₂H₇₀N₂O₁₈·0.5H₂O: C, 63.47; H, 6.14; N, 2.37. Found C, 63.15; H, 5.95; N, 2.34. MS: Cald M+Na 1154.22. Found M+Na 1152.80.

7-[N-\alpha\text{-}\text{tert-Butoxy-L-serine}\text{paclitaxel (39)}

7-Boc-Ser-OBn-PAC 38 (30 mg, 0.027 mmol) was dissolved in methanol and the solution was cooled to -20°C. 10% Pd/C (3 mg) were added, and the heterogeneous mixture was subjected to hydrogenation (25 psi) for 18 hours. The solution was then filtered over celite bed, and the filtrate was evaporated under vacuum. The desired product 39 was dissolved in DCM (1 ml) and precipitated using hexane (50 ml). 24 mg of
7-L-Serine-paclitaxel (40)

7-Ser(Boc)-PAC 39 (10 mg, 0.01 mmol) was treated with a 1:1 DCM:formic acid mixture (4 ml) at room temperature for 12 hours. The volatiles were evaporated under vacuum and the residue was dissolved in minimal amount of DCM. The desired product was precipitated out of the DCM by the addition of n-hexanes (20 ml). The precipitate was filtered and dried under high vacuum. 8 mg of 40 were collected (90%). Mp: 187-190°C (darkens). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$: 8.11 (d, 1H), 7.72 (d, 1H), 7.36 (m, 15H), 6.89 (d, 1H), 6.37 (s, 1H), 6.28 (m, 1H), 5.95 (d, 1H), 5.70 (d, 1H), 5.57 (m, 2H), 5.51 (d, 1H), 4.96 (d, 1H), 4.31 (m, 1H), 4.17 (m, 2H), 3.94 (d, 1H), 3.36 (d, 1H), 2.62 (m, 1H), 2.43 (s, 2H), 2.17 (s, 3H), 2.15 (s, 3H), 2.36 (m, 1H), 2.03 (s, 1H), 1.99 (s, 3H), 1.80 (s, 3H), 1.43 (s, 9H), 1.19 (s, 3H), 1.13 (s, 3H). Anal. Cald for C$_{55}$H$_{64}$N$_2$O$_{18}$. 0.75 H$_2$O: C, 62.64, H, 6.26, N, 2.66. Found C, 62.33, H, 6.25, N, 2.84. MS: Cald M+Na 1154.28. Found M+Na 1152.80.

2'-Carbobenzyloxy-7-[N-$\alpha$-tert-butoxy-L-serine, O-Benzyl Ether]paclitaxel (42)

Boc-Ser(OBn) (30 mg, 0.10 mmol) was dissolved in DCM (15 ml) and preactivated with DIC (19.2 mg, 0.15 mmol) for 1 hour. 2'-Cbz-PAC (50 mg, 0.0506 mmol) and catalytic
amount of DMAP (5 mg, 0.041 mmol) were then added, and the reaction mixture was stirred at room temperature for 10 hours. The reaction mixture was washed with 2% aqueous HCl solution (20 ml) and brine (20 ml). The organic layer was collected and dried over anhydrous sodium sulfate. Following the evaporation of the volatiles, the residue was purified by column chromatography (EtOAC:Hexanes, 2:1) and 54 mg of the desired product were collected (85%). Mp: 128-131°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 8.11 (d, 1H), 7.71 (d, 1H), 7.59 (t, 1H), 7.36 (m, 20H), 6.89 (d, 1H), 6.28 (s, 1H), 6.21 (m, 1H), 5.94 (dd, 1H), 5.67 (d, 1H), 5.64 (m ,1H), 5.43 (d, 1H), 5.15 (m, 4H), 4.92 (d, 1H), 4.49 (m, 2H), 4.31 (d, 1H), 4.17 (d, 1H), 3.93 (d, 1H), 3.72 (m, 1H), 3.60 (m, 1H), 2.58 (m, 1H), 2.41 (s, 3H), 2.37 (m ,1H), 2.18 (m, 1H), 2.07 (s, 2H), 1.99 (s, 3H), 1.45 (s, 9H), 1.19 (s, 3H), 1.14 (s, 3H). Anal. Cald for C$_{70}$H$_{76}$N$_2$O$_{20}$.0.25H$_2$O: C, 66.21, H, 6.07, N, 2.21. Found C, 65.89, H, 6.07, N, 2.29. MS: Cald M+Na 1288.35. Found M+Na 1287.80.

Neutral Rhodamine 6G Aminoethyl Amide (D2)

R6G D1 (0.25 g, 0.52 mmol), dissolved in DCM, was treated with 10 equivalents of ethylene diamine (1.2 g, 5.2 mmol). The reaction mixture was stirred at room temperature for 4 hours after which the volatiles were evaporated under reduced pressure. The residue was purified by column chromatography using DCM:MeOH (10:1). 230 mg of the D2 were collected (95%). Mp: 251-253°C. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$: 7.92 (m, 1H), 7.44 (m, 2H), 7.05 (m, 1H), 6.33 (s, 2H), 6.21 (s, 2H), 3.51 (m, 2H), 3.19 (m, 6H), 2.41 (t, 2H), 1.90 (s, 6H), 1.32 (t, 6H). Anal. Cald for C$_{28}$H$_{32}$N$_4$O$_2$.0.15 H$_2$O. 0.15 CH$_2$Cl$_2$: C, 71.63, H, 6.96, N, 11.87, O 7.29. Found C, 71.83, H, 6.94, N, 11.94, O, 7.67.

Boc-Glu-OBn (1.62 g, 4.82 mmol) was reacted with CDI (0.78 g, 4.82 mmol) in DCM (20 ml) for 30 minutes. Rhodamine dye D2 (2.2 g, 4.82 mmol) was then added. The reaction mixture was allowed to stir at room temperature for 18 hours, after which the volatiles were evaporated, and the resulting residue was purified by column chromatography using EtOAc:hexane (3:1) as an eluent. 3.37 g of the desired product D3 was collected (90%). Mp: 112-114°C. \( ^1\text{H NMR (CDCl}_3, 600 \text{ MHz}) \delta: 7.90 \text{ (m, 1H)}, 7.46 \text{ (m, 2H)}, 7.32 \text{ (m, 5H)}, 7.03 \text{ (m, 1H)}, 6.47 \text{ (s, 1H)}, 6.33 \text{ (d, 2H)}, 6.18 \text{ (d, 2H), 5.52 (d, 1H)}, 5.14 \text{ (m, 2H)}, 4.25 \text{ (m, 1H)}, 3.56 \text{ (bs, 2H)}, 3.20 \text{ (m, 6H)}, 3.00 \text{ (m, 2H)}, 2.06 \text{ (m, 3H)}, 1.87 \text{ (s, 3H)}, 1.85 \text{ (s, 1H)}, 1.41 \text{ (s, 9H)}, 1.29 \text{ (m, 6H).} \) Anal. Cald for C_{45}H_{53}N_{5}O_{7}: C, 69.66, H, 6.88, N, 9.03. Found C, 69.37, H, 6.97, N, 8.79. MS: Cald M+Na 798.93. Found M+Na 798.50


Rhodamine-6G-Glu(Boc,OBn) D3 (2 g, 2.58 mmol) was dissolved in a 1:1 DCM/TFA mixture (40 ml). The reaction mixture was stirred for 12 hours, after which the volatiles were evaporated under vacuum. The resulting residue was dissolved in DCM (100 ml) and washed with a saturated NaHCO\textsubscript{3} solution till all the TFA is neutralized. The organic layer was collected and the volatiles were evaporated. The resulting residue was purified by column chromatography (EtOAc:MeOH, 25:1) to afford 1.65 g of the desired product D4 (95%). Mp: 87-90°C. \( ^1\text{H NMR (CDCl}_3, 600 \text{ MHz}) \delta: 7.90 \text{ (m, 1H)}, 7.46 \text{ (m, 2H)},

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R6G-Glu D4 (200 mg, 0.3 mmol) was dissolved in MeOH, and 6 drops of AcOH were added. The reaction mixture was cooled to -20°C, after which 10% Pd/C (20 mg) was added. The heterogeneous mixture was subjected to hydrogenation (H₂, 20 psi) for 12 hours, after which it was filtered through a celite bed. The volatiles were evaporated, and the resulting residue was washed with hexane (3 x 25ml). 0.14 g of D5 were collected as a red solid (75%). Mp: 177-179°C. ¹H NMR (CD₃OD, 600 MHz) δ: 7.86 (m, 1H), 7.52 (m, 1H), 7.00 (m, 1H), 6.33 (s, 2H), 6.13 (s, 2H), 3.49 (m, 1H), 3.32 (s, 1H), 3.17 (m, 6H), 2.94 (m, 2H), 2.21 (m, 2H), 1.99 (m, 4H), 1.87 (m, 6H), 1.29 (m, 6H). Anal. Calcd for C₃₃H₃₉N₅O₅·AcOH: C, 65.10, H, 6.71, N, 10.85. Found C, 65.06, H, 7.05, N, 10.90. MS: Calcd M+Na 608.96. Found M+Na 608.40.


Boc-Glu-OBn (1.12 g, 3.55 mmol) was dissolved in anhydrous DCM (20 ml) and DMF (2 ml), to which PyBOP (1.9 g, 3.55 mmol) and HOBt (0.5 g, 3.55 mmol) were added, followed by 2 equivalents of DIPEA (0.9 g, 7.1 mmol). The reaction mixture was stirred at room temperature for 1 hour, after which Rhodamine 6G-Glu D4
(2 g, 2.96 mmol) was added. The resulting reaction mixture was stirred for 12 hours, after which it was washed with water (20 ml x 2). The organic layer was collected, and the volatiles were evaporated. The resulting residue was purified by column chromatography using EtOAc as an eluent. 2.35 g of the desired product D6 were collected (80%). Mp: 99-101°C. ¹H NMR (CDCl₃, 400 MHz) δ: 7.90 (m, 1H), 7.45 (m, 2H), 7.32 (m, 10H), 7.04 (m, 1H), 6.79 (m, 1H), 6.33 (s, 2H), 6.19 (s, 2H), 5.47 (d, 1H), 5.14 (m, 4H), 4.46 (m, 1H), 4.31 (m, 1H), 3.61 (m, br, 2H), 3.20, (m, 6H), 2.94 (d, 2H), 2.30 (m, 2H), 2.11 (m, 6H), 1.95 (m, 6H), 1.40 (s, 9H), 1.29 (m, 6H). Anal. Cald for C₅₇H₆₆N₆O₁₀: C, 68.79, H, 6.68, N, 8.44. Found C, 68.52, H, 6.78, N, 8.33.


A solution of D6 (2 g, 2 mmol) in a 1 : 1 DCM/TFA mixture (40 ml) was stirred at room temperature for 12 hours, after which the volatiles were evaporated under vacuum. The resulting residue was dissolved in DCM (100 ml) and washed with a saturated NaHCO₃ solution till all the TFA is neutralized. The organic layer was collected, the volatiles were evaporated, and the resulting residue was purified by column chromatography using EtOAc:MeOH (25:2) as an eluent. 1.62 g of D7 were collected (90%). Mp: 87-89°C. ¹H NMR (CDCl₃, 400 MHz) δ: 7.90 (m, 1H), 7.45 (m, 2H), 7.32 (m, 10H), 7.04 (m, 1H), 6.84 (m, 1H), 6.33 (s, 2H), 6.19 (s, 2H), 5.14 (m, 4H), 4.48 (m, 1H), 4.31 (m, 1H), 3.66 (m, br, 2H), 3.20, (m, 6H), 2.93 (d, 2H), 2.39 (m, 2H), 2.14 (m, 6H), 1.95 (m, 6H), 1.29 (m, 6H). Anal. Cald for C₅₂H₅₈N₆O₈ . 0.75 H₂O: C, 68.74, H, 6.60, N, 9.25. Found C, 68.60, H, 6.78, N, 9.16.

D7 (300 mg, 0.34 mmol) was dissolved in MeOH, and 6 drops of AcOH were added. The reaction mixture was cooled to -20°C, and 10% Pd/C (30 mg) was added. The heterogeneous mixture was subjected to hydrogenation (H2, 20 psi) for 18 hours, after which it was filtered through a celite bed. The volatiles were evaporated, and the resulting residue was washed with hexane (3 x 25 ml). 0.21 g of D8 were collected as a red solid (78%). Mp: 146-149°C. ¹H NMR (CD3OD, 600 MHz) δ: 7.86 (m, 1H), 7.49 (m, 2H), 6.99 (m, 1H), 6.32 (s, 2H), 6.13 (s, 2H), 4.26 (m, 1H), 3.68 (m, 1H), 3.29 (m, 2H), 3.18 (m, 6H), 2.94 (m, 2H), 2.48 (m, 2H), 2.12 (m, 6H), 1.99 (m, 3H), 1.87 (m, 6H), 1.25 (m, 6H). Anal. Cald for C38H46N6O8 · AcOH · 0.25 H2O: C, 61.65, H, 6.53, N, 10.78. Found C, 61.44, H, 6.72, N, 10.49. MS: Cald M+H 715.81. Found M+H 715.80.


To a solution of Boc-Glu-OBn (0.38 g, 1.11 mmol) in anhydrous DCM (15 ml) and DMF (2 ml), was added PyBOP (0.58 g, 1.11 mmol) and HOBT (0.15 g, 1.11 mmol), followed by 2 equivalents of DIPEA (0.28 g, 2.22 mmol). The reaction mixture was stirred at room temperature for 1 hour, after which R6G-Glu-Glu D7 (1 g, 1.11 mmol) was added. The resulting reaction mixture was stirred for 12 hours, after which it was washed with water (2 x 20 ml). The organic layer was collected and dried over anhydrous sodium sulfate, and the volatiles were evaporated. The resulting residue was
purified by column chromatography using EtOAc as an eluent. 1.2 g of the desired product **D9** were collected (88%). Mp: 100-103°C. $^1$H NMR (CDCl$_3$, 400 MHz) δ: 7.90 (m, 1H), 7.55 (d, 1H), 7.45 (m, 2H), 7.34 (m, 15H), 7.04 (m, 1H), 6.92 (d, 1H), 6.84 (m, 1H), 6.33 (s, 2H), 6.19 (s, 2H), 5.53 (d, 1H), 5.16 (m, 6H), 4.52 (m, 1H), 4.45 (m, 1H), 4.32 (m, 1H), 3.60 (m, br, 2H), 3.21 (m, 6H), 2.93 (d, 2H), 2.22 (m, 12H), 1.86 (m, 6H), 1.42 (s, 9H), 1.28 (m, 6H). Anal. Cald for C$_{69}$H$_{79}$N$_7$O$_{13}$: C, 67.31, H, 6.48, N, 7.93. Found C, 67.33, H, 6.39, N, 8.00. MS: Cald M+Na 1237.40. Found M+Na 1236.70.


R6G-Glu-Glu-Glu **D9** (1 g, 0.82 mmol) was dissolved in a 1:1 DCM/TFA mixture (40 ml). The reaction mixture was stirred for 10 hours, after which the volatiles were evaporated under vacuum. The resulting residue was dissolved in DCM (100 ml) and washed with a saturated NaHCO$_3$ solution till all the TFA is neutralized. The organic layer was collected, the volatiles were evaporated. Purifying the resulting residue on a silica column (EtOAc:MeOH, 25:2) afforded 0.84 g of **D10** (92%). Mp: 107-110°C. $^1$H NMR (CDCl$_3$, 600 MHz) δ: 7.88 (m, 1H), 7.66 (d, 1H), 7.45 (m, 2H), 7.32 (m, 15H), 7.16 (m, 1H), 7.03 (m, 1H), 6.87 (m, 1H), 6.32 (s, 2H), 6.17 (s, 2H), 5.12 (m, 6H), 4.51 (m, 1H), 4.42 (m, 1H), 3.59 (m, br, 2H), 3.51 (m, 1H), 3.21 (m, 6H), 2.90 (s, 2H), 2.22 (m, 12H), 1.85 (m, 6H), 1.28 (m, 6H). Anal. Cald for C$_{64}$H$_{71}$N$_7$O$_{14}$·0.5 H$_2$O: C, 68.43, H,

Three drops of acetic acid were added to a solution of D10 (48 mg, 0.043 mmol) in MeOH (50 ml). The reaction mixture was cooled to -20\(^\circ\)C, after which 10\% Pd/C (15 mg) was added. The mixture was shaken under hydrogen gas (20 psi) for 10 hours, after which it was filtered through a celite bed. The filtrate was collected and the volatiles were evaporated. The resulting residue was washed with hexane (3 x 15 ml). 30 mg of the desired product D11 were collected as a red solid (76\%). Mp: 186-189\(^\circ\)C. \(^1\)H NMR (CD\(_3\)OD, 600 MHz) \(\delta\): 7.86 (m, 1H), 7.52 (m, 1H), 7.00 (m, 1H), 6.34 (m, 2H), 6.14 (m, 2H), 4.36 (m, 1H), 4.28 (m, 1H), 3.69 (m, 1H), 3.20 (m, 6H), 2.96 (m, 2H), 2.50 (m, 2H), 2.34 (m, 2H), 2.08 (m, 7H), 1.89 (m, 6H), 1.28 (m, 6H). Anal. Cald for C\(_{45}\)H\(_{57}\)N\(_7\)O\(_{13}\).AcOH. 0.5 CH\(_2\)Cl\(_2\). H\(_2\)O: C, 56.66, H, 6.27, N, 10.17. Found C, 56.84, H, 6.15, N, 10.33.

L-Glutamic acid, \(\alpha\)-Benzyl Ester Hydrochloride (44)

Boc-Glu-OBn 43a (450 mg, 1.35 mmol) was dissolved in EtOAc (25 ml) and the solution was cooled to 0\(^\circ\)C. The solution was saturated with HCl (g) for 5 minutes after which it was stirred for 1 hour. The volatiles were evaporated and 0.37 g of the desired product were collected as a white solid after triturating with hexane (100\%). Mp: 125-127\(^\circ\)C. \(^1\)H NMR (D\(_2\)O, 400 MHz) \(\delta\): 7.25 (m, 5H), 5.10 (s, 2H), 4.03 (t, 1H), 2.31 (m, 2H), 2.01 (m, 2H). Anal. Cald for C\(_{12}\)H\(_{15}\)NO\(_4\). HCl. 0.25 EtOAc: C, 52.80, H, 6.13, N, 4.74. Found C, 53.13, H, 5.93, N, 5.00.
N-α-tert-Butoxycarbonyl-L-glutamic Acid, α-Benzyl Ester γ-[L-Glutamic Acid, α-Benzyl Ester]amide (45a)

Boc-Glu-OBn 43a (500 mg, 1.48 mmol) was dissolved in DCM (20 ml) and CDI (240 mg, 1.48 mmol) was added. The solution was stirred at room temperature for 30 minutes after which 44 (400 mg, 1.48 mmol), dissolved in DCM (10 ml) and treated with DIPEA (200 mg, 1.48 mmol), was added. The reaction mixture was stirred at room temperature for 6 hours after which it was washed with water (20 ml). The organic layer was collected and dried over anhydrous sodium sulfate, and the volatiles were evaporated under vacuum. The resulting residue was purified by column chromatography (EtOAc: Hexane, 3:2). 0.68 g of 45a were collected as a white solid (82%). Mp: 92-94°C. \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\): 7.34 (m, 10H), 6.46 (d, 1H), 5.16 (m, 4H), 4.63 (m, 2H), 4.33 (m, 2H), 3.63 (s, 1H), 2.20 (m, 8H), 1.42 (s, 9H). Anal. Cald for C\(_{29}\)H\(_{36}\)N\(_2\)O\(_9\). 0.25 H\(_2\)O: C, 62.18, H, 6.55, N, 5.00. Found C, 61.92, H, 6.65, N, 5.35. MS: Cald M+Na 579.60. Found M+Na 579.40.

N-α-Carbobenzyloxy-L-glutamic Acid, α-Benzyl Ester γ-[L-Glutamic Acid, α-Benzyl ester]amide (45b)

To a solution of Boc-Glu-OBn 43b (500 mg, 1.35 mmol) in DCM (20 ml) was added CDI (220 mg, 1.35 mmol). The solution was stirred at room temperature for 30 minutes after which 44 (370 mg, 1.35 mmol), dissolved in DCM (10 ml) and treated with DIPEA (200 mg, 1.48 mmol), was added. The reaction mixture was stirred at room temperature for 10 hours after which it was washed with water (30 ml). The organic layer was collected and dried over anhydrous sodium sulfate. The volatiles were evaporated under
vacuum and the resulting residue was purified by column chromatography (EtOAc:hexane, 3:2) where 0.64 g of 45b were collected as a white solid (80%). Mp: 124-126°C. ¹H NMR (CDCl₃, 400 MHz) δ: 7.33 (m, 15H), 6.38 (d, 1H), 5.68 (d, 1H), 5.09 (m, 6H), 4.65 (m, 1H), 4.38 (m, 1H), 2.36 (t, 2H), 2.24 (t, 2H), 1.87 (m, 4H). Anal. Cald for C₃₂H₃₄N₂O₉. 0.1 H₂O: C, 64.88, H, 5.82, N, 4.73. Found C, 64.56, H, 5.80, N, 4.84. MS: Cald M+Na 613.62. Found M+Na 613.30.

N-α-tert-butoxycarbonyl-L-Glutamic Acid, α-Benzyl Ester γ-[[L-Glutamic Acid, α-Benzyl Ester γ-[L-Glutamic Acid, α-Benzyl Ester]amide]amide] (46a)

45a (622 mg, 1.12 mmol) was dissolved in DCM (15 ml) and activated with CDI (180 mg, 1.12 mmol) at room temperature for 30 minutes. 44 (306 mg, 1.12 mmol), dissolved in DCM (5 ml) and freed by DIPEA (144 mg, 1.12 mmol), was added and the reaction mixture was stirred at room temperature for 12 hours. The reaction mixture was then washed with water (25 ml) and the organic layer was collected and dried over anhydrous sodium sulfate. The volatiles were evaporated and the residue was purified by column chromatography (EtOAc:hexane, 3:2) yielding 0.63 g of the desired tripeptide 46a (72%). Mp: 137-139°C. ¹H NMR (CD₃OD, 600 MHz) δ: 7.30 (m, 15H), 5.11 (m, 6H), 4.41 (m, 2H), 4.14 (m, 1H), 2.27 (m, 9H), 1.99 (s, 1H), 1.87 (m, 3H), 1.40 (s, 9H). Anal. Cald for C₄₁H₄₉N₃O₁₂: C, 63.47, H, 6.37, N, 5.42. Found C, 63.28, H, 6.28, N, 5.33. MS: Cald M+Na 579.60. Found M+Na 579.30.
N-\(\alpha\)-Carbobenzyloxy-L-glutamic Acid, \(\alpha\)-Benzy Ester \(\gamma\)\-[L-Glutamic Acid, \(\alpha\)-Benzy Ester \(\gamma\)\]-L-glutamic Acid, \(\alpha\)-Benzy Ester \(\gamma\)\-[L-Glutamic Acid, \(\alpha\)-Benzy Ester]amide]amide] (46b)

Dipeptide 45b (500 mg, 0.847 mmol) and CDI (0.15 g, 0.931 mmol) were reacted in DCM (40 ml) for 30 minutes. 44 (232 mg, 0.847 mmol) was dissolved in DCM (5 ml) and treated with DIPEA (110 mg, 0.847 mmol), and the resulting solution was added over the activated dipeptide. The reaction mixture was stirred for 12 hours after which it was washed with water (35 ml). After collecting and drying the organic layer over anhydrous sodium sulfate, the volatiles were evaporated and the residue was purified on a silica column (EtOAc:Hexane, 2:1). 0.51 g of 46b were collected as a white solid (74%).

Mp: 87-90°C (amorphous solid). \(^1\)H NMR (CD\(_3\)OD, 400 MHz) \(\delta\): 7.34 (m, 20H), 5.14 (m, 8H), 4.75 (m, 1H), 4.42 (m, 1H), 4.25 (m, 1H), 2.27 (m, 12H). Anal. Cald for C\(_{44}\)H\(_{47}\)N\(_3\)O\(_{12}\): C, 65.26, H, 5.85, N, 5.19. Found C, 65.60, H, 5.88, N, 5.21. MS: Cald M\(+\)Na 832.86. Found M\(+\)Na 832.50.

2’-Carbobenzyloxy-7\-[N-\(\alpha\)-Carbobenzyloxy-L-glutamic Acid, \(\alpha\)-Benzy Ester \(\gamma\)\]-L-glutamic Acid, \(\alpha\)-Benzy Ester]amide]paclitaxel (47)

The glutamate dipeptide 45b (71.7 mg, 0.121 mmol) was dissolved in DCM (10 ml) and DIC (30.5 mg, 0.242 mmol) was added. The reaction mixture was stirred at room temperature for 1 hour after which 2’-Cbz-PAC 27 (60 mg, 0.0607 mmol) and DMAP (15 mg, 0.121 mmol) were added and stirring was continued for 10 hours. The reaction mixture was washed with 2% aqueous HCl solution (10 ml) and brine (10 ml). The volatiles were evaporated after drying the organic layer over anhydrous sodium sulfate. The residue was cleaned by column chromatography (EtOAc: Hexane, 2:1) and 46 was
collected. 46 was dissolved in DCM (1 ml) and hexane (50 ml) was added causing the precipitation of 46. The white solid was filtered and 69 mg of the desired product were obtained (73%). Mp: 125-128°C. 1H NMR (CDCl3, 600 MHz) δ: 8.11 (d, 1H), 7.71 (d, 2H), 7.60 (t, 1H), 7.49 (m, 3H), 7.38 (m, 27H), 6.89 (d, 1H), 6.51 (d, 1H), 6.25 (t, 1H), 6.16 (s, 1H), 5.95 (d, 1H), 5.71 (d, 1H), 5.66 (d, 1H), 5.58 (m, 1H), 5.43 (s, 1H), 5.11 (m, 9H), 4.93 (d, 1H), 4.52 (m, 1H), 4.31 (d, 1H), 4.15 (d, 1H), 3.92 (d, 1H), 2.47 (s, 4H), 2.55 (m, 1H), 2.43 (s, 3H), 2.20 (m, 8H), 2.08 (s, 3H), 1.98 (s, 3H), 1.77 (s, 3H), 1.20 (s, 3H), 1.14 (s, 3H). Anal. Cald for C84H89N3O24: 0.1 H2O: C, 66.88, H, 5.75, N, 2.69. Found C, 66.54, H, 5.86, N, 2.90. MS: Cald M+Na 1583.64. Found M+Na 1582.90.

7-[Glutamic acid, γ-[L-Glutamic acid]amide]paclitaxel (48)

47 (20 mg, 0.013 mmol) was dissolved in methanol (25 ml) and 3 drops of acetic acid were added. The solution was cooled in a dry ice/acetone bath, and 10% Pd/C (8 mg) were added. The mixture was shaken under hydrogen (25 psi) for 12 hours after which it was filtered over a celite bed. The filtrate was collected and methanol was evaporated. Hexane (2 x 20 ml) was added to wash the resulting residue. Hexane was decanted and the residue was dissolved in DCM (1 ml). Hexane (25 ml) was added causing the precipitation of a 48. The white solid was collected by filtration. 14 mg of 48 were obtained (93%). Mp: 1H NMR (CD3OD, 600 MHz) δ: 8.08 (d, 2H), 7.84 (d, 2H), 7.58 (d, 1H), 7.44 (m, 9H), 7.27 (t, 1H), 6.20 (s, 1H), 6.13 (t, 1H), 5.61 (m, 3H), 4.97 (d, 1H), 4.72 (d, 1H), 4.17 (m, 2H), 3.87 (d, 1H), 3.64 (m, 1H), 2.49 (m, 5H), 2.34 (s, 1H), 2.14 (s, 3H), 1.95 (m, 1H), 1.86 (s, 1H), 1.75 (s, 3H), 1.27 (s, 3H), 1.13 (s, 3H), 1.09 (s, 3H). MS: Cald M+Na 1135.13. Found M+Na 1133.70.
2'-Carbobenzyloxy-7-[N-α-tert-butoxy-L-Glutamic acid, α-benzyl ester γ-[L-Glutamic acid, α-benzyl ester]amide]paclitaxel (49)

Dipeptide 45a (90 mg, 0.162 mmol) was dissolved in DCM (15 ml) and DIC (24.5 mg, 0.194 mmol) was added. The reaction mixture was stirred at room temperature for 1 hour after which 2'-Cbz-PAC 27 (80 mg, 0.081 mmol) and DMAP (20 mg, 0.162 mmol) were added and stirring was continued for 10 hours. The reaction mixture was washed with 2% aqueous HCl solution (10 ml) and brine (10 ml). The organic layer was dried over anhydrous sodium sulfate and the volatiles were evaporated. The residue was purified by column chromatography (EtOAc:hexane, 2:1) and 48 was collected. 48 was precipitated by dissolving it in DCM (1 ml) followed by the addition of hexane (50 ml). The white solid was filtered and 86 mg of the desired product 48 was obtained (70%). Mp: 135-138°C. \(^1\)H NMR (CDCl\(_3\), 600 MHz) \(\delta\): 8.12 (d, 1H), 7.71 (d, 2H), 7.58 (t, 1H), 7.38 (m, 20H), 6.89 (d, 1H), 6.23 (m, 1H), 6.16 (s, 1H), 5.95 (m, 1H), 5.67 (m, 1H), 5.67 (d, 1H), 5.58 (m, 1H), 5.42 (s, 1H), 5.11 (m, 6H), 4.97 (d, 1H), 4.51 (m, 1H), 4.45 (m, 1H), 4.31 (m, 1H), 4.15 (d, 1H), 3.92 (d, 1H), 3.79 (d, 2H), 2.54 (m, 1H), 2.44 (s, 3H), 2.27 (s, 3H), 2.20 (m, 8H), 2.08 (s, 3H), 1.99 (s, 3H), 1.78 (s, 3H), 1.41 (s, 9H), 1.20 (s, 3H), 1.14 (s, 3H). MS: Cald M+Na 1549.63. Found M+Na 1549.00.

2'-Carbobenzyloxy-7-[N-α-tert-butoxy-L-Glutamic acid, α-benzyl ester γ-[L-Glutamic acid, α-benzyl ester]amide]amide]paclitaxel (51a)

To a solution of 41a (35 mg, 0.0451 mmol) in DCM (10 ml) was added DIC (8.5 mg, 0.0677 mmol). The solution was stirred for 1 hour after which Z-PAC 23 (22.2 mg,
0.0226 mmol) was added. The reaction mixture was stirred for 10 hours. DCM (20 ml) was added and the solution was washed with 2% aqueous HCl solution (15 ml) and brine (15 ml). The organic layer was collected and dried over anhydrous sodium sulfate. DCM was evaporated under vacuum and the residue was purified on a silica column (EtOAc: Hexane, 3:2). The desired product was collected and re-dissolve in DCM (1ml). Hexane (40 ml) was added and a white solid precipitated out. The precipitate was filtered. 28 mg of 51a were collected (71%). Mp: 117-120°C. \(^1\)H NMR (CDCl\(_3\), 600 MHz) \(\delta\): 8.13 (d, 1H), 7.71 (d, 2H), 7.58 (t, 1H), 7.38 (m, 30H), 6.90 (d, 1H), 6.23 (m, 2H), 5.96 (d, 1H), 5.68 (d, 1H), 5.42(s, 1H), 5.13 (m, 8H), 4.96 (d, 1H), 4.52 (m, 1H), 4.50 (m, 1H), 4.44 (m, H), 4.31 (d, 1H), 4.19 (d, 1H), 3.79(d, 1H), 3.06 (s, 1H), 2.56(m, 1H), 2.49 (d, 1H), 2.44 (s, 3H), 2.37 (m, 2H), 2.23 (s, 3H), 2.19 (m, 6H), 2.09 (s, 1H), 1.98 (s, 1H), 1.91 (s, 3H), 1.77 (s, 3H), 1.67 (s, 3H), 1.40 (m, 9H), 1.20 (s, 3H), 1.14 (s, 3H). Anal. Cald for C\(_{96}\)H\(_{104}\)N\(_4\)O\(_27\). 0.4 EtOAc: C, 65.82, H, 6.07, N, 3.15. Found C, 65.96, H, 6.10, N, 2.75. MS: Cald M+Na 1768.86. Found M+Na 1767.70.

**N-Fmoc-Methionyl-glycine, tert-Butyl Ester (55)**

To a solution of N-Fmoc-methionine 53 (0.64g, 1.72 mmol) in DCM (10 ml) was added 1.2 equivalents of CDI (0.33g, 2.07 mmol). The reaction mixture was allowed to stir at room temperature for 20 minutes. In a separate flask, glycine, tert-butyl ester acetate salt 54 (0.3 g, 1.56 mmol) was treated with 2 equivalents of DIPEA (0.4 g, 4.14 mmol). The second reaction was stirred at room temperature for 20 minutes, after which it was added as a single portion to the activated methionine. The combined reaction mixture was stirred at room temperature for 2 hours, followed by dilution with ethyl ether (70 ml).
The reaction mixture was filtered and the filtrate was successively washed with 5% aqueous HCl solution (25 ml), saturated NaHCO$_3$ (20 ml) and brine (20 ml). After drying over anhydrous sodium sulfate, the volatiles were evaporated under reduced pressure and the residue was purified by column chromatography using DCM:MeOH (50:1) to provide 0.72 g of the desired product as a white solid (95% yield). Mp: 108-110°C. $^1$H NMR (CDCl$_3$, 600 MHz) δ: 7.75 (d, 2H), 7.56 (d, 2H), 7.38 (t, 2H), 7.29 (t, 2H), 6.56 (s, 1H), 5.53 (d, 1H), 4.39 (d, 2H), 4.19 (m, 1H), 3.86 (m, 2H), 2.56 (m, 2H), 2.10 (s, 3H), 1.95 (m, 1H), 1.61 (s, 2H), 1.44 (s, 9H). $^{13}$C NMR (CDCl$_3$, 100 MHz) δ: 171.16, 168.52, 156.08, 143.78, 143.67, 141.29, 127.73, 127.08, 125.07, 119.99, 82.48, 67.05, 53.67, 47.12, 42.03, 31.61, 29.98, 28.02, 15.16. Anal. Cald for C$_{26}$H$_{32}$N$_2$O$_5$S: 0.15 CH$_2$Cl$_2$: C, 63.14, H, 6.54, N, 5.63, O, 16.08. Found C, 63.24, H, 6.59, N, 5.65, O, 16.27.

**N-Fmoc-(D)-(O-tert-Butyl)-tyrosyl-methionyl-glycine, tert-Butyl Ester (57)**

N-Fmoc-Met-Gly-O$^t$Bu 55 (1 g, 2.01 mmol) was dissolved in THF (15ml) and treated with 10 equivalents of 1-octanethiol (3.0 g, 20.6 mmol). A catalytic amount of DBU dissolved in THF (5 ml) was added dropwise over a period of 30 minutes. The reaction mixture was stirred for 3 hours at room temperature after which the volatiles were evaporated under reduced pressure. The resulting residue was treated with saturated oxalic acid:EtOAc solution (50 ml) and stirred at room temperature for 3 hours. The desired Met-Gly oxalate salt 56 was obtained as a white precipitate, which was collected and washed with cold EtOAc.

Met-Gly oxalate 56 (0.175 g, 0.49 mmol), dissolved in DCM (10 ml), was treated with 2.5 equivalents of DIPEA (0.154 g, 1.22 mmol) and the reaction mixture stirred at room
temperature for 30 minutes. In a separate flask, Fmoc-(D)-Tyr-(O\text{\textprime}Bu)-OH (0.25 g, 0.54 mmol) was treated with 1.3 equivalents of DCC (0.13 g, 0.64 mmol), and the reaction mixture stirred at room temperature for 30 minutes. The free Met-Gly was added to the activated tyrosine as a single portion, and the combined reaction mixture was stirred at room temperature for 5 hours, after which it was filtered. The filtrate was washed successively with 5% aqueous HCl solution (25 ml), saturated NaHCO$_3$ solution (20 ml) and brine (20 ml). The organic layer was collected, dried over anhydrous sodium sulfate, and the volatiles evaporated under reduced pressure. The resulting residue was purified by column chromatography (DCM:MeOH, 50:1) to afford 214 mg (62%) of the desired (D)-Tyr-Met-Gly tripeptide 57 as a white solid. Mp: 113-115°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 7.73 (d, 2H); 7.50 (m, 2H); 7.36 (t, 2H), 7.28 (m, 2H), 7.06 (d, 2H), 7.02 (s, 1H), 6.88 (d, 2H), 5.66 (d, 1H), 4.63 (q, 1H), 4.33 (m, 2H), 4.24 (m, 2H), 4.11 (m, 1H), 4.09 (q, 1H), 3.81 (dq, 2H), 3.44 (m, 1H), 3.00 (dq, 2H), 2.40 (m, 2H), 2.02 (s, 3H), 1.96 (m, 1H), 1.89 (d, 1H), 1.81 (m, 1H), 1.65 (m, 1H), 1.55 (m, 1H), 1.39 (s, 9H), 1.32 (m, 1H), 1.27 (s, 9H), 1.23 (t, 1H), 1.08 (m, 2H). $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$: 171.35, 170.99, 168.83, 156.34, 154.73, 143.93, 141.49, 131.18, 130.03, 129.95, 127.97, 127.36, 127.32, 125.33, 125.29, 124.60, 124.54, 120.21, 82.45, 78.66, 67.45, 57.02, 52.29, 49.41, 47.26, 42.20, 37.98, 34.16, 31.04, 30.14, 29.05, 28.23, 28.83, 25.17, 15.28. Anal. Cald for C$_{39}$H$_{49}$N$_3$O$_7$S: C, 66.55, H, 7.02, N, 5.97, O, 15.91. Found C, 66.59, H, 7.13, N, 6.17, O, 15.66.
N-[4-(2-Thienyl)butyryl]-(D)-(O-tert-Butyl)tyrosyl-methionyl-glycine,tert-Butyl Ester (59)

To a stirred solution of Fmoc-(D)-Tyr-Met-Gly-O\textsubscript{Bu} 57 (0.5 g, 0.710 mmol) dissolved in THF (20 ml) was added 10 equivalents of 1-octanethiol (1.04 g, 7.10 mmol). A catalytic amount of DBU dissolved in THF (5 ml) was added dropwise over a period of 20 minutes. The reaction mixture was stirred for 4 hours at room temperature, after which the volatiles were evaporated under reduced pressure. The resulting residue was treated with oxalic acid:EtOAc (5 g:25 ml), from which the Fmoc-deprotected tripeptide oxalate salt 58 was collected as a white solid after being filtered and washed with cold EtOAc (3 x 10 ml).

4-(2-Thienyl)butyric acid (0.38 g, 2.22 mmol), dissolved in CH\textsubscript{2}Cl\textsubscript{2} (10 ml), was activated using 1.2 equivalents of CDI (0.43 g, 2.66 mmol). In a separate flask, Tyr-Met-Gly oxalate salt 58 was treated with DIPEA (0.86 g, 6.66 mmol) and the reaction mixture stirred at room temperature for 30 minutes. The free tripeptide was then added to the activated butyric acid and the combined reaction mixture stirred at room temperature for 5 hours. The reaction mixture was treated successively with 5 % aqueous HCl solution (25 ml), saturated NaHCO\textsubscript{3} solution (20 ml) and brine (20 ml). After drying the organic layer over anhydrous Na\textsubscript{2}SO\textsubscript{4} (5 g), the solvent was evaporated under reduced pressure and the resulting residue purified by column chromatography (DCM:MeOH, 50:1) to provide 1 g of the desired product 59 as a crystalline solid (75 %). Mp: 124-126\degree C. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) \textdelta: 7.11 (m, 3H), 6.92 (m, 4H), 6.75 (s, 1H), 6.04 (d, 1H), 6.09(d, 1H), 4.56 (m, 2H), 3.86 (dq, 2H), 3.00 (t, 2H), 2.81 (t, 2H), 2.41 (t, 2H), 2.20 (m, 2H), 2.06 (s, 3H), 1.92 (m, 3H), 1.85 (m, 1H), 1.44 (s, 9H), 1.31 (s, 9H). \textsuperscript{13}C NMR
(CDCl₃, 100 MHz) δ: 173.17, 171.32, 170.90, 168.89, 154.78, 144.20, 131.12, 129.85, 127.04, 124.85, 124.59, 123.48, 82.45, 78.71, 55.48, 52.29, 42.19, 37.50, 35.32, 30.19, 29.23, 29.05, 28.26, 27.38, 15.28. Anal. Cald for C₃₂H₄₇N₃O₆S₂: C, 60.64, H, 7.47, N, 6.63. Found C, 60.45, H, 7.40, N, 6.72.

**N-[4-(2-Thienyl)butyryl]-(D)-tyrosyl-methionyl-glycine (CD3-246)**

(Thienyl)butyryl-(D)-tyr(O-tBu)-Met-Gly(O-tBu) **59** (1 g, 1.57 mmol) was treated with CH₂Cl₂:TFA (1:1) (8 ml). The reaction mixture was stirred at room temperature for 2 hours. After evaporating the volatiles under reduced pressure, the resulting residue was purified using CH₂Cl₂:Acetone:AcOH (1:1:0.1). 0.74 g of the target compound **CD3-246** was collected in 90% yield as a crystalline solid (m.p. 137-139°C). ¹H NMR (DMSO; 400 MHz) δ: 9.14 (s, 1H), 8.23 (d, 2H), 8.09 (d, 1H), 7.27 (d, 1H), 6.92 (d, 2H), 6.89 (t, 1H), 6.76 (d,1H), 6.59 (d, 2H), 4.41 (q, 1H), 4.28 (1, 1H), 3.70 (d, 2H), 2.81 (m, 1H), 2.67 (t, 3H), 2.24 (m, 2H), 2.08 (t, 2H), 1.96 (s, 3H), 1.84 (m, 2H), 1.69 (m, 3H). ¹³C NMR (DMSO, 100 MHz) δ: 221.03, 172.63, 172.16, 172.11, 171.63, 156.46, 145.03, 130.75, 128.23, 127.55, 125.52, 125.16, 124.13, 115.46, 55.39, 52.17, 41.35, 37.51, 36.57, 34.87, 32.23, 30.01, 29.21, 27.99, 15.18. Anal. Cald for C₂₄H₃₁N₃O₆S₂ 0.25 H₂O: C, 54.73, H, 5.98, N, 7.98, O, 19.00. Found C, 54.49, H, 5.82, N, 8.12, O, 18.96.


Boc-Asp-OBn (0.9 g, 2.85 mmol) was dissolved in DCM, and reacted with CDI (0.55 g, 3.42 mmol) for 1 hour. Rhodamine analogue **D2** (1.3 g, 2.85 mmol) was added, and the
reaction mixture was stirred at room temperature for 12 hours. The volatiles were then evaporated, and the residue was purified by column chromatography using EtOAc as an eluent to yield 1.84 g (85%) of the desired product. Mp: 111-113°C. $^1$H NMR (CDCl$_3$, 400 MHz) δ: 7.91 (m, 1H), 7.48 (m, 2H), 7.30 (m, 5H), 7.05 (m, 1H), 6.34 (s, 2H), 6.19 (d, 2H), 5.52 (d, 1H), 5.50 (m, 1H), 5.13 (s, 2H), 4.79 (m, 1H), 3.85 (dq, 2H), 3.31 (m, 1H), 3.20 (m, 6H), 2.96 (m, 2H), 2.78 (dd, 1H), 2.51 (dd, 1H), 1.89 (s, 6H), 1.43 (s, 9H), 1.30 (m, 6H). Anal. Cald for C$_{44}$H$_{55}$N$_5$O$_7$. 0.5 H$_2$O: C, 68.55, H, 6.80, N, 9.08. Found C, 68.60, H, 6.78, N, 9.16.

**Neutral Rhodamine 6G** [2’-L-Aspartic Acid, α-Benzyl Esteramide]ethyl-amide (D13)

R6G-Asp(Boc,OBn) D12 (1 g, 1.31 mmol) was dissolved in a 1:1 DCM:TFA mixture (40 ml). The reaction mixture was stirred for 10 hours, after which the volatiles were evaporated under vacuum. The resulting residue was dissolved in DCM (100 ml) and washed with a saturated NaHCO$_3$ solution until all the TFA was neutralized. The organic layer was collected, the volatiles were evaporated, and the resulting residue was purified by column chromatography using EtOAc:MeOH (25:1) as an eluent. 0.8 g (93%) of the desired product D13 were collected. Mp: 92-95°C. $^1$HNMR (CDCl$_3$, 600 MHz) δ: 7.90 (m, 1H), 7.46 (m, 2H), 7.30 (m, 5H), 7.03 (m, 2H), 6.32 (s, 2H), 6.20 (s, 2H), 5.13 (s, 2H), 3.82 (m, 1H), 3.51 (s, br, 2H), 3.33 (m, 6H), 2.97 (m, 1H), 2.91 (m, 1H), 2.58 (dd, 1H), 2.35 (m, 1H), 1.87 (s, 6H), 1.30 (m, 6H). Anal. Cald for C$_{39}$H$_{43}$N$_5$O$_5$: C, 70.82, H, 6.55, N, 10.59. Found C, 70.66, H, 6.69, N, 10.40.

Boc-Gly-OH (0.18 g, 1.06 mmol) was dissolved in anhydrous DCM (10 ml) and DMF (2 ml), to which PyBOP (0.55 g, 1.06 mmol) and HOBt (0.14 g, 1.06 mmol) were added, followed by 2 equivalents of DIPEA (0.27 g, 2.16 mmol). The reaction mixture was stirred at room temperature for 1 hour, after which R6G-Asp D13 (0.70 g, 1.06 mmol) was added. The resulting reaction mixture was stirred for 12 hours, after which it was washed with water (20 ml x 2). The organic layer was collected and the volatiles were evaporated. The resulting residue was purified by column chromatography (EtOAc) and 0.52 g (60%) of the desired product were collected. Mp: 120-122°C ¹H NMR (CDCl₃, 600 MHz) δ: 7.90 (m, 1H), 7.47 (m, 2H), 7.29 (m, 5H), 7.04 (m, 1H), 6.32 (s, 2H), 6.18 (d, 2H), 5.47 (m, 1H), 5.12 (s, 2H), 4.78 (m, 1H), 3.89 (dd, 2H), 3.79 (dd, 1H), 3.31 (m, 1H), 3.20 (m, 6H), 2.98 (m, 1H), 2.86 (m, 1H), 2.78 (dd, 1H), 2.51 (dd, 1H), 1.89 (s, 6H), 1.42 (s, 9H), 1.30 (m, 6H). Anal. Cald for C₄₆H₅₄N₆O₈·0.25H₂O: C, 67.10, H, 6.67, N, 10.21. Found C, 66.86, H, 6.67, N, 10.34.


R6G-Asp(OBn)-Gly D14 (0.3 g, 0.37 mmol) was dissolved in a 1:1 DCM:TFA mixture (40 ml). The reaction mixture was stirred for 10 hours, after which the volatiles were evaporated under vacuum. The resulting residue was dissolved in DCM (100 ml) and washed with a saturated NaHCO₃ solution until all the TFA was neutralized. The organic layer was collected, the volatiles were evaporated, and the resulting residue was purified
by column chromatography using EtOAc:MeOH (25:1) as an eluent. 0.24 g (90%) of the desired product **D15** were obtained. Mp: 130-133°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 8.08 (d, 1H), 7.89 (m, 1H), 7.46 (m, 2H), 7.29 (m, 5H), 7.04 (m, 1H), 6.48 (m, 1H), 6.32 (s, 2H), 6.18 (s, 2H), 5.12 (m, 2H), 4.81 (m, 1H), 3.55 (s, br, 1H), 3.35 (s, 2H), 3.20 (m, 6H), 2.99 (m, 1H), 2.94 (m, 1H), 2.77 (dd, 1H), 2.51 (dd, 1H), 1.88 (s, 6H), 1.30 (m, 6H).


Cbz-Arg(di-cbz) (0.24 g, 0.42 mmol) was dissolved in anhydrous DCM (10 ml) and DMF (2 ml), to which PyBOP (0.22 g, 0.42 mmol) and HOBt (56 mg, 0.42 mmol) were added, followed by 2 equivalents of DIPEA (0.11g, 0.84 mmol). The reaction mixture was stirred at room temperature for 1 hour, after which R6G-Asp-Gly **D15** (0.3 g, 0.42 mmol) was added. The resulting reaction mixture was stirred for 12 hours, after which it was washed with water (20 ml x 2). The organic layer was collected and the volatiles were evaporated. The resulting residue was purified by column chromatography (EtOAc) to afford 0.37 g (70%) of **D16a**. Mp: 129-132°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 9.34 (d, 2H), 7.87 (m, 1H), 7.44 (m, 2H), 7.31(m, 20H), 7.22 (d, 1H), 7.18 (m, 1H), 7.03 (m, 1H), 6.37 (m, 1H), 6.32 (s, 2H), 6.18 (m, 2H), 5.08 (m, 8H), 4.73 (m, 1H), 4.31 (m, 1H), 4.00 (m, 1H), 3.84 (m, 1H), 3.62 (m, 1H), 3.55 (m, 3H), 3.25 (m, 1H), 3.16 (d, 4H), 3.11 (m, 1H), 2.86 (m, 1H), 2.71 (dd, 1H), 2.41 (dd, 1H), 1.85 (m, 6H), 1.70 (m, 3H), 1.64 (s, 3H),
1.30 (m, 6H). Anal. Calcd for C\textsubscript{72}H\textsubscript{77}N\textsubscript{10}O\textsubscript{13}: C, 66.82, H, 6.01, N, 10.79.

Found C, 66.58, H, 6.06, N, 10.02.


Dye-RGD D\textsubscript{16a} (50 mg, 0.039 mmol) was dissolved in MeOH, and 6 drops of AcOH were added. The reaction mixture was cooled to -20\textdegree C, after which Pd/C (80 mg) was added. The heterogeneous mixture was subjected to hydrogenation (H\textsubscript{2}, 20 psi) for 48 hours at room temperature, after which it was filtered through a celite bed. The volatiles were evaporated and the resulting residue was washed with hexane (3 x 25ml). 28 mg (80\%) of the desired product D\textsubscript{17} were obtained. Mp: 174-177\textdegree C. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 600 MHz) \(\delta\): 7.86 (m, 1H), 7.51 (m, 2H), 7.00 (m, 1H), 6.32 (s, 2H), 6.12 (s, 2H), 4.33 (m, 1H), 4.00 (d, 1H), 3.76 (d, 1H), 3.59 (s, br, 1H), 3.29 (m, 4H), 3.18 (m, 8H), 2.87 (m, 2H), 2.51 (m, 2H), 1.90 (s, 6H), 1.87 (m, 6H), 1.67 (m, 4H), 1.25 (m, 6H).

**N-\textalpha-Carbobenzyloxy-N\textsuperscript{G}-4-methoxy-2,3,6-trimethylbenzenesulfonyl-L-arginyl-glycine, \textalpha-tert-Butyl Ester (62a)**

To a solution of Glu-O\textsuperscript{\textbeta}Bu acetate 61 (80 mg, 0.61 mmol) treated by DIPEA (12 mg, 0.92 mol) in DCM (10 ml) was added a solution of Cbz-Arg(Mtr) 60a (0.35 g, 0.67 mmol) preactivated with DCC (0.21 g, 1.0 mmol) in DCM (10 ml). A catalytic amount of DMAP (5 mg, 0.041 mmol) was added and the reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was filtered and the filtrate was treated successively with 5\% aqueous HCl solution (15 ml), saturated NaHCO\textsubscript{3} solution (15 ml),
and brine (15 ml). The organic layer was collected and the volatiles were evaporated. The resulting oily residue was purified by column chromatography (DCM: MeOH, 25:1). 0.31 g (82%) of product 62a were collected as a white solid after evaporating the volatiles. Mp: 84-86°C (amorphous solid). 1H NMR (CDCl3, 400 MHz) δ: 7.44 (s, 1H), 7.29 (m, 5H), 6.50 (s, 1H), 6.33 (s, 2H), 6.02 (d, 1H), 5.04 (s, 2H), 4.31 (m, 1H), 3.91 (dd, 1H), 3.80 (s, 3H), 3.76 (dd, 1H), 3.18 (m, 2H), 2.65 (s, 3H), 2.58 (s, 3H), 2.10 (s, 3H), 1.87 (m, 1H), 1.67 (m, 2H), 1.41 (s, 9H). 13C NMR (CDCl3, 100 MHz) δ: 169.56, 158.74, 156.64, 138.81, 136.44, 128.73, 128.35, 128.18, 125.07, 111.93, 82.46, 67.20, 55.67, 53.70, 42.15, 30.33, 28.27, 24.39, 18.58, 12.22. Anal. Cald for C30H43N5O8S. H2O: C, 5.523; H, 6.9; N, 10.74. Found C, 55.38; H, 6.44; N, 10.54.

**N-α- Carbobenzyloxy -N^G^-nitro-L-arginyl-glycine, α-tert-Butyl Ester (62b)**

Cbz-Arg(NO2) 60b (1 g, 2.83 mmol) was dissolved in DCM (20 ml) and activated by CDI (0.68 g, 4.25 mmol) for 30 minutes. Gly-OtBu acetate 61 (0.65 g, 3.42 mmol) was dissolved in DCM and treated with DIPEA (0.66 g, 5.13 mmol). The resulting solution was added to the activated arginine residue 60b. The reaction mixture was stirred at room temperature for 6 hours and then washed with a 2% aqueous HCl solution (15 ml). The organic layer was collected and DCM was evaporated under vacuum. The residue was purified by column chromatography (DCM: MeOH, 25:1) to yield 1.24 g (94%) of the desired product 62b. Mp: 84-86°C (amorphous solid). 1H NMR (CDCl3, 600 MHz) δ: 7.34 (m, 5H), 5.93 (s, 1H), 5.06 (m, 1H), 4.33 (m, 1H), 3.83 (m, 3H), 3.24 (m, 2H), 1.72 (m, 5H), 1.43 (s, 9H). Anal. Cald for C20H30N6O7 . 0.25 H2O: C, 51.00, H, 6.53, N, 17.84. Found C, 51.39, H, 6.36, N, 17.53.
N-α-Carbobenzyloxy-N\textsuperscript{G}-4-methoxy-2,3,6-trimethylbenzenesulfonyl-L-arginyl-
glycine (63a)

Cbz-Arg(Mtr)-Gly-O\textsuperscript{t}Bu 62a (1 g, 1.58 mmol) was treated with a 1:1 DCM:TFA mixture (20 ml) for 2 hours. The volatiles were evaporated and the resulting residue was purified by column chromatography using DCM:MeOH (25:1) as an eluent to provide 0.86 g (95%) of 63a as a white solid. Mp: 101-103°C. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) δ: 7.78 (s, 1H), 6.48 (s, 1H), 5.03 (m, 2H), 3.79 (s, 3H), 3.20 (m, 2H), 2.60 (s, 2H), 2.54 (m 2H), 2.07 (s, 3H), 1.55 (m, 6H), 1.26 (s, 1H). Anal. Cald for C\textsubscript{26}H\textsubscript{35}N\textsubscript{5}O\textsubscript{8}S. 0.75 H\textsubscript{2}O . 0.2 DCM: C, 51.75, H, 6.12, N, 11.52, O, 23.02. Found C, 51.84, H, 5.85, N, 11.23, O, 22.87. MS: Cald M+Na 600.65. Found M+Na 600.20.

N-α-Fmoc-L-Aspartic Acid, α-Benzyl Ester β-tert-Butyl Ester (65)

To a solution of Fmoc-Asp-O\textsuperscript{t}Bu 64 (0.25 g, 0.607 mmole) dissolved in DCM (15 ml) was added 10 equivalents of benzyl alcohol (0.656 g, 6.07 mmol), DCC (0.16 g, 0.789 mmol) and a catalytic amount of DMAP (20 mg, 0.16 mmol). The reaction mixture was allowed to stir at room temperature for 2 hours after which it was diluted by ethyl ether (50 ml). The reaction mixture was filtered and the filtrate was treated successively with 5% aqueous HCl solution (20 ml), saturated aqueous NaHCO\textsubscript{3} solution (20 ml), and brine solution (20 ml). The organic layer was collected and the solvents were evaporated. The resulting residue was purified by column chromatography (DCM:MeOH, 50:1) and 0.28 g (95%) of the desired product 65 were collected as a white solid. Mp: 87-89°C (amorphous solid). \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) δ: 7.75 (d, 2H), 7.42 (d, 2H), 7.30 (m,
$^1$H NMR (CDCl$_3$, 100 MHz) $\delta$: 171.01, 169.78, 156.19, 144.12, 143.99, 141.51, 135.67, 128.86, 128.69, 128.61, 127.95, 127.32, 125.42, 120.23, 67.45, 67.03, 67.45, 67.03, 51.14, 47.34, 37.19, 28.08. Anal. Cald for C$_{30}$H$_{31}$NO$_{16}$. 0.25 H$_2$O: C, 71.24, H, 6.23, N, 2.77, O, 19.78. Found C, 71.34, H, 6.38, N, 2.78, O, 19.55. MS: Cald M+Na 524.57. Found M+Na 524.30.

**L-Aspartic Acid, $\alpha$-Benzyl Ester $\beta$-tert-Butyl Ester Oxalate (66)**

Fmoc-Asp(OBn)-O$^1$Bu 58a (0.6 g, 1.19 mmol) was dissolved in THF (20 ml) to which 10 equivalents of 1-octanethiol (1.75g, 11.9 mmol) was added. A catalytic amount of DBU (3 drops) dissolved in THF was added dropwise over a period of 20 minutes. The reaction mixture was stirred at room temperature for 6 hours. The volatiles were evaporated and the resulting residue was treated with saturated oxalic acid in EtOAc (10 ml). The mixture was stirred for 1 hour causing the precipitation of the desired product as a white solid that was collected by filtration and stirred in DCM for 12 hours. 0.26 g (80 %) of the washed solid 58b were collected by filtration. Mp: 156-158°C. $^1$H NMR (CD$_3$OD, 600 MHz) $\delta$: 7.31 (m, 5H), 5.14 (q, 2H), 4.22 (t, 1H), 2.95 (dq, 2H), 1.42 (s, 9H). $^{13}$C NMR (CD$_3$OD, 100 MHz) $\delta$: 169.84, 167.13, 165.43, 135.76, 128.45, 128.40, 84.33, 67.11, 49.68, 34.05, 26.77. Anal. Cald for C$_{15}$H$_{21}$NO$_4$. C$_2$H$_2$O$_4$. 0.6 H$_2$O: C, 53.66, H, 6.36, N, 3.68, O, 36.19. Found C, 53.66, H, 6.40, N, 3.66, O, 36.41. MS: Cald M+Na 302.33. Found M+Na 302.00.
N-\(\alpha\)-Carbobenzyloxy-N\(^G\)-4-methoxy-2,3,6-trimethylbenzenesulfonyl-L-arginyl-glycyl-L-aspartic acid, \(\alpha\)-Benzyl Ester \(\beta\)-tert-Butyl Ester (67a)

Cbz-Arg(Mtr)-Gly 63a (0.56g, 0.97 mmol) was activated using 1.3 equivalents of CDI (0.2 g, 1.26 mmol). Asp(OBn)-O\(^t\)Bu oxalate 66 was suspended in DCM (10 ml) and DIPEA (0.48 g, 3.48 mmol) was added. The resulting solution was added to the activated Arg-Gly dipeptide. The reaction mixture was stirred for 4 hours at room temperature after which it was successively washed with 5% aqueous HCl solution (20 ml), saturated aqueous NaHCO\(_3\) solution (20 ml), and brine (20 ml). The organic layer was dried over anhydrous sodium sulfate and the volatiles were evaporated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH, 20:1). The desired fraction was collected and 0.48 g (60%) of the desired product 67a were obtained as a white solid following the evaporation of the organic solvent. Mp: 82-84\(^o\)C (amorphous solid). \(^1\)H NMR (CDCl\(_3\), 600 MHz) \(\delta\): 7.47 (s, 1H), 7.30 (m, 10H), 7.03 (d, 1H), 6.47 (s, 1H), 6.22 (s, 2H), 5.83 (d, 1H), 5.04 (m, 4H), 4.65 (m, 1H), 4.32 (m, 1H), 3.98 (dq, 2H), 3.78 (s, 3H), 3.30 (s, 1H), 3.14 (m, 1H), 2.81 (dq, 2H), 2.64 (s, 3H), 2.58 (s, 3H), 2.09 (s, 3H), 1.86 (m, 2H), 1.66 (m, 6H), 1.33 (s, 9H). \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\): 171.39, 171.00, 156.65, 138.83, 136.77, 136.48, 135.87, 135.69, 133.88, 128.79, 128.72, 128.26, 128.55, 128.52, 128.33, 128.17, 124.94, 111.90, 83.06, 81.92, 67.18, 67.09, 66.80, 55.62, 49.49, 36.48, 34.09, 28.13, 28.02, 25.16, 24.35, 18.58, 12.17, 0.217. Anal. Cald for C\(_{41}\)H\(_{54}\)N\(_6\)O\(_{11}\)S: 0.5 H\(_2\)O: C, 57.77, H, 6.50, N, 9.85. Found C, 57.91, H, 6.51, N, 9.84.
N-α- Carbobenzyloxy -N\textsuperscript{G}-4-nitro-L-arginyl-glycinyl-L-aspartic acid, α-Benzyl Ester β-tert-Butyl Ester (67b)

Cbz-Arg(NO\textsubscript{2})-Gly-O\textsuperscript{t}Bu 62b (0.96 g, 2.07 mmol) was stirred in a 1:1 DCM:TFA mixture (30 ml) for 3 hours. The volatiles were evaporated and the residue was washed with hexanes (3 x 50 ml). The resulting Cbz-Arg(NO\textsubscript{2})-Gly 63b was dried on a high vacuum pump. \(^1\text{H} \text{NMR} \text{ (CD}_3\text{OD, 600 MHz)} \delta: 1.68 \text{ (m, 5H)}, 3.23 \text{ (m, 2H)}, 3.86 \text{ (m, 2H)}, 4.18 \text{ (m, 1H)}, 5.06 \text{ (m, 2H)}, 7.28 \text{ (m, 5H)}. \text{MS: Calculated M+Na 433.38. Found M+Na 432.80.}

The dried dipeptide 63b was then dissolved in DCM (20 ml) and CDI (0.5 g, 3.11 mmol) was added. The mixture was stirred at room temperature for 30 minutes after which a mixture of Asp(OBn)-O\textsuperscript{t}Bu oxalate 66 (1.14 g, 3.11 mmol) and DIPEA (1.21 g, 9.33 mmol) in DCM (10 ml) was added. After stirring for 12 hours at room temperature, the reaction mixture was washed with brine (20 ml). The organic layer was collected and dried over anhydrous sodium sulfate. DCM was evaporated under vacuum and the resulting residue was purified by column chromatography (DCM:MeOH, 40:1) to yield 0.97 g (70%) of the desired tripeptide 67b. \text{Mp: 105-108°C. \(^1\text{H} \text{NMR} \text{ (DMSO, 400 MHz)} \delta: 8.47 \text{ (s, br, 1H)}, 8.28 \text{ (m, 1H)}, 8.21 \text{ (m, 1H)}, 7.50 \text{ (d, 1H)}, 7.29 \text{ (m, 10H)}, 5.04 \text{ (m, 4H)}, 4.66 \text{ (m, 1H)}, 3.96 \text{ (m, 1H)}, 3.72 \text{ (d, 2H)}, 3.10 \text{ (m, 2H)}, 2.72 \text{ (m, 1H)}, 2.58 \text{ (m, 1H)}, 1.48 \text{ (m, 4H)}, 1.32 \text{ (s, 9H)}. \text{Anal. Cald for C}_{31}\text{H}_{41}\text{N}_{7}\text{O}_{10} \cdot 0.5 \text{H}_{2}\text{O: C, 54.70, H, 6.22, N, 14.40. Found C, 54.67, H, 6.18, N, 14.18. MS: Cald M+Na 694.70. Found M+Na 694.50.}
N-α-Carbobenzyloxy-NG-4-methoxy-2,3,6-trimethylbenzenesulfonyl-L-arginyl-glycinyl-L-aspartic Acid, α-Benzyl Ester (68a)

RGD tripeptide 67a (0.5 g, 0.60 mmol) was treated with 1:1 DCM: TFA mixture (30 ml) for 2 hours at room temperature. The volatiles were then evaporated under reduced pressure and the residue was washed with ethyl ether (30 ml) to yield 0.43 g (92%) of 68a as a white solid. Mp: 82-84°C (amorphous solid). 1H NMR (CDCl3, 600 MHz) δ: 7.90 (s, 1H), 7.70 (s, 1H), 7.29 (m, 10H), 6.47 (s, 1H), 6.22 (m, 2H), 5.04 (m, 4H), 4.75 (m, 1H), 4.32 (m, 1H), 3.98 (m, 2H), 3.77 (s, 3H), 3.08 (m, 1H), 2.89 (m, 2H), 2.60 (s, 3H), 2.53 (s, 3H), 2.06 (s, 3H), 1.66 (m, 6H). Anal. Cald for C37H46N6O11S: C, 54.87, H, 6.10, N, 10.38. Found C, 54.64, H, 5.76, N, 10.06. MS: Cald M+Na 805.83. Found M+Na 804.80.

N-α-tert-Butoxycarbonyl-L-glycinyl-L-aspartic Acid, α,β-Dibenzyl Ester (71)

Boc-Gly 69 (0.36 g, 2.06 mmol) was dissolved in DCM (15 ml) and reacted with CDI (0.4, 2.47 mmol) for 30 minutes. A solution of Asp(OBn)-OBn tosylate 70 (1.55 g, 3.19 mmol) in DCM (15 ml), pretreated with DIPEA (0.41 g, 3.19 mmol), was added to the activated Gly solution, and the mixture was stirred at room temperature for 6 hours. The reaction mixture was washed with brine (50 ml) and the DCM layer was dried over anhydrous sodium sulfate. After evaporating the volatiles, the residue was purified by column chromatography (DCM:MeOH, 50:1) to provide 0.91 g (94%) of 71 as an oil after evaporation of eluent. 1H NMR (CDCl3, 400 MHz) δ: 7.33 (m, 10H), 6.96 (d, 1H), 5.14 (s, 2H), 5.05 (m, 2H), 4.89 (m, 1H), 3.79 (m, 2H), 3.07 (dd, 1H), 2.88 (dd, 1H), 2.04 (ss, 1H), 1.45 (s, 9H). MS: Cald M+Na 493.51. Found M+Na 493.30.
N-α-tert-Butoxycarbonyl- N\(^G\), N\(^G\)-dicarbobenzyloxy-L-arginyl-L-glycinyl-L-aspartic Acid, α,β-Dibenzyl Ester (74)

Boc-Gly-Asp(OBn)-OBn 71 (0.76 g, 1.62 mmol) was stirred in a 1:1 mixture of DCM:TFA (30 ml) at room temperature for 5 hours until the TLC indicated the disappearance of the starting material. The volatiles were evaporated and the resulting residue was dissolved in DCM (50 ml) and then washed successively with a saturated NaHCO\(_3\) solution (25 ml) and brine (25 ml). The organic layer was collected, DCM was evaporated, and the desired product 72 was obtained in the neutral form as confirmed by mass spectroscopy. MS: Calculated M+H 371.4. Found M+H 371.20. A solution of 72 in DCM (10 ml) was added to a solution of Boc-Arg(di-Z)-OH 73 (1.05 g, 1.94 mmol) in DCM (20 ml) preactivated with CDI (0.38 g, 2.33 mmol). The reaction mixture was stirred at room temperature for 12 hours after which it was washed with brine (30 ml). The organic layer was dried over anhydrous sodium bicarbonate and the DCM was evaporated under vacuum. The residue was purified on a silica column (DCM:MeOH, 75:1) and 1.0 g of tripeptide 74 was obtained (72% yield over the two steps). Mp: 117-119\(^°\)C. \(^1\)H NMR (CDCl\(_3\), 600 MHz) δ: 9.37 (s, 2H), 7.38 (m, 20H), 7.03 (m, 1H), 6.87 (s, 1H), 5.84 (s, 1H), 5.09 (m, 8H), 4.82 (m, 2H), 4.24 (s, 1H), 4.06 (m, 1H), 3.81 (m, 1H), 3.45 (s, 2H), 2.96 (dd, 1H), 2.80 (dd, 1H), 2.03 (s, 1H), 1.71 (m, 6H), 1.44 (s, 9H). Anal. Cald for C\(_{47}\)H\(_{54}\)N\(_6\)O\(_{12}\): C, 63.08, H, 6.08, N, 9.39. Found C, 62.81, H, 6.00, N, 9.06. MS: Cald M+Na 917.96. Found M+Na 917.50.

The RGD analogue 68a (85.7 mg, 0.11 mmol) was dissolved in DCM (10 ml) and activated using 1.3 equivalents of CDI (23 mg, 0.327 mmol). R6G (50 mg, 0.11 mmol) was added to the activated RGD tripeptide and the reaction was stirred at room temperature for 4 hours. DCM was evaporated and the residue was purified by column chromatography using DCM:MeOH (25:1) to give 110 mg (85%) of the desired product D16b. Mp: 140-142°C. ^1H NMR (CDCl₃, 400 MHz) δ: 8.30 (m, 1H), 7.94 (m, 1H), 7.77 (dd, 2H), 7.41 (m, 6H), 7.29 (m, 10H), 7.07 (t, 2H), 6.48 (s, 1H), 4.69 (m, 1H), 4.32 (m, 2H), 3.82 (dd, 1H), 3.77 (s, 3H), 3.66 (dd, 1H), 3.56 (s, 3H), 3.39 (t, 1H), 3.20 (m, 6H), 2.81 (m, 6H), 2.65 (s, 3H), 2.59 (s, 3H), 2.08 (s, 3H), 1.87 (m, 6H), 1.82 (s, 2H), 1.74 (s, 6H), 1.33 (t, 6H). Anal. Calcd for C₆₅H₇₆N₁₀O₁₂S. 0.75H₂O: C, 63.22, H, 6.33, N, 11.34. Found C, 63.11, H, 6.32, N, 11.07.


The RGD tripeptide 67b (0.5 g; 0.74 mmol) was treated with a 1:1 DCM:TFA (10 ml) solution for 3 hours at room temperature, after which the volatiles were evaporated and the resulting residue was triturated with hexane (2 x 50 ml) for 30 minutes to remove any remaining TFA. Hexane was discarded and the formation of intermediate 68b was confirmed by mass spectroscopy (Calculated M+Na 638.59, found M+Na 638.30). 68b was activated using CDI (0.16g; 0.96 mmol) for 30 minutes, after which R6G dye D2 (0.43g; 0.95 mmol) was added. The reaction mixture was allowed to stir at room
temperature for 18 hours after which the volatiles were evaporated. The resulting residue was purified by column chromatography (DCM:MeOH, 50:1) to produce 0.6 g (76%) of the desired product D16b. Mp: 136-139°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 7.79 (m, 2H), 7.42 (m, 2H), 7.25 (m, 10H), 7.02 (m, 1H), 6.34 (s, 2H), 6.17 (s, 1H), 6.13 (s, 1H), 5.05 (m, 4H), 4.74 (s, 1H), 4.44 (d, 1H), 3.65 (m, 2H), 3.17 (m, 6H), 2.86 (m, 3H), 2.49 (m, 1H), 1.90 (s, 6H), 1.64 (d, 2H), 1.31 (t, 6H). Anal. Cald for C$_{55}$H$_{63}$N$_{11}$O$_{11}$.0.75 H$_2$O: C, 61.87, H, 6.09, N, 14.43. Found C, 61.75, H, 5.99, N, 14.38. MS: Cald M+Na 1077.16. Found M+Na 1076.70.


RGD-Rhodamine 6G D16b (0.25 g; 0.2 mmol) was dissolved in MeOH (30 ml), and the solution was cooled to 0°C. 10% Pd/ C (50 mg) was then added, and the reaction mixture was subjected to catalytic hydrogenation (H$_2$, 30 psi) for 24 hours at room temperature. The reaction mixture was then filtered on a celite bed and the methanol was evaporated. The resulting residue was purified by column chromatography (DCM: MeOH, 3:1) producing 0.17 g (85%) of the desired product D18. Mp: 165-169°C. $^1$H NMR (CDCl$_3$, 600 MHz) $\delta$: 7.94 (m, 1H), 7.42 (m, 2H), 7.05 (m, 1H), 6.46 (s, 2H), 6.29 (s, 2H), 6.12 (m, 1H), 3.71 (s, 3H), 3.15 (m, 6H), 1.84 (s, 6H), 1.27 (s, 6H). Anal. Cald for C$_{50}$H$_{64}$N$_{10}$O$_{10}$S. 1.5 H$_2$O: C, 58.64, H, 6.59, N, 13.68. Found C, 58.36, H, 6.78, N, 13.48.

R6G-RGD D16b (0.25g; 0.24 mmol) was dissolved in MeOH (30 ml), and the solution was cooled to 0°C. 10% Pd/C (75 mg) was then added, and the reaction mixture was subjected to catalytic hydrogenation (H₂, 30 psi) for 24 hours at room temperature. The reaction mixture was then filtered on a celite bed and the filtrate was concentrated to 5 ml. Methanolic HCl (5ml) was added and the reaction mixture stirred at room temperature for 10 minutes after which volatiles were evaporated. The resulting residue was washed with ethyl ether (2 x 25 ml) and 0.19 g (87%) of the desired product D19 were collected as a red solid. Mp: 175-178°C. ¹H NMR (CDCl₃, 600 MHz) δ: 8.75 (m, 1H), 7.94 (m, 1H), 7.42 (m, 2H), 7.05 (m, 1H), 6.46 (s, 2H), 6.29 (s, 2H), 6.12 (m, 1H), 3.71 (s, 3H), 3.15 (m, 6H), 2.05 (m, 8H), 1.84 (s, 6H), 1.27 (s, 6H). Anal. Cald for C₄₀H₆₂Cl₆N₁₀O₉.6HCl. 2H₂O: C, 46.21, H, 6.01, N, 13.47. Found C, 45.93, H, 6.11, N, 13.52.

[2’-Carbobenzyloxy-7-[O-Succinyl-(N⁵,N⁵-dicarbobenzyloxy-L-arginyll-L-glycinyl-L-aspartic Acid, α,β-Dibenzyl Ester)amide]-paclitaxel (78)

Boc-Arg(di-cbz)-Gly-Asp(OBn)-OBn 74 (34 mg, 0.038 mmol) was treated with a 1:1 DCM:TFA (10 ml) for 2 hours. The volatiles were evaporated and the residue was redissolved in DCM and washed with a saturated NaHCO₃ solution. The organic layer was dried over anhydrous sodium sulfate and the DCM was evaporated. Intermediate 75 was obtained as oil. Its production was confirmed by mass spectroscopy (MS: Calculated M+H 795.85. Found M+H 795.50). 75 was then added to a solution of 2’-Cbz-7-
Succinyl-PAC 26 (20 mg, 0.0184 mmol) dissolved in DCM (5 ml) and prereacted with DIC (4.8 mg, 0.038 mmol). The reaction mixture was stirred at room temperature for 12 hours after which it was diluted with DCM (20 ml) and successively washed with 2% HCl solution (15 ml) and brine (15 ml). The organic layer was collected and dried over anhydrous sodium sulfate, and the volatiles were evaporated. The residue was purified on a silica column (EtOAc: Hexane, 3:2) to provide 18 mg (52%) of conjugate 78. Mp: 108-111°C. ¹H NMR (CDCl₃, 600 MHz) δ: 8.11 (d, 2H), 7.71 (d, 2H), 7.59 (m, 1H), 7.48 (m, 3H), 7.34 (m, 20H), 7.04 (d, 1H), 6.87 (d, 11), 6.21 (m, 2H), 5.95 (d, 1H), 5.56 (m, 1H), 5.43 (m, 5.43), 5.00 (m, 10H), 4.38 (m, 1H), 4.30 (d, 1H), 4.15 (d, 1H), 4.00 (m, 1H), 3.91 (d, 1H), 3.85 (m, 1H), 3.57 (m, 2H), 2.93 (m, 1H), 2.82 (m, 1H), 2.52 (m, 4H), 2.42 (s, 3H), 2.11 (s, 3H), 1.94 (s, 3H), 1.78 (s, 3H), 1.68 (s, 3H), 1.37 (m, 1H), 1.16 (m, 6H). Anal. Cald for C₁₀₁H₁₀₅N₇O₂₈·0.5 CH₂Cl₂: C, 63.91, H, 5.60, N, 5.14. Found C, 63.67, H, 5.61, N, 5.15. MS: Cald M+Na 1887.94. Found M+Na 1887.40.

N-Benzyl-N-(3,4,5,6,7,8,9,10-Heptadecafluoro-decanyl-amine Hydrochloride (82)

3,4,5,6,7,8,9,10-Heptadecafluoro-decanyl-iodide 80 (10 g, 17.4 mmol) and benzyl amine (5.6 g, 52.2 mmol) were refluxed in EtOAc for 24 hours. The solution was washed with a saturated aqueous sodium bicarbonate solution (50 ml). The organic layer was collected and dried under vacuum. EtOAc was removed under reduced pressure and the resulting residue was purified by column chromatography (EtOAc:Hexane, 1:3) to provide intermediate 81 as on oil. 81 was then dissolved in methanol (100 ml) and the solution was cooled to ca. -20°C. 10% Pd/C (1 g) was carefully added to the cooled solution, and the mixture was shaken under hydrogen gas (25 psi) for 18 hours at room temperature.
The rapid consumption of hydrogen gas during the first 1 hour caused a significant drop in hydrogen pressure wherein the pressure had to be readjusted to 25 psi. Following hydrogenation, methanol (100 ml) was added, and the heterogeneous mixture refluxed for an additional 45 minutes. This mixture was then filtered while still hot. The filtrate was first cooled in an ice bath and then saturated with HCl gas. After stirring for 1 hour, methanol was evaporated under vacuum and 4.8 g (55%) were afforded as a white solid. Mp: 220-222°C. $^1\text{H NMR}$ (DMSO, 600 MHz) $\delta$: 4.04 (s, 1H), 3.07 (m, 2H), 2.64 (m, 2H). $^{19}\text{F NMR}$ (DMSO, 200 MHz) $\delta$: -80.76 (3F), -113.55 (2F), -122.02 (6H), -122.85 (2F), -123.61 (2F), -126.09 (2F).

**N-$\alpha$-Carbobenzyloxy-$N^\text{G}$-4-nitro-L-arginyl-glycinyl-$\beta$-[N-(3,4,5,6,7,8,9,10-heptadeca-fluorodecanyl)amide]L-aspartic Acid, $\alpha$-Benzyl ester (83)**

To a solution of cbz-Arg(NO$_2$)-Gly-Asp-OBn 68b (1.23 g, 2.00 mmol) in DCM (15 ml) was added CDI (0.48 g, 3.00 mmol) and the mixture was stirred for 30 minutes. A solution of 75 (1.49 g, 3.00 mmol) in DCM treated with DIPEA (0.47 g, 3.60 mmol) was added to the activated tripeptide solution, and the reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was washed with brine and the volatiles were then evaporated. The residue was purified on a silica column (DCM:MeOH, 100:1) and 1.6 g of product 83 (76%) were obtained. Mp: 127-129°C. $^1\text{H NMR}$ (CD$_3$OD, 600 MHz) $\delta$: 7.28 (m, 10H), 5.12 (m, 4H), 4.09 (m, 2H), 3.87 (m, 2H), 3.69 (s, 2H), 3.59 (s, 1H), 3.39 (m, 1H), 3.22 (m, 2H), 2.83 (m, 1H), 2.72 (m, 1H), 2.34 (m, 1H), 1.68 (m, 6H). Anal. Calcd for C$_{37}$H$_{37}$F$_{17}$N$_8$O$_9$: 0.15 H$_2$O: C, 41.79, H, 3.54, N, 10.54, F, 30.37. Found C, 41.42, H, 3.47, N, 10.47, F, 30.76. MS: Cald M+Na 1083.71. Found M+Na 1083.40.
L-Arginyl-glycyl-β-[N-(3,4,5,6,7,8,9,10-heptadeca-fluorodecanyl)amide]L-aspartic Acid (84)

83 (200 mg, 0.19 mmol) was dissolved in methanol (50 ml) and the solution was cooled to -20°C. 10% Pd/C (15 mg) was added and the mixture was shaken under hydrogen gas (30 psi) for 2 days at room temperature. The heterogeneous mixture was filtered over a celite bed, and the methanol was evaporated under vacuum to provide 0.15 g of product 84 (85%). Mp: > 210°C (darkens). 1H NMR (CD3OD, 400 MHz) δ: 4.37 (m, 1H), 4.01 (m, 2H), 3.81 (m, 1H), 3.43 (m, 2H), 3.18 (m, 2H), 2.69 (m, 2H), 2.42 (m, 2H), 1.70 (m, 6H). Anal. Cald for C22H26F17N7O5.1.5H2O.0.5MeOH: C, 32.38, H, 3.74, N, 11.75. Found C, 32.77, H, 3.47, N, 11.38. MS: Cald M+H 791.46, Found M+H 792.50.

N-α-Carbobenzyloxy-N-4-methoxy-2,3,6-trimethylbenzene-sulfonyl-L-arginyl-glycyl-N-(3,4,5,6,7,8,9,10-heptadeca-fluorodecanyl)ethyl-succinimide (85a)

To a solution of Cbz-Arg(Mtr)-Gly-Asp 68a (0.5 g, 0.64 mmol) in DCM (20 ml) was added CDI (0.12 g, 0.77 mmol) and the mixture was stirred for 30 minutes. A solution of 75 (0.48 g, 0.96 mmol) in DCM treated with DIPEA (0.33 g, 2.56 mmol) was added to the activated tripeptide solution, and the reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was washed with brine and the volatiles were then evaporated. The residue was purified on a silica column (DCM:MeOH, 75:1) and 0.58 g of product 85a (81%) were obtained. Mp: 120-123°C. 1H NMR (CDCl3, 600 MHz) δ: 7.98 (s, br, 1H), 7.82 (s, br, 1H), 6.49 (s, 1H), 6.27 (m, 3H), 5.01 (s, 1H), 4.43 (m, 1H), 4.22 (m, 1H), 3.89 (m, 2H), 3.79 (s, 1H), 3.72 (m, 2H), 3.14 (m, 1H), 2.89 (m, 1H), 2.67
N-α-Carbobenzyloxy-N\textsuperscript{G}-4-nitro-L-arginyl-glycinyl-N-(3,4,5,6,7,8,9,10-heptadeca-fluorodecanyl)ethyl-succinimide (85b)

To a solution of cbz-Arg(NO\textsubscript{2})-Gly-Asp 68b (0.25 g, 0.41 mmol) in DCM (15 ml) was added CDI (0.08 g, 0.49 mmol) and the mixture was stirred for 30 minutes. A solution of 75 (0.31 g, 0.62 mmol) in DCM treated with DIPEA (0.32 g, 2.48 mmol) was added to the activated tripeptide solution, and the reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was washed with brine and the volatiles were then evaporated. The residue was purified on a silica column (DCM:MeOH, 75:1) and 0.30 g of product 85b (78%) were obtained. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 600 MHz) \(\delta\): 7.66 (s, 2H), 7.32 (m, 5H), 5.47 (s, 1H), 5.03 (m, 2H), 4.52 (m, 1H), 3.82 (m, 4H), 3.33 (s, 1H), 3.23 (m, 2H), 2.97 (m, 1H), 2.64 (dd, 1H), 2.48 (m, 1H), 1.68 (m, 4H). MS: Calcd M+Na 975.57. Found M+Na 975.40.

N\textsuperscript{G}-4-methoxy-2,3,6-trimethylbenzene-sulfonyl-L-arginyl-glycinyl-N-(3,4,5,6,7,8,9,10-heptadeca-fluorodecanyl)ethyl-succinimide acetate (86)

85a (0.6 g, 0.54 mmol) was dissolved in methanol and 6 drops of acetic acid were added. The solution was cooled in a dry ice/acetone bath and 10% Pd/C (60 mg) were slowly added. The mixture was shaken under hydrogen gas (25 psi) for 12 hours at room temperature. The mixture was filtered over a celite bed, and the filtrate was collected. Methanol was evaporated and the resulting residue was washed with hexane (2 x 50 ml)
to remove the excess acetic acid. The residue was dried under vacuum, and 0.54 g of 86 were collected as an amorphous solid (95%). Mp: 115-116°C ¹H NMR (CD₃OD, 600 MHz) δ: 6.64 (s, 1H), 4.50 (m, 1H), 3.86 (m, 2H), 3.81 (s, 3H), 3.79 (s, 1H), 3.33 (s, 2H), 3.30 (m, 2H), 3.17 (m, 2H), 3.02 (m, 1H), 2.65 (m, 3H), 2.58 (s, 1H), 2.46 (m, 2H), 2.10 (s, 3H), 1.90 (s, 3H), 1.71 (m, 6H). Anal. Cald for C₃₄H₄₀F₁₇N₇O₉S: C, 39.05, H, 3.86, N, 9.38. Found C, 38.77, H, 3.95, N, 9.42. MS: Cald M+H 986.30. Found M+H 985.71.

[2-Dimethyl(amo no)ethyl]hydrazine (89)

N,N-Dimethylaminoethyl chloride hydrochloride 88 (50 g, 0.35 mol) was added over a period of 1 hour to a solution of hydrazine monohydrate 87 (59.6 g, 1.19 mol) in water (80 ml). The solution was stirred for 15 minutes, after which 2 equivalents of potassium carbonate (96.6 g, 0.7 mol) were added and the reaction mixture refluxed for 7 hours. After cooling to room temperature, the reaction mixture was made strongly basic (pH~12) by adding solid sodium hydroxide (120 g, 3.0 mol). The reaction mixture was then extracted diethyl ether (1 L). The organic layers were collected and the solvent was evaporated under vacuum. The resulting residue was distilled under 10 mm-Hg (water aspirator) and 22.4 g (62%) of compound 80 were collected as a colorless liquid at 74-75°C. Bp: 75°C at 10 mm-Hg (lit.¹⁴⁰ bp: 71-72°C at 18 mm-Hg). ¹H NMR (CDCl₃, 600 MHz) δ: 3.22 (bs, 3H), 2.65 (t, 2H), 2.232 (t, 2H), 2.031 (6H, s). ¹³C NMR (CDCl₃, 100 MHz) δ: 45.82, 53.35, 57.53.
2-[2-(Dimethyl(amo)no)ethyl]-5-(chloro)indazolo-[4,3-gh]isoquinolin-6(2H)-one (92)

A solution of 89 (3 g, 29 mmol) in anhydrous THF (10 ml) was added dropwise over a period of 30 minutes to a mixture of anthraquinone 90 (2.53 g, 9.6 mmol) and DIPEA (1.24 g, 9.62 mmol) in THF (50 ml). The reaction mixture was stirred at room temperature for 1 hour after which 150 ml of water were added. The reaction mixture was then extracted with methylene chloride (10 x 50 ml). The organic layers were collected and dried over anhydrous sodium sulfate. The solvent was evaporated under vacuum, and the resulting solid was purified by column chromatography using DCM: MeOH (50: 1) as eluent. 2.35 g of the desired product 92 were collected as a yellow solid (75%). Mp: 154-155°C. 1H NMR (CDCl₃, 400 MHz) δ: 9.42 (s, 1H), 8.77 (d, 1H), 8.10 (d, 1H), 7.61 (d, 1H), 7.50 (d, 1H), 4.567 (t, 2H), 2.90 (t, 2H), 2.30 (s, 6H). 13C NMR (CDCl₃, 100 MHz) δ: 180.63, 149.87, 145.85, 138.04, 137.90, 135.67, 131.53, 129.84, 124.98, 121.52, 121.37, 116.73, 59.14, 49.00, 45.88. Anal. Calcd for C₁₇H₁₅ClN₄O: C, 62.48, H, 4.63, N, 17.15, O, 4.90. Found C, 62.49, H, 4.58, N, 17.20, O, 4.73.

2-[2-(Dimethyl(amo)no)ethyl]-5-[[2-(dimethylamo)no)ethyl]amino]indazolo-[4,3-gh]isoquinolin-6(2H)-one (93, CD3-1)

A mixture of 92 (0.74 g, 2.26 mmole) and [2-(dimethylamo)no)ethyl]amine (1.4 g, 15.82 mmol) in dry pyridine (20 ml) was heated between 85°C-90°C under a nitrogen for 4.5 hours. Pyridine was then evaporated, and the resulting dark residue partitioned between brine (50 ml) and ethyl acetate (11 x 50 ml). The organic layers were collected, dried over sodium sulfate, and concentrated to 5ml. Hexane (20 ml) was added, and the mixture stirred for 1 hour, after which the desired product 93 was collected as an impure
orange solid. The orange solid was purified by column chromatography using CH$_2$Cl$_2$:MeOH as eluent starting with 10:1 ratio in a gradient elution process by gradually increasing the MeOH content until the lowest TLC $R_f$ material eluted. 0.5 g of pure 93 were collected (60%). Mp: 159-160°C (Lit. $^{136}$ mp: 150-152°C). $^1$H NMR (CDCl$_3$, 400 MHz) δ: 9.64 (s, 1H), 9.31 (t(br), 1H), 8.77 (d, 1H), 8.29 (d, 1H), 7.66 (d, 1H), 6.95 (d, 1H), 4.61 (t, 2H), 3.53 (q, 2H), 2.71 (t, 2H), 2.68 (t, 2H), 2.35 (s, 6H), 2.31 (s, 6H). $^{13}$C NMR (CDCl$_3$, 100 MHz) δ: 181.08, 150.23, 147.91, 145.95, 138.37, 134.43, 131.19, 125.54, 123.34, 120.66, 120.52, 113.81, 105.34, 59.53, 58.29, 49.20, 45.98, 45.80, 41.39. Anal. Cald for C$_{21}$H$_{26}$N$_6$O. 0.25 H$_2$O: C, 65.80, H, 6.91, N, 21.9, O, 5.22; Found C, 65.59, H, 6.75, N, 21.85, O, 5.14. MS: Cald M+H 379.47. Found M+H 379.29.

(Dimethyl(amo)no)ethyl]-5-[(2-(dimethylamino)ethyl]amino]indazo-lo-[4,3-gh]isoquinolin-6(2H)-one-bis-N-oxide trihydrochloride (95)

Compound 93 (0.3 g, 0.79 mmol) was dissolved in a mixture of methylene chloride and methanol (3:1) and kept at 0°C-5°C. 2.5 equivalents of oxaziridine 94 (0.67 g, 1.98 mmol) dissolved in methylene chloride, were added dropwise over a period of 1 hour. The reaction mixture was allowed to stir for 6 hours at 0°C-5°C in the dark. HCl gas was then passed through the reaction mixture until the pH was ca.1, wherein a dark-red solid appeared. The reaction mixture was then diluted with 30 ml of ethyl acetate and allowed to stir for 30 minutes. The solvent was evaporated under vacuum, and the dark-red residue was then stirred for 30 minutes in a mixture of EtOAc:CH$_2$Cl$_2$:MeOH (10:4:1). The dark red solid 95 was then filtered and dried for 10 hours under vacuum. 0.36 g of 95 were collected (87.6%). Mp: 204-206°C. $^1$H NMR (D$_2$O, 400 MHz) δ: 9.20 (s, 1H), 8.61
(Dimethyl(amo)no)ethyl]-5-[[2-(dimethylamino)ethyl]amino]indazolo-[4,3gh]-isoquinolin-6(2H)-one-N-oxide (96) and (97)

Anthrapyrazole 93 (0.1 g 0.26 mmol) was dissolved in a 3:1 mixture of CH₂Cl₂:MeOH, to which an equimolar amount of oxaziridine 94 (0.089 g; 0.26 mmol) dissolved in CH₂Cl₂, was added dropwise over a period of 30 minutes. The reaction mixture was allowed to stir at 0°C-5°C in the darkness for 3 hours, after which the solvent was evaporated under reduced pressure. The resulting orange residue was purified by column chromatography using a 3:1:0.5 mixture of MeOH:CH₂Cl₂:Isopropylamine as eluent. The product was collected as a 50:50 mixture of N-oxide 96 and 97 (0.051g, 49%). Mp: 141-142°C. ¹H NMR (CD₃OD, 400 MHz) δ: 9.20 (d, 2H), 8.63 (t, 2H), 8.08 (t, 2H), 7.81 (t, 2H), 7.10 (d, 1H), 6.96 (d, 1H), 5.03 (t, 2H), 4.60 (t, 2H), 4.06 (t, 2H), 3.99 (t, 2H), 3.69 (t, 2H), 3.59 (t, 2H), 3.30 (s, 3H), 3.22 (s, 3H), 2.95 (t, 2H), 2.73 (t, 2H), 2.40 (s, 3H), 2.32 (s, 3H). ¹³C NMR (CD₃OD, 100 MHz) δ: 179.22, 150.24, 149.47, 146.81, 146.65, 144.53, 144.47, 138.36,138.21, 133.91,133.24, 131.35, 130.92,125.47, 125.19, 122.49, 122.45, 121.04, 120.94, 120.71, 120.63, 114.36, 113.70, 104.78, 104.11, 68.15, 68.01, 58.63, 58.31, 58.15, 57.79, 44.55, 44.48, 44.01, 40.52, 37.39. Anal. Cald for
N-(4-Bromo-benzylidene)-benzenesulfonamide (100)

An equimolar mixture of p-bromobenzaldehyde diethyl diacetal 99 (2 g, 7.7 mmol) and benzenesulfonamide 98 (1.21 g, 7.7 mmol) was heated between 160°C-170°C. The reaction proceeded with the evolution of ethanol and the formation of 2.4 g of 100 as a white solid (99%). Mp: 205-207°C. ¹H NMR (CDCl₃, 400 MHz) δ: 7.56 (t, 2H), 7.63 (m, 3H), 7.78 (d, 2H), 7.99 (d, 2H), 9.01 (s, 1H). ¹³C NMR (CDCl₃, 100 MHz) δ: 169.48, 133.92, 132.87, 132.67, 131.40, 130.65, 129.44, 128.32, 127.76. Anal. Cald for C₁₃H₁₀BrNO₂S: C, 48.16, H, 3.11, N, 4.32, O, 9.87, S, 9.89. Found C, 48.00, H, 3.15, N, 4.48, O, 9.9, S, 9.97.

2-Benzensulfonyl-3-(4-bromo-phenyl)-oxaziridine (94)

Benzyldiene 100 (1.12g, 2.44 mmol), a saturated solution of sodium bicarbonate (10 ml), and 0.11 equivalent of benzyltriethylammonium chloride (BTEAC) (0.06 g, 0.26 mmole) were placed in a 500 ml three-necked flask equipped with magnetic stirrer. The reaction mixture was cooled to 0°C-5°C in an ice bath. A solution of 1.3 equivalent of m-chloroperbenzoic acid (0.54 g, 3.12 mmol) in chloroform was added dropwise over a period of 30 minutes, after which it was stirred for an additional 20 minutes. The chloroform layer was separated, washed successively with water (20 ml), 10% aqueous sodium sulfite (20 ml), and brine solution (10 ml). The chloroform layer was dried over sodium sulfate, and the solvent was evaporated under vacuum. 0.95 g (90%) of the
desired oxaziridine 94 were collected as a white solid after being crystallized from methanol. Mp: 124-125°C. \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\): 8.03 (d, 2H), 7.77 (t, 1H), 7.62 (t, 2H), 7.53 (d, 2H), 7.30 (d, 2H), 5.46 (s, 1H). \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\): 135.34, 134.82, 132.30, 130.03, 129.78, 129.65, 129.60, 126.26, 75.861. Anal. Cald for C\(_{13}\)H\(_{10}\)BrNO\(_3\)S: C, 45.90, H, 2.96, N, 4.12, O, 14.11. Found: C, 46.31, H, 2.92, N, 3.99, O, 14.22.

**Growth Inhibition Assay**

Growth inhibition assays were conducted based on the method employed in the NCI *in vitro* anticancer screening program.\(^{143}\) MCF7 and NCI/ADR-RES cells were grown in T-75 flasks containing RPMI 1640 medium with 2 mM L-glutamine, 2 mg/ml sodium bicarbonate, 25 mM HEPES, 5% fetal bovin serum (FBS), 5% NuSerum IV, and 50 µg/ml gentamicin. MCF12A cells were incubated in a 1:1 mixture of DMEM and F-12 media containing 2.5 mM L-glutamine, 2 mg/ml sodium bicarbonate, 15 mM HEPES, 10mg/L insulin, 500 µg/L hydrocortisone, 20 µg/L Epidermal Growth Factor, 100 µg/L cholera toxin, and 5% FBS. Cultured cells were passaged weekly and incubated at 37°C in a humidified 95% air/5% CO\(_2\). Following their removal from culture flasks by trypsinization,, the cells were counted on a hemocytometer and loaded into 96 well plates via 100 µl media per well at cell densities of 1800 cells per well (cpw) for MCF7, 3300 cpw for NCI/ADR-RES, and 1100 cpw for MCF12A. Following the overnight culturing of cells that allowed them to attach, test agents were added via a 100 µl media addition at five 10-fold serially diluted concentrations, while equivalent levels of DMSO (final level 0.25% DMSO) were added to control wells. Plates were cultured for 48 hours with test
agents, and then fixed with 50 µl per well of cold trichloroacetic acid (TCA). Fixed plates were refrigerated for 1-2 hours, after which they were rinsed five times with chilled deionized water and allowed to dry. Cultures were then stained with 50 µl per well of an aqueous solution containing 0.4% (wt/vol) sulforhodamine B (SRB) and 1% acetic acid in deionized water. Unbound dye was removed by five washes with 1% acetic acid in deionized water, and cultures were allowed to dry. The bound dye was extracted with 150 µl per well of 10 mM unbuffered Tri-base [tris(hydroxymethyl)aminomethane], and optical density was measured at 565 nm on a Molecular Devices M5 plate reader. One plate was fixed immediately after agent addition and then stained with other plates to provide a reading of the optical density at the start of the agent exposure period. The measure of the cell growth was determined by the difference between the optical density at the beginning and end of the agent exposure period. The measure of the fraction of control cell growth was obtained from the optical density ratio for a given test agent versus control wells. The test agent concentration that caused a 50% reduction in cell growth versus the control wells (GI$_{50}$) was estimated for each agent with each cell line from a Hill Equation fit of the fraction of control growth versus agent concentration.
Chapter 8

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Chapter 9

Appendix
The Boc group was removed from 16a to give 16b.