SYNTHESIS, CHARACTERIZATION, AND APPLICATIONS OF A MELAMINE BASED DENDRIMER WITH TWELVE CYSTEINE GROUPS ON THE PERIPHERY

A Thesis

by

BRANDON MARK VITTUR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Chemistry

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Approved by:

Chair of Committee,	Eric E. Simanek
Committee Members,	Jean-Philippe Pellois
	Daniel Singleton
Head of Department,	David Russell

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ABSTRACT

Synthesis, Characterization, and Applications of a Melamine Based Dendrimer with Twelve Cysteine Groups on the Periphery. (December 2009)

Brandon Mark Vittur, B.S., University of Houston

Chair of Advisory Committee: Dr. Eric E. Simanek

A potential drug delivery vehicle based on a melamine dendrimer with twelve free thiols on the periphery for constructing bio-labile disulfides has been synthesized. Under ideal conditions for the native chemical ligation reaction, attempts for attaching the cell penetrating peptide TAT, via native chemical ligation proved difficult due to the low solubility of the dendrimer. A camptothecin derivative containing a reactive disulfide was prepared for disulfide exchange with the melamine dendrimer. Up to 7 exchange reactions were achieved as determined by mass spectroscopy. NMR and mass spectroscopy was used to characterize all of the intermediates. Capping groups to replace the hydrophobic piperidine with more water-soluble groups to aid the ligation reaction and optimization of the disulfide exchange step to give 12 substitutions have been proposed for future studies. The end target is a peptide dendrimer containing a cell penetrating peptide to mediate endocytosis and a bio-labile linker connecting an anti-tumor drug to the dendrimer, which would ultimately be released inside the cancerous cell.

DEDICATION

To Suhyung Park, a tremendous friend and colleague who passed too soon.

ACKNOWLEDGEMENTS

I would like to thank my mother for keeping me out of trouble long enough for me to make it this far; my wife and the rest of my family for their support during the tough times; and my committee chair, Dr. Simanek, and my committee members, Dr. Singleton, and Dr. Pellois, for their guidance and support throughout the course of this research.

Thanks also go to my friends, colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

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CHAPTER I

INTRODUCTION

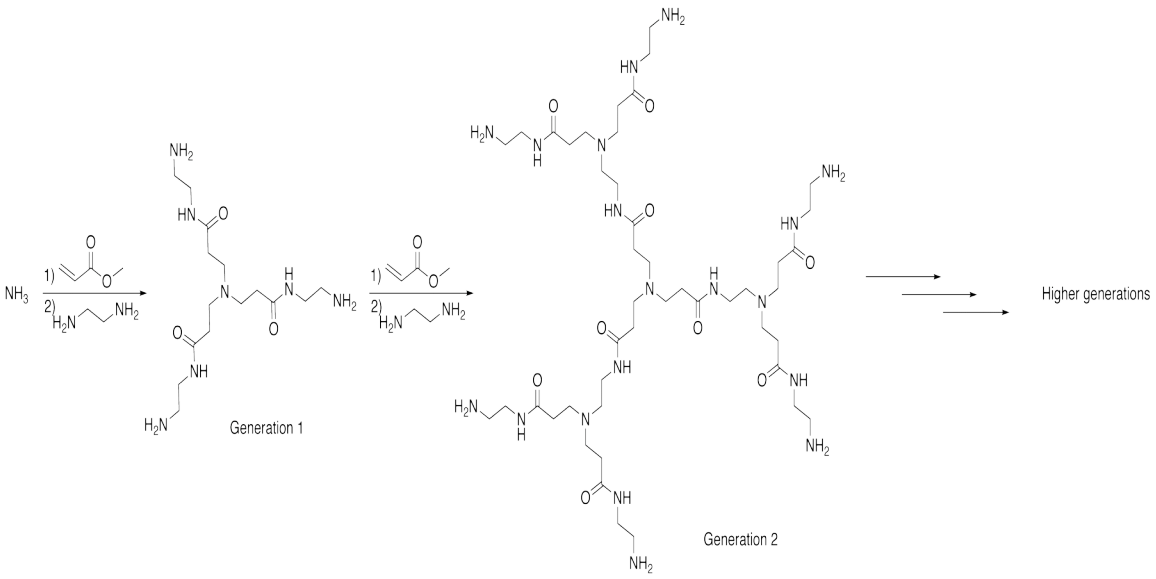
Dendrimers: A Brief Historical Perspective

"Few revolutionary findings in science are ever accepted without a predictable period of rejection"[1]

The above quote was an attempt by Professor Flory to console Dr. Tomalia during the infancy of dendrimers. In 1985 a new class of macromolecules emerged termed starburst polymers.[2] The building blocks to these novel polymers were referred to as dendrimers. Donald Tomalia coined this term due to their dendritic topology. The first dendrimers were named PAMAM or polyamido amine, and were synthesized by Michael Addition of ammonia or ethylenediamine with methyl acrylate followed by amidation with ethylene diamine (Scheme 1.1).

This thesis follows the style of *Journal of the American Chemical Society.*

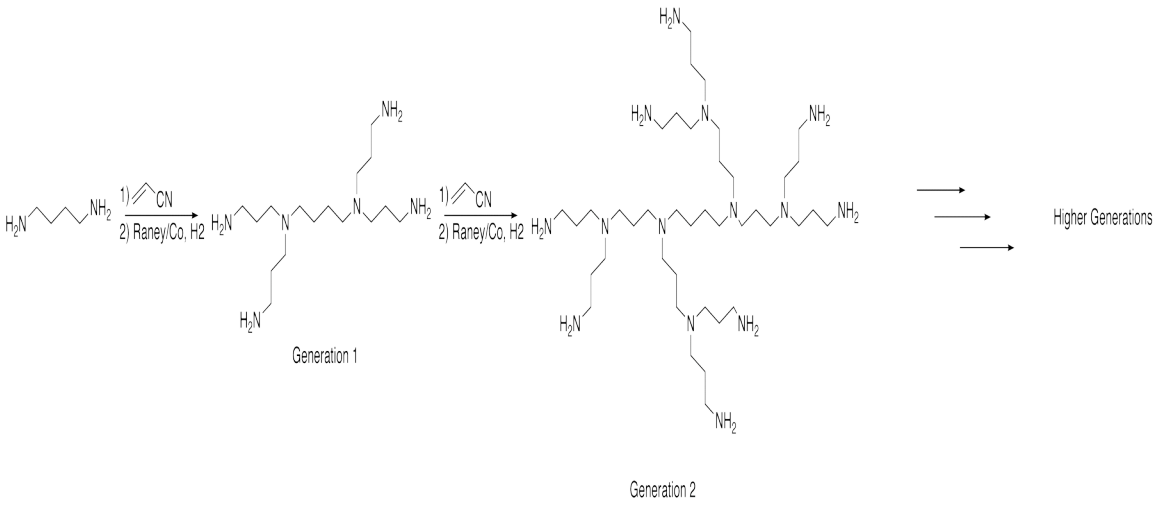
Scheme 1.1. Synthetic scheme for the synthesis of PAMAM dendrimers leading to higher generations.



PAMAM dendrimers were plagued in the lab with incomplete and retro Michael reactions, intramolecular cyclizations, and solvolysis of the terminal functionality as well as in the scientific community with doubts of monodispersity and their ability to exhibit host-guest properties.[1]

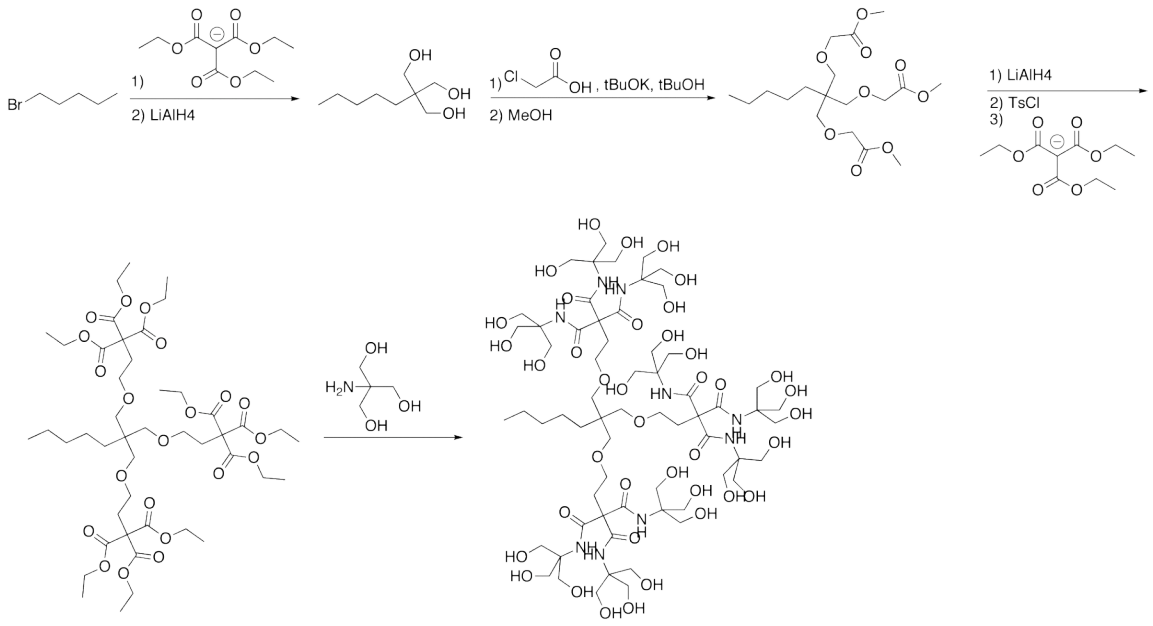
Dendrimer synthesis actually began in 1978 by Vogtle.[3] In his paper, he describes the synthesis of cascade molecules now known as poly(propylene imine) (PPI) dendrimers (Scheme 1.2). The difference being the core, where Vogtle originally used pyridine-2,6-diyldimethanamine, 1,3-phenylenedimethanamine, and ethane-1,2-diamine, and now butane-1,4-diamine is commonly used.

Scheme 1.2. Synthetic scheme for PPI dendrimers leading to higher generations.[3]



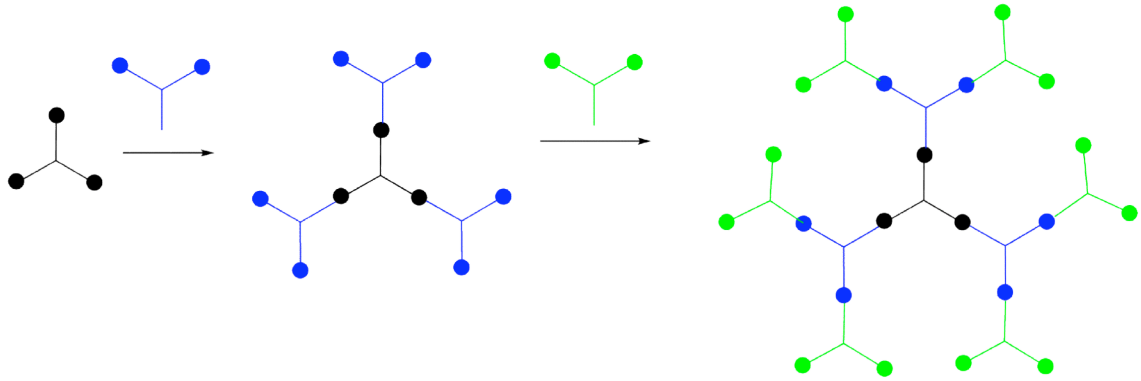
In the same year Tomalias' seminal paper was published, a paper by Newkome[4] describing molecules called aborols was published (Scheme 1.3) displaying a dendritic structure. Newkome predicted expanding the synthesis of these one-directional molecules to two-directional would give a symmetrical molecule with a cavity capable of host-guest interactions.

Scheme 1.3. Synthetic scheme for aborols.[4]



There are essentially two ways to synthesize dendrimers: divergently or convergently. Less common are the accelerated and orthogonal approaches. The first dendrimers relied on the divergent approach,[2,4] which consist of branching out from a central core by addition of a monomer (Scheme 1.4).

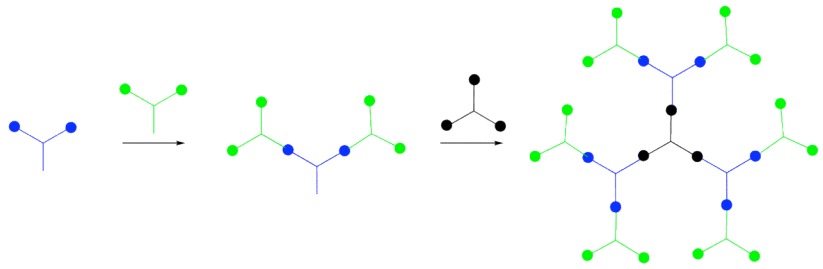
Scheme 1.4. Generation growth by the divergent route.



Each round of monomer addition yields a new generation (i.e. addition of the monomer to the core yields generation 1 (G1) of that dendrimer, addition of the monomer to G1 yields generation 2 (G2) of that dendrimer, etc.) As the generation is increased by one, the number of surface groups increases exponentially. In order to form a dendritic structure, each peripheral reactive site must react during successive generation synthesis. This attribute has some drawbacks, particularly at higher generations where reactions become increasingly more difficult, giving incomplete reactions (structural defects) due to steric hindrance between the peripheral groups.

The other most common route is the convergent approach[5] where the peripheral groups are built first and the core is installed in the final step of dendrimer synthesis (Scheme 1.5).

Scheme 1.5. Generation growth by the convergent route.

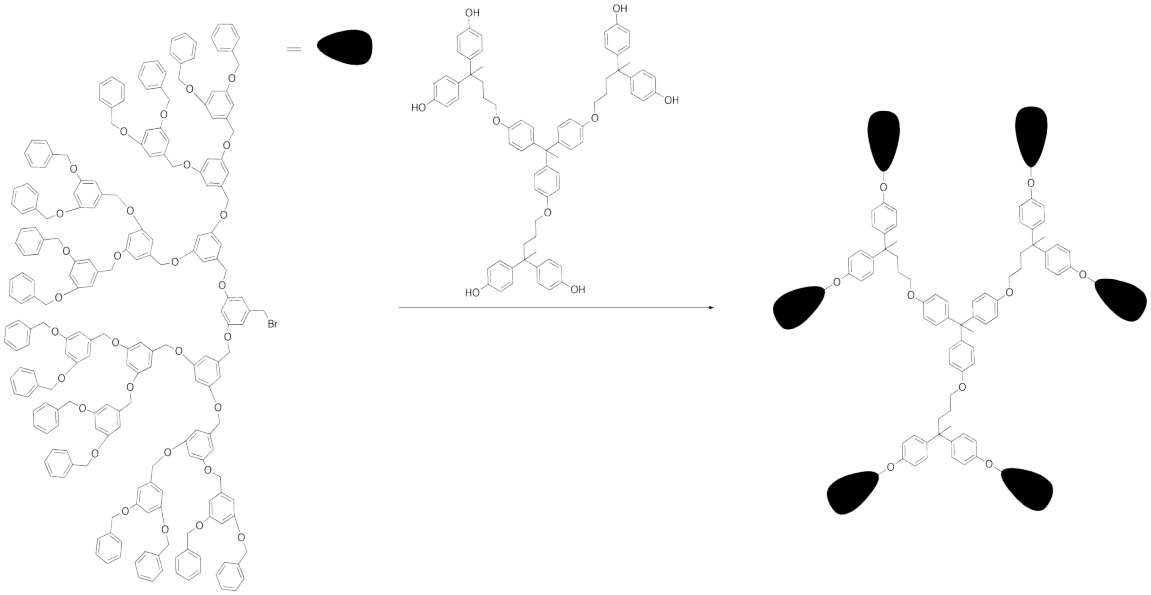


This approach has some advantages (as well as disadvantages) over the divergent approach including fewer structural defects and easier purification.

Mass spectrometry has become an invaluable tool for dendrimer chemist when probing sample purity. Grayson and Frechet[6] have noted that divergent approaches to dendrimer synthesis generally gives a mixture of similar products, usually due to incomplete reactions. Convergent approaches however show a high degree of monodispersity.

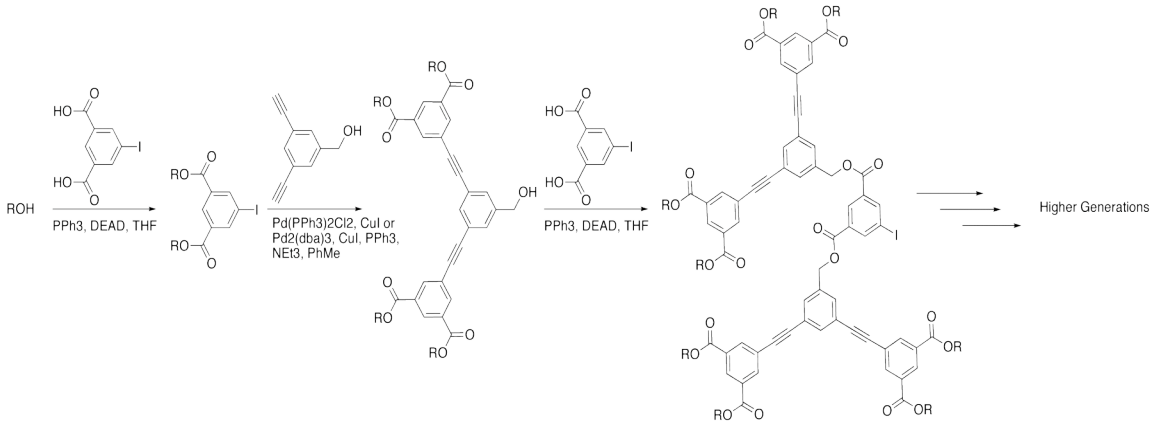
Employing both routes, referred to as an accelerated approach, can often times accentuate the advantages of both routes and yield higher generation dendrimers (divergent) with fewer steps (convergent). For example, a G7 poly (aryl ether)[7] was constructed (Scheme 1.6) by reacting a G3 dendrimer, referred to as a hypercore synthesized divergently, with a G4 dendron (a fraction of a dendrimer or the molecule attached to the core in the last step) synthesized convergently.

Scheme 1.6. Dendrimer synthesis by the accelerated approach.[7]



Using monomers with differential reactivity give rise to orthogonal approaches to dendrimer synthesis. A lack of reactivity control necessitates the need for activation and deprotection steps, however if one can take advantage of the differential reactivity of different sites orthogonal to each other, one can bypass these steps. Zeng and Zimmerman[8] were the first to demonstrate this approach using (4-*tert*-Butylphenoxy)ethanol, 5-iodoisophthalic acid and (3,5-diethynylphenyl)methanol (Scheme 1.7). These building blocks were chosen to synthesize dendrimers by the Mitsunobu and Sonogashira reactions.

Scheme 1.7. Dendrimer synthesis by the orthogonal approach.[8]

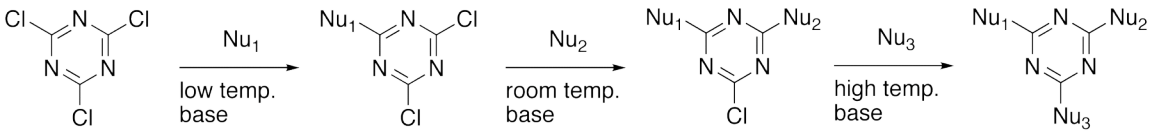


The ability to control these macromolecules with such elegance has led to a wide variety of applications particularly in the medical field as drug delivery vehicles.[9-28] Of particular attractiveness is the ability to release these small molecules through changes in the environment (i.e. pH and red/ox). This exceptional control along with the enhanced permeability and retention (EPR) effect[29] holds great potential for dendrimers in targeted drug delivery.

Melamine Based Dendrimers

The synthesis of dendrimers based on melamine relies on iterative reactions of cyanuric chloride and diamine linkers.[30] Scheme 1.8 shows how nucleophilic aromatic substitution of cyanuric chloride offers a chemoselective synthetic strategy where the degree of substitution is controlled by temperature.

Scheme 1.8. Temperature controlled chemoselectivity of cyanuric chloride.[30]



Both convergent and divergent strategies were used to synthesize our first dendrimer (Figure 1.1), a G3 dendrimer based on melamine with *p*-aminobenzylamine as the substituent used to propagate to higher generations.[31] The substituent was chosen so as to circumvent the use of protecting groups or functional group manipulations during the convergent approach. This is possible due to the differential reactivity of the diamine with the benzylic amine being far more reactive than the aniline amine. For this particular dendrimer the convergent approach gave higher yields and purity compared to the divergent approach.

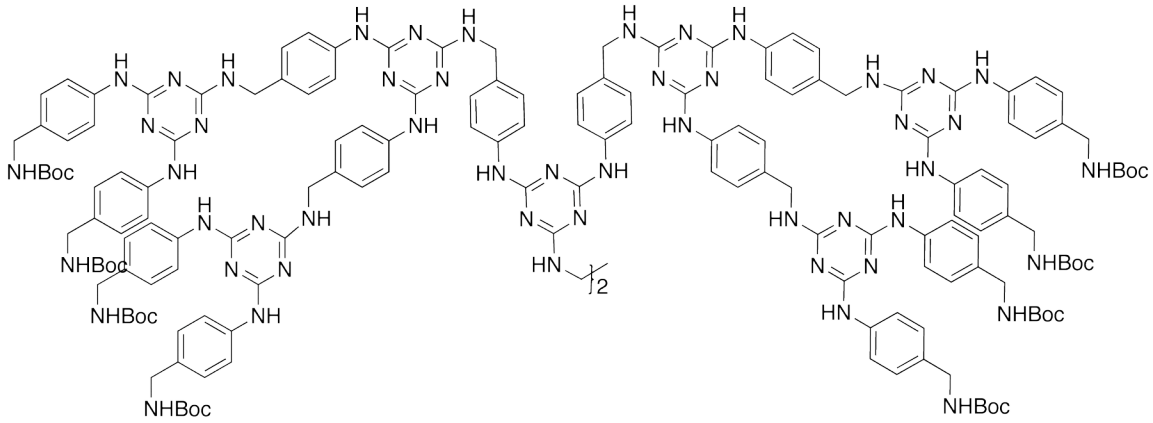


Figure 1.1. G3 dendrimer based on melamine with *p*-aminobenzylamine as the substituent used to propagate to higher generations.[31]

In order to expand the utility of our dendrimers, an orthogonally protected dendrimer, which could be manipulated upon selective removal of protecting groups was constructed.[32] This "fruit salad tree" bears 16 Boc-protected amines, 4 free hydroxyls, 4 TBDPS protected alcohols, 4 pyridyl disulfides and 4 levulinic acid groups (Figure 1.2). Though this degree of diversity is excessive, it serves as a proof of concept for the complexity achieved with triazine dendrimers.

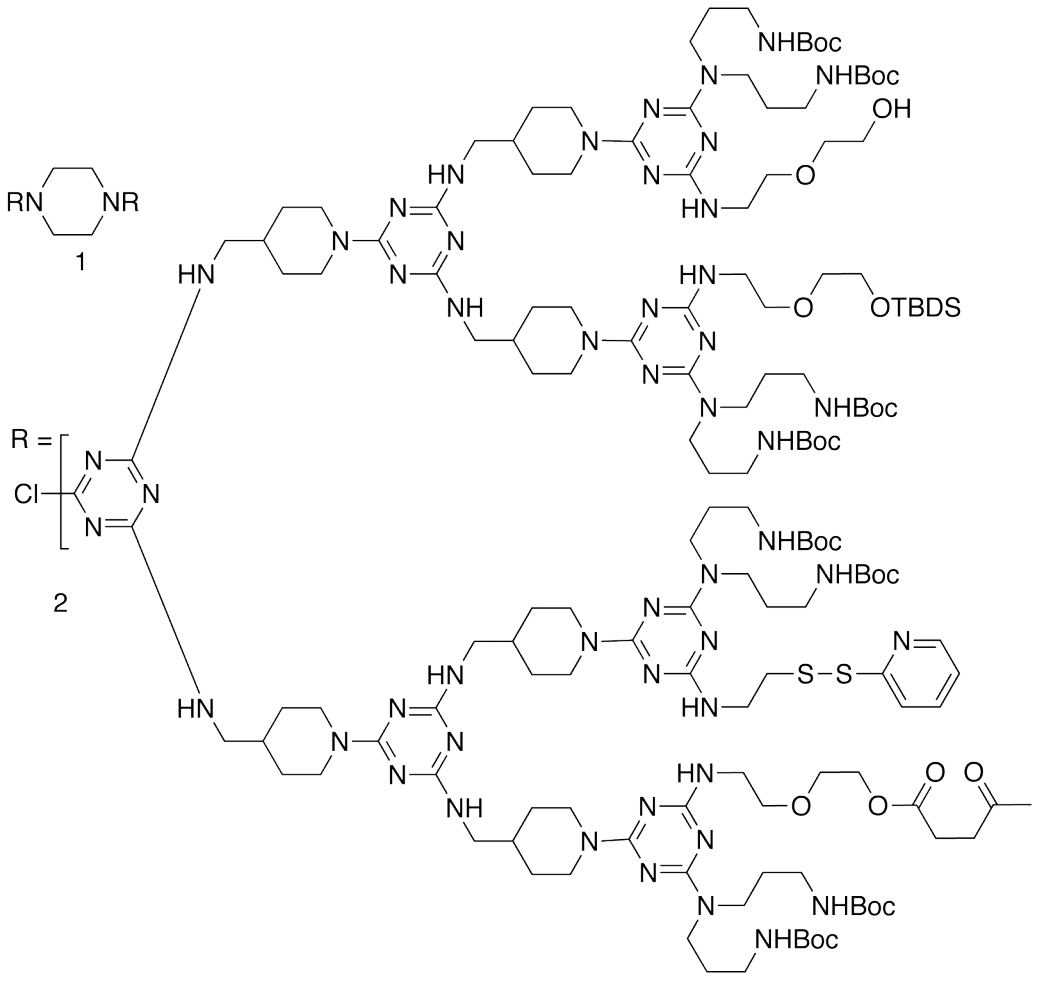
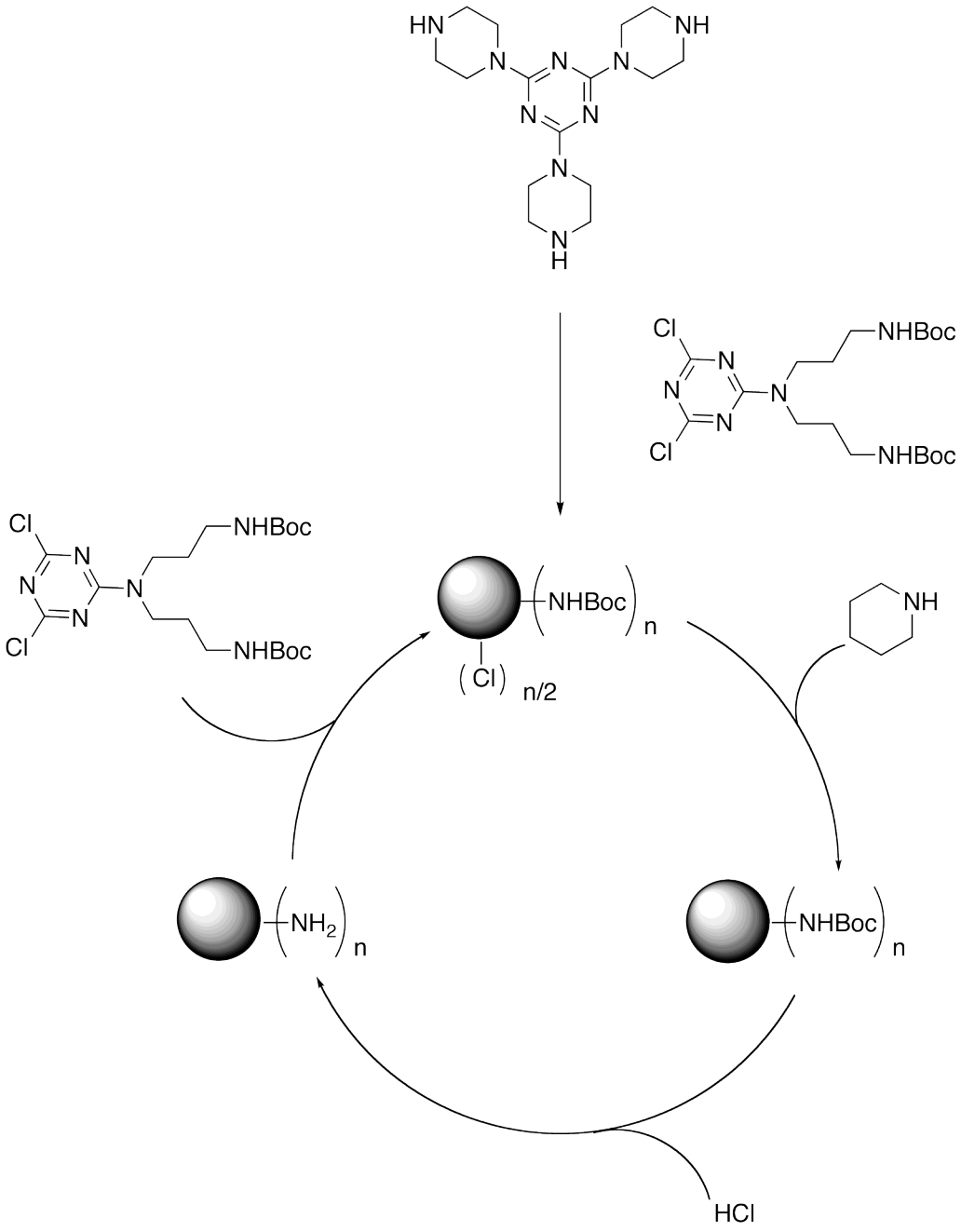


Figure 1.2. Melamine dendrimer with 16 Boc-protected amines, 4 free hydroxyls, 4 TBDPS protected alcohols, 4 pyridyl disulfides and 4 levulinic acid groups on the periphery.[32]

Using an iterative divergent approach G1-5 dendrimers based on melamine were synthesized.[33] As expected, higher generations gave impure products. The divergent route consisted of addition of the core to a dichlorotriazine monomer, capping with piperazine, then Boc-deprotection to yield the first generation. Subsequent addition, capping, and deprotection yield higher generation dendrimers (Scheme 1.9).

Scheme 1.9. Generation propagation scheme used to generate up to G5.[33]



Efforts in drug conjugation began with a dendrimer having three orthogonally reactive groups on the surface and one on the interior totaling twenty six reactive sites for manipulation.[34] These sites are optimal for attaching drugs, enhancing solubility and biocompatibility, attaching biodistribution tags, and targeting ligands. A Bolton-Hunter type reagent, used as a biodistribution tag, was attached to the internal monochlorotriazine (Figure 1.3).

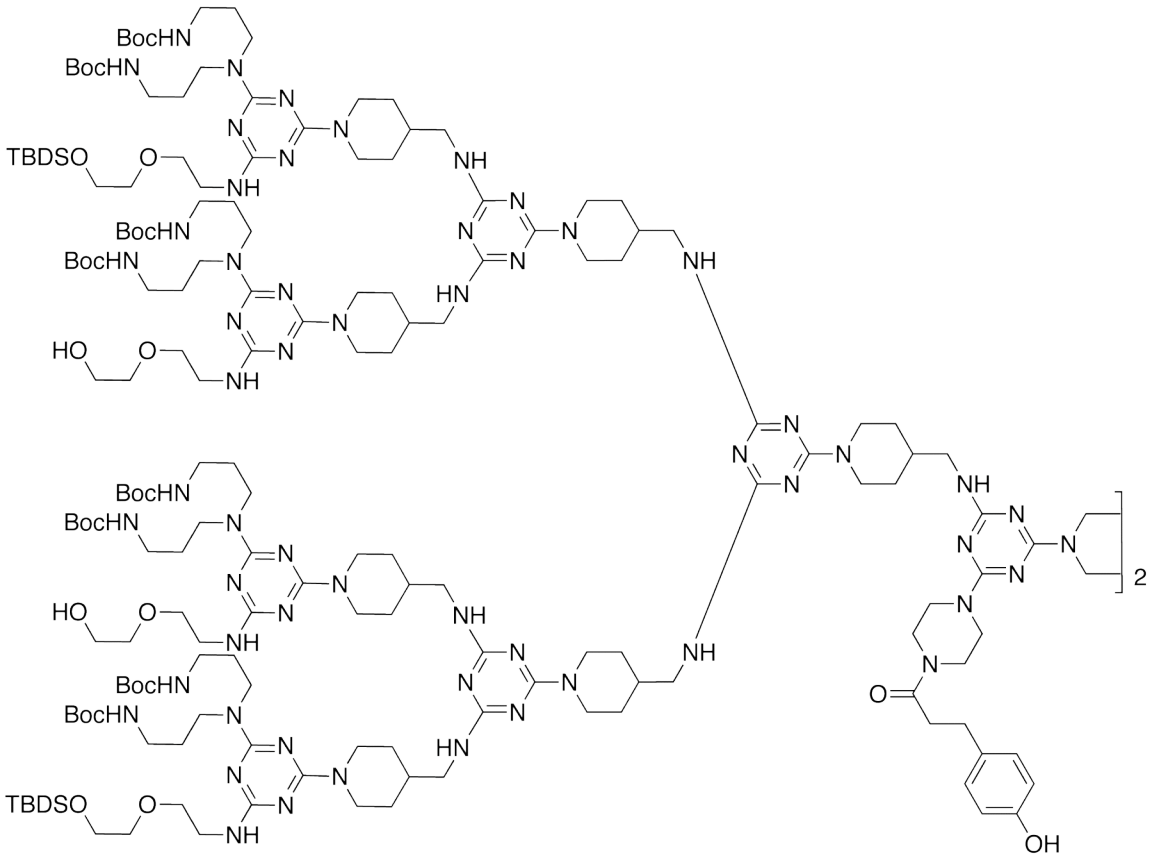


Figure 1.3. Melamine dendrimer with three orthogonally reactive groups on the surface and one on the interior totaling twenty six reactive sites for manipulation.[34]

A small library of dendrimers was prepared from a G3 dendrimer with AB_4 groups on the periphery and a G1 super core (Figure 1.4).[35] Seven different dendrimers were synthesized with 48 peripheral sites, containing a primary amine, guanidine, sulfonate, phosphonate, carboxylate, or poly(ethylene glycol) chains on each site. The various dendrimers were prepared in order to study the cytotoxicity, hemolysis, and acute in vivo toxicity of dendrimers based on melamine, to assess their practical use as drug delivery vehicles. The cationic dendrimers were more cytotoxic and hemolytic than their anionic and PEGylated counterparts. No significant toxicity *in vivo* for the PEGylated dendrimer was observed.

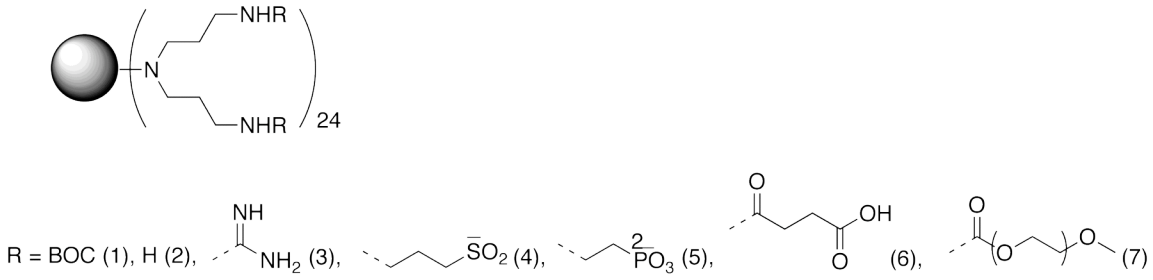


Figure 1.4. A melamine dendrimer with 48 peripheral sites, containing either a primary amine, guanidine, sulfanate, phosphonate, carboxylate, or poly(ethylene glycol) chains on each site.[35]

A dendrimer with 24 Boc-protected amines and 12 Dde-protected amines was synthesized and modified by site selective deprotection followed by acylation to give 12 pyridyl protected thiols and 24 PEG chains.[36] The protected thiols allow for attachment of thiol containing molecules through disulfide exchange while the PEG chains enhance their water solubility, biocompatibility, and biodistribution. Steric hindrance prevented

the synthesis of the target dendrimer using the convergent approach whereas the target dendrimer was obtained in 66% overall yield using an accelerated approach.

The solubility enhancement of drugs including indomethacin, methotrexate,10-hydroxycamptothecin, and bisindolemethane with melamine dendrimers was studied.[37] Melamine dendrimers did not increase the solubility of indomethacin or methotrexate. Increased solubility of 10-hydroxycamptothecin (3.7 molecules solubilized/dendrimer) and bisindolemethane (4.5 molecules solubilized/dendrimer) was observed however. Of particular interest was the dendrimer-bisindolemethane complex is as active as the drug solubilized by DMSO in cell culture. The dendrimers alone did not show any apparent toxicity in mice at 1mg/kg, 2.5mg/kg, or 10mg/kg.

A mixture of thirteen-sixteen Paclitaxel (Taxol) molecules - a clinically relevant anticancer drug insoluble in water - were attached to a melamine dendrimer through a labile ester (Figure 1.5).[38] The dendrimer also contained two Bolton-Hunter type groups, used for biodistribution studies. The resulting dendrimer drug conjugate is water soluble with minimum PEG chains of 2kDa.

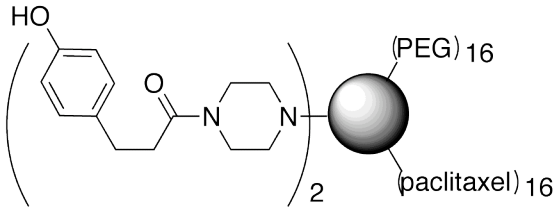


Figure 1.5. A melamine dendrimer bearing 16-paclitaxel drugs, 16-PEG chains, and a Bolton-Hunter group for biodistribution studies.[38]

Attachment of peptides to melamine dendrimers was performed on three G2 and G3 dendrons having either one or two pyridyl protected thiols (Figure 1.6).[39] The DNA oligonucleotide used to simultaneously probe multiple target oligonucleotides contains a terminal cysteine residue and was attached via disulfide exchange to the dendrons.

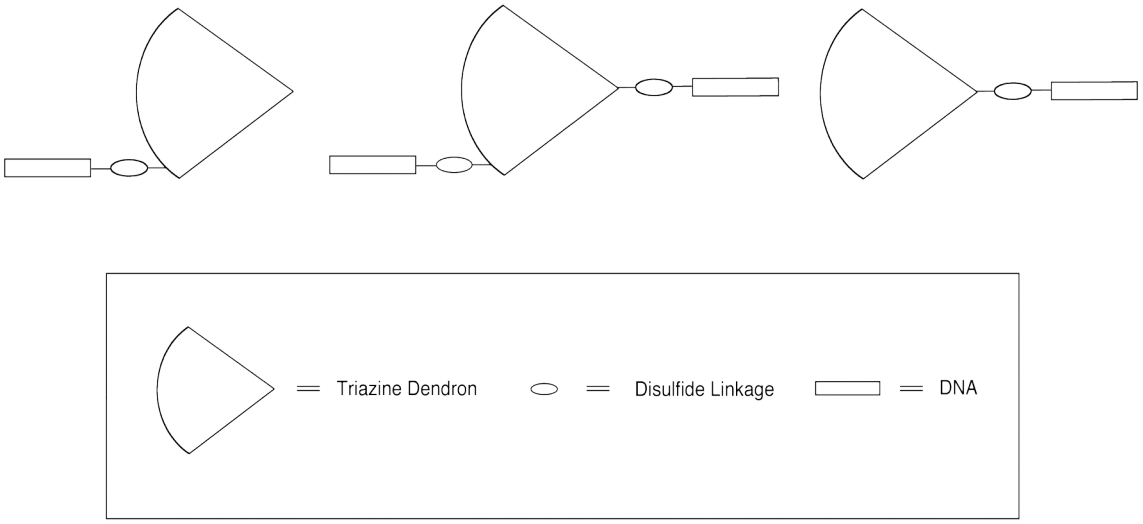


Figure 1.6. Melamine dendron with a DNA oligonucleotide attached either on the periphery, at the focal point or both.[39]

In another effort to attach peptides to our dendrimers via disulfide exchange the cysteine-terminated peptide CLKKDRA was used.[40] Captopril, a small drug with a thiol functional group, was attached in the same manner. The dendron (tetravalent scaffold) gives almost quantitative yields with the peptide however the dendrimer (octavalent scaffold) only gives the hexavalent product even after 10 days in excess of the peptide, presumably due to sterics. Captopril however proceeds cleanly to the tetravalent and the octavalent products.

To study the disulfide exchange mechanism with our dendrimers, studies with five different dendrimers differing in size, valency, and placement of the disulfide was performed.[41] The rate of exchange decreases upon increasing dendrimer size. Disulfides closer to the core have slower exchange rates compared with those on the periphery. Particularly interesting was the fact that exchange is a bimolecular process between dithiothreitol and the dendrimer and no intramolecular exchange was observed.

Using the same divergent strategy for preparing up to G5 dendrimers, our group has prepared a G2 dendrimer on kilogram scale.[42] This paper has put our dendrimers one step closer to becoming clinically relevant molecules. The next step, one in which this thesis describes, is the manipulation of this dendrimer to prepare a drug delivery vehicle.

CHAPTER II

CYSTEINE FUNCTIONALIZED DENDRIMERS

Introduction

Cysteine is a ubiquitous molecule in biology and biochemical applications. Cysteine contains three functional groups offering a wide range of attachment to various biologically relevant molecules. Dendrimers possessing cysteine are generally derivatized through the reactive thiol by forming a thioether, sulfoxide, disulfide, or thiosulfoxide.

A general method for preparing peptide dendrimers with various linkages including oxime, hydrazone, and thiazolidine was developed using a lysine dendron with a reactive aldehyde at the focal point.[43] A peptide with an aminooxy, hydrazide, or cysteine on the terminus was reacted with the lysine dendron to form an oxime, hydrazone, or thiazolidine linkage respectively. The strategy described uses base mediated ligation of native peptides in aqueous media to form peptide dendrimers of high molecular weight. The rates of ligation can be varied by changes in pH and temperature, and also by the presence of organic co-solvents. This strategy is applicable to the synthesis of cyclic peptides, the semi-synthesis of proteins, and the attachment of biologically relevant molecules to proteins.

A biocompatible and biosensitive G4 PAMAM dendrimer was prepared by coupling a G4 PAMAM dendrimer to Boc-Cys(Acm).[44] After Boc-deprotection, the amines were coupled to PEG chains. Upon removal of the Acm groups, the free thiols

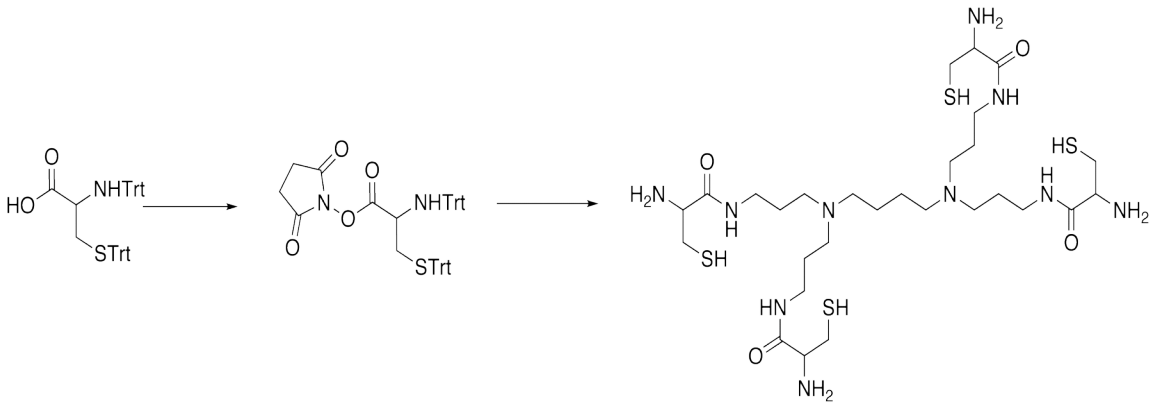
were thought to form disulfides as determined by Ellman's method due to the presence of I_2 (used to remove Acm groups). To determine the utility of this dendrimer as a drug delivery vehicle, rose bengal was used as a small molecule representative. Using dithiothreitol (DTT) to invoke reductive conditions, a substantial increase in the amount of rose bengal molecules incorporated into the interior of the dendrimer was observed compared to the oxidized form of the dendrimer. The network of disulfides on the periphery of the dendrimer effectively controlled the access of small molecules due to its sensitivity to the red/ox conditions of the environment, indicating its use as an effective drug delivery vehicle.

Glycopeptide dendrimers were prepared with various glycoside moieties (β-glucose, α-galactose, α-N-acetyl-galactose, or lactose) on the periphery and a cysteine residue at the core.[45] A derivative of colchicine, an inhibitor of mitosis, was attached to the dendrimers via a disulfide bond. The peripheral glycoside moieties were chosen to mimic the uptake of glycoproteins by glycoprotein receptors on the cellular surface. All dendrime-colchicine conjugates displayed cytotoxicity and the glycosylated dendrimers were found to be more active than their non-glycosylated counterparts. The dendrimers showed higher selectivity for tumor cells over normal cells compared to free colchicine. Of interest to our strategy is the link between the dendrimer and the drug by a thioether, sulfoxide, disulfide, or thiosulfoxide bond does not have a pronounced effect on the bioactivity of the conjugates.

In 2005, Baal et al. coupled cysteine to G1, 2, and 3 PPI dendrimers (Scheme 2.1) in order to construct multivalent dendrimers through native chemical ligation of the

dendrimer with peptides and proteins.[46] The G1 and 2 dendrimers were fully ligated with the peptide LYRAG giving single components, as determined by mass spec. G3 however, gave mixtures of 12-16 ligations presumably due to steric hindrance. Ligation between G1 and the green fluorescent protein gave a mixture of products with 1-4 proteins attached. Full conversion is presumed to be inhibited by limited solubility of the protein under the conditions required for native chemical ligation as well as sterics. The authors have also prepared a G1 protein-dendrimer construct with the protein occupying one of the four terminal cysteine groups while peptides (GRGDSGG-MPAL) where ligated to the remaining three.

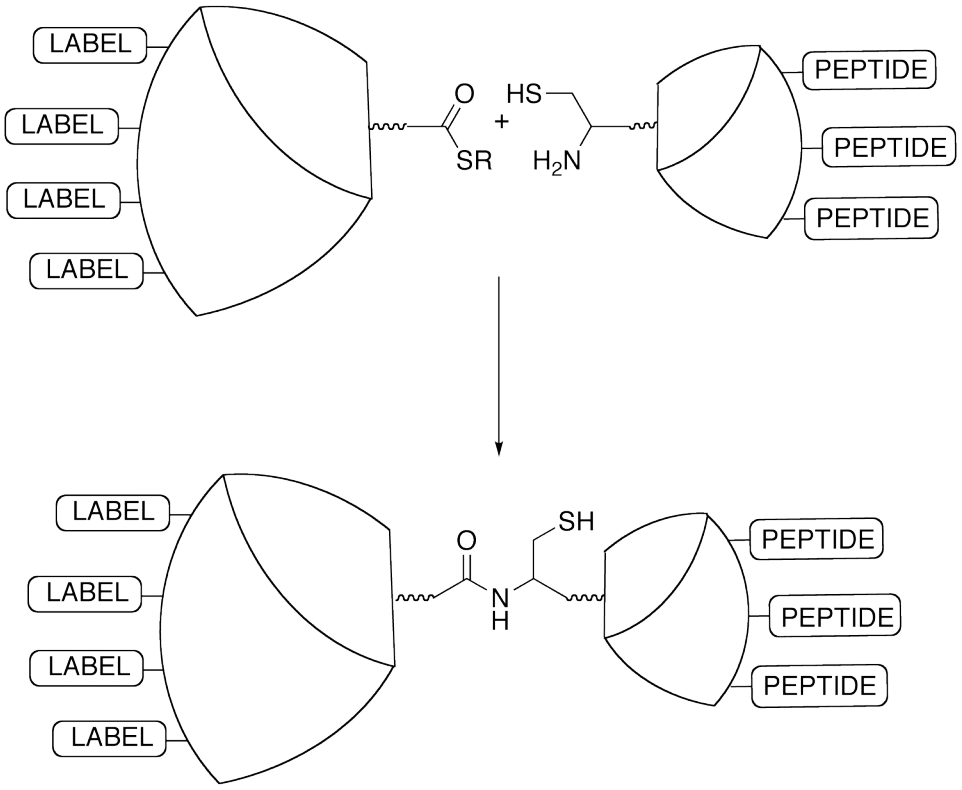
Scheme 2.1. Synthetic scheme for a G1 PPI dendrimer coupled to cysteine for native chemical ligation.[46]



In 2006 Baal et al. also successfully coupled two poly(lysine) dendrons to develop multivalent target-specific MRI contrast agents for *in vivo* imaging (Scheme 2.2).[47] One dendron contained an oligopeptide (RGDS) sequence which binds to $\alpha_v\beta_3$ integrins and the other a chelating agent (diethylenetriaminepentaacetic acid) used in

magnetic resonance imaging. The labeled dendron contained a thioester at the focal point and the RGDS dendron contained a cysteine residue at its focal point, allowing native chemical ligation between the dendrons.

Scheme 2.2. Poly(lysine) dendrons coupled by native chemical ligation for multivalent target-specific MRI contrast agents for in vivo imaging.[47]



Using a tris-alkene triazine core and 1-thiolglycerol, a G4 dendrimer was divergently synthesized using thiol-ene click chemistry.[48] The synthesis was performed in a metal catalyst and solvent free environment with trace amounts of the photo initiator 2,2-dimethoxy-2-phenylacetophenone. Four functional thiols including N-fmoc-cysteine,

were coupled to the dendrimer through thiol-ene click chemistry as proof of concept for functionalizing the periphery. These terminal reactions were less than optimal requiring some solvent and an increase of the photo initiator from 2-mol % to 10-mol %.

A library of cobalamin peptide dendrimers was synthesized as potential delivery vehicles for vitamin B_{12}.[49] The peptide dendrimers contained either a cysteine or histidine residue near the core and glutamate residues on the periphery. The thiol from the cysteine residue coordinates to the cobalt in vitamin B_{12}, however it rapidly forms disulfides with free cysteine or glutathione allowing release of the cargo.

In an effort to develop a vaccine against Alzheimer's dementia (AD), β-Amyloid epitopes extended by a cysteine molecule were attached to oligopeptides through a thiol ether bond.[50] Epitopes with an N-terminus cysteine residue provided higher flexibility and antibody accessibility compared to their C-terminus counterparts. A lysine dendrimer was among the carriers studied and was found that the different carrier types did not show a pronounced influence on antibody recognition.

Two G4 PAMAM dendrimers coupled to S-nitrosothiols were synthesized as delivery vehicles for nitric oxide.[51] Nitrosothiols are the main carriers of NO *in vivo* and regulate several biological processes including vasodilation, platelet activation, neurotransmission, and tissue inflammation. The dendrimer was coupled to either N-acetyl-D,L-penicillamine (NAP) or N-acetyl-L-cysteine (NACys), and subsequently converted to their nitrosothiol analogues by reacting the free thiol with $NaNO_2$ under acidic conditions. The G4-NACysNO dendrimer was more resilient with regard to changes in light intensity than the NAPNO dendrimer. These findings lead to more

resilient strategies of NO storage and handling, such as the use of higher generation dendrimers or the attachment of other biologically relevant materials.

A G4 PAMAM dendrimer was coupled to pyridylmercaptopropionic acid for use in delivery of N-Acetyl-L-cysteine (NAC), an anti-inflammatory agent used for treatment of neuroinflammation, stroke, and cerebral palsy.[52,53] NAC was attached through a bio-labile disulfide through disulfide exchange with the pyridyl group. The dendrimer effectively transported and released NAC inside the cell. This method has improved the efficacy of NAC by up to one order of magnitude.

A G1 dendron developed by Newkome was manipulated to include a protected thiol at the focal point and guanidine groups on the periphery to act as a molecular transporter.[54] The thiol was deprotected and attached to a polymeric scaffold through a maleimide group. This transporter was further engineered by attaching protected thiols in the form of pyridylmercaptopropionic acid through free amines on the scaffold. The authors reported the attachment of 24 copies of the peptidic cargo giving a 30% drug load. Labeling studies showed cleavage and subsequent release of the cargo peptides from the scaffold and the dendron was critical for intracellular delivery of the cargo.

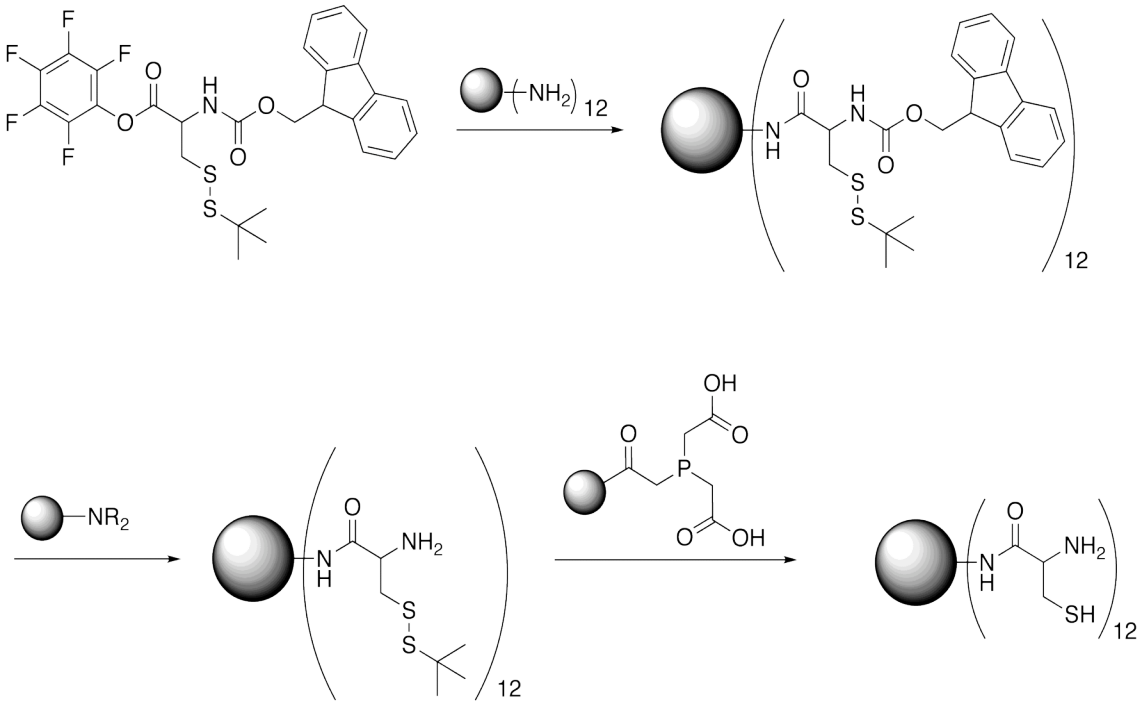
Results and Discussion

Synthesis

Following a similar procedure developed by Baal et. al.[46] a melamine based cysteine dendrimer was synthesized for use in thiol assisted acylation reactions including native peptide ligation. The initial strategy (Scheme 2.3) included two solid phase

deprotection steps in order to provide an unprotected cysteine dendrimer with a simplified purification protocol compared to solution phase. This approach however was quickly derailed by the fmoc deprotection step using polymer-supported amines (Figure 2.1). Full fmoc deprotection proved difficult possibly due to sterics or other factors that were not explored. Solid supported bases attempted were piperizine, morpholine, BEMP, NH_2, and TBD though to no avail.

Scheme 2.3. Initial synthetic strategy for melamine dendrimers with cysteine groups on the periphery.



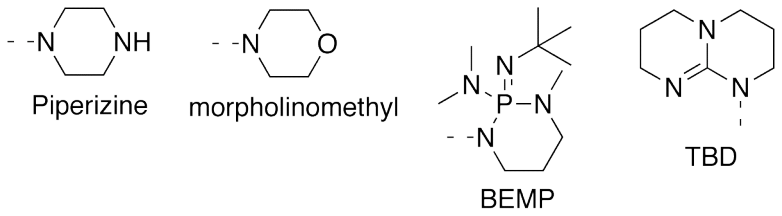


Figure 2.1. Polymer supported amines used for fmoc deprotection.

Due to the unavailability of the penta fluoro phenolic ester used initially, the non-activated cysteine precursor was employed. DCC proved to be the coupling reagent of choice giving full substitution on the dendrimer. Figure 2.2 shows a peak at 7941.96 (calc. 7918) corresponding to full substitution.

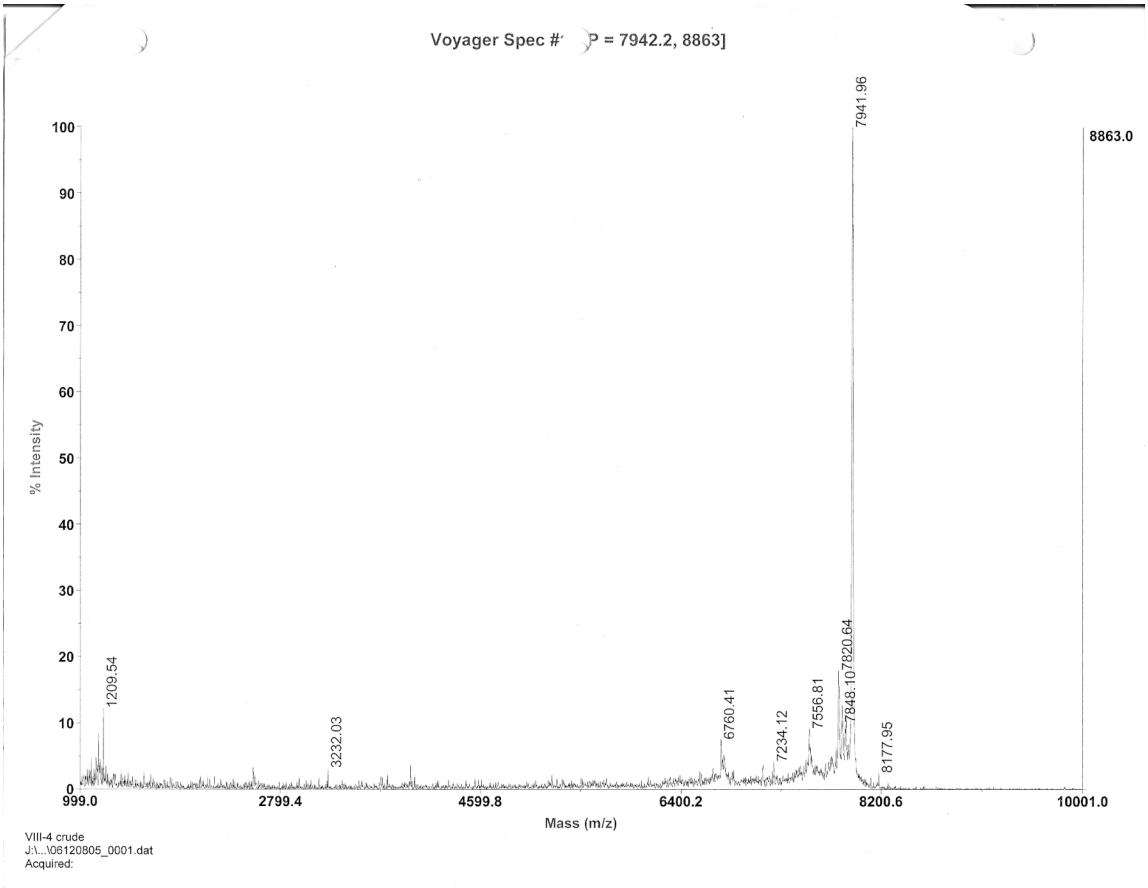


Figure 2.2. Mass Spectrum of G2-Cys-NHFmoc(SStbutyl).

Solution phase deprotection of the fmoc group with piperidine and DBU gave full conversion to the amine. Figure 2.3 shows a peak at 5267.09 (calc. 5252) corresponding to full deprotection.

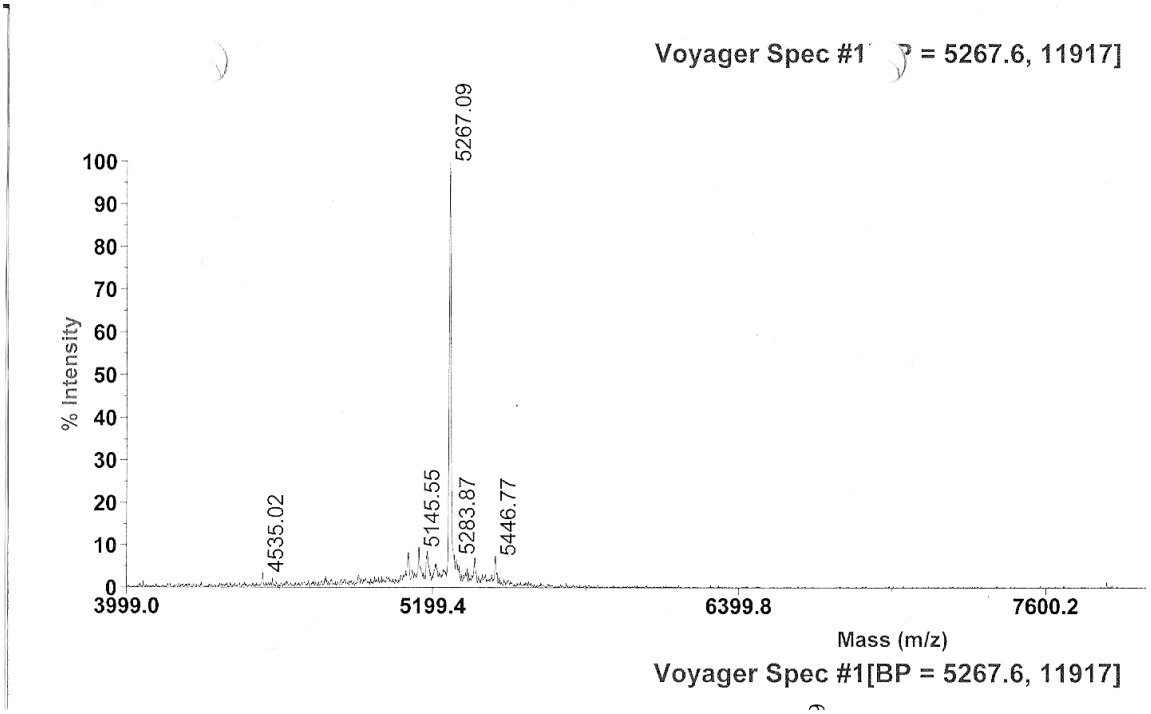


Figure 2.3. Mass Spectrum of G2-Cys-NH_2(Stbuthio).

TCEP was added and gave full reduction of the disulfide. Figure 2.4 shows a peak at 4206.89 (calc. 4194) corresponding to full deprotection. Solid phase reduction with immobilized TCEP was not attempted.

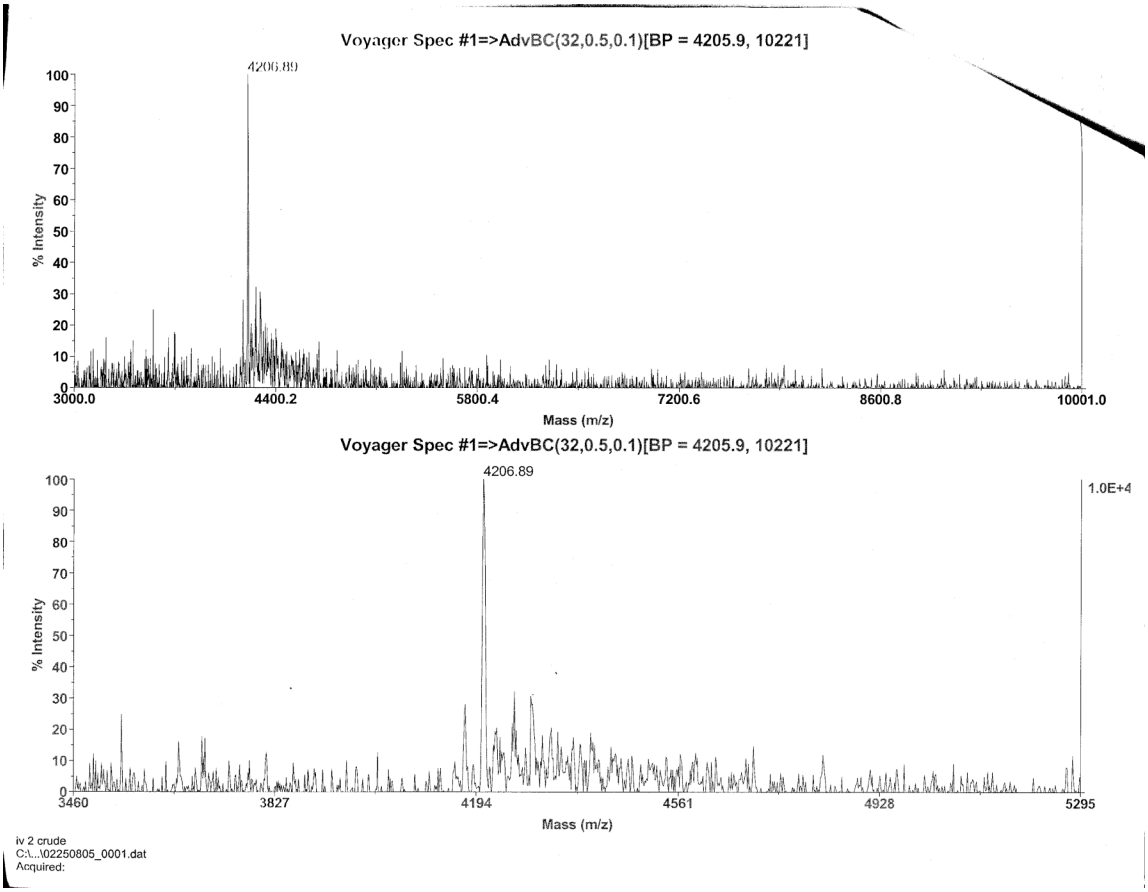
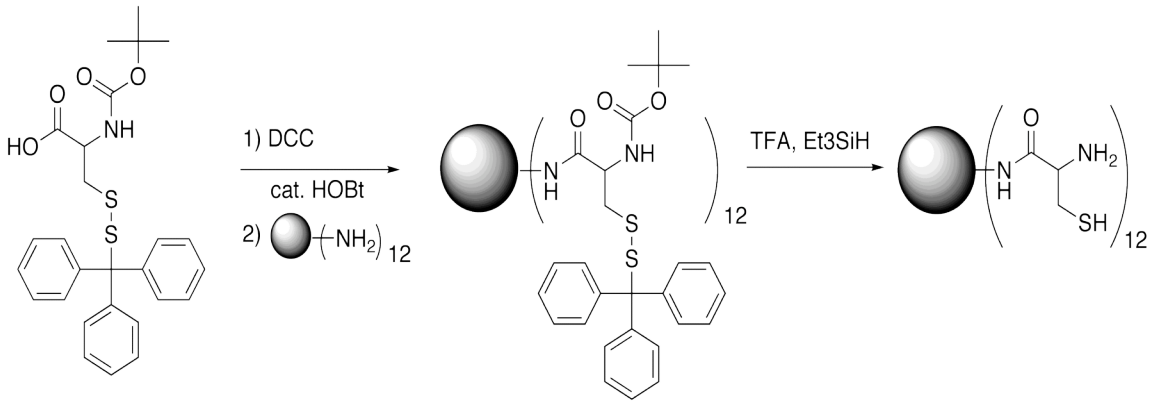


Figure 2.4. Mass Spectrum of G2-Cys.

We opted not to deprotect the disulfide after our initial success, as this would prove unnecessary due to the reductive conditions used in native chemical ligation. Difficulty during the native ligation step gave rise to our current strategy (Scheme 2.4), which relies instead, on a global solution phase deprotection of the Boc and Trt groups by TFA and scavenging of the Trt cation by Et_3SiH.

Scheme 2.4. Current synthetic strategy for a cysteine dendrimer based on melamine.



1g batches of the protected dendrimer have been prepared with mass spec and NMR showing full substitution after 24h in 14% yield. Deprotection has been performed on 100mg scale giving full deprotection based on mass spec and NMR after 48h to give the product (Figure 2.5) in 98% yield.

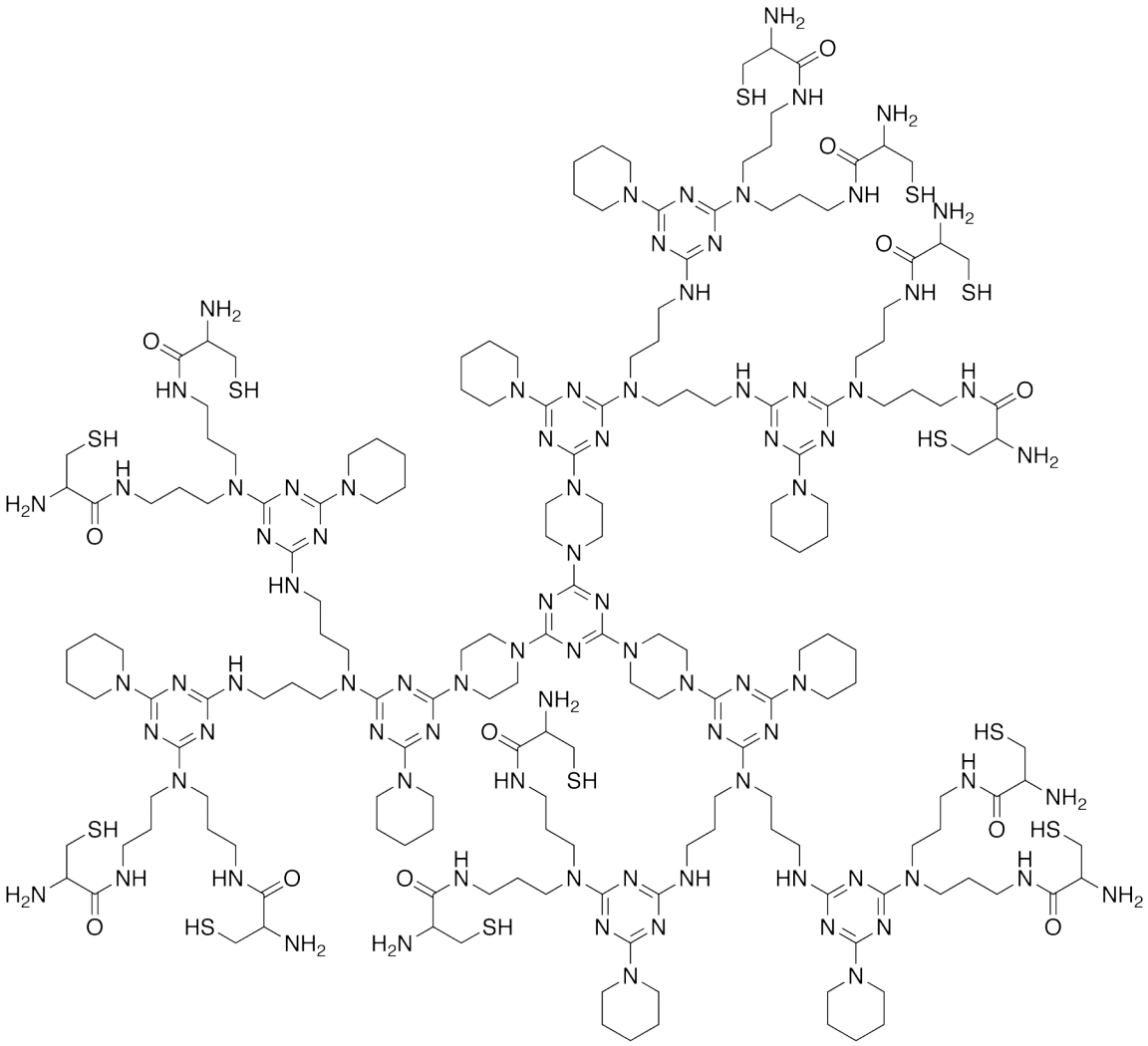


Figure 2.5. Full chemical embodiment of a G2 cysteine dendrimer based on melamine.

Characterization

Characterization of the protected cysteine dendrimer is relatively straightforward. There are four major peaks from the cysteine derivative in ^{1}H NMR (Figure B.1): 7.5-7.15 corresponds to the protons on the trityl protecting group; 1.39 corresponds to the

methyl groups on the boc protecting group; 4.01 corresponds to the α-proton; and 2.55 corresponds to the β-protons. These assignments were made by comparing the product to G2-NH_2, assigned previously,[33] and the cysteine precursor, N-Boc-Cys(Trt)-OH. The β-protons are diastereotopic, however they overlap giving a single peak, which integrates accordingly. ^{13}C assignment is also straightforward (Figure B.2): 170.36 corresponds to the carbonyl carbon of cysteine; 144.38 corresponds to the carbonyl carbon on the boc protecting group; 129.5, 127.97, and 126.75 correspond to the phenyl carbons on the trityl protecting group; 80.06 corresponds to the tertiary carbon on the boc protecting group; 66.88 corresponds to the α-carbon; 33.92 corresponds to the β-carbon; and 28.31 corresponds to the methyl groups on the boc protecting group. Mass spectroscopy shows a peak at 8308.35 (Figure B.3) corresponding to twelve substitutions (calcd. 8290.86), a peak at 8066.99, corresponds to twelve substitutions with fragmentation by loss of a trityl group (M – 244), and a peak at 7862, corresponds to eleven substitutions (M – 446).

Confirmation of the deprotection step was made by a lack of peaks in the aromatic region and at 1.39 ppm of the proton spectra indicating full deprotection of the trityl-protecting group and the boc protecting group respectively (Figure B.4). The peak at 4.05 corresponds to the α-proton. The peak at 2.95 corresponds to the β-protons. ^{13}C (Figure B.5) shows a peak at 155.35, that may correspond to the carbonyl carbon of the Boc group, however a lack of peak at 80.06 corresponding to $C(CH_3)_3$ indicates full deprotection. Mass spectroscopy shows a peak at 4193 (Figure B.6) corresponding to

full deprotection (calcd. 4194), a peak at 4215 corresponds to M + Na, a peak at 4248 (M + 55) and 4270 (M + 77) both are currently unassigned.

Experimental Section

General

All reagents were used without purification. The procedure for synthesis of G2-NH_2 has been reported previously.[33] N-Boc-Cys(Trt)-OH was purchased from Nova-Biochem.

Synthesis

G2-CysProt - 8.41g of cysteine was dissolved in 40ml of $CHCl_3$ and cooled to 0 ^oC. 3.74g of DCC was added to the cooled solution and stirred at 0 ^oC for 30min. The solution was then added to a solution of G2-NH_2 (2.99g) in 40ml $CHCl_3$. 6ml of Et_3N and 3mg of HOBt was subsequently added. The reaction was removed from the ice bath and stirred at RT for 24hrs. The urea precipitate was removed by filtration. The resulting solution was concentrated and the product was precipitated with MeOH to give 1.17g (14%) of product. ^1H NMR (300 MHz, $CDCl_3$) δ 7.49-7.15 (br, 180H, Trityl), 7.0-4.9 (br, 30H, NH), 4.01 (br, 12H, α-H Cysteine), 3.8 (br, 24H, CH_2, piperazine), 3.68 (br, 36H, $C_5H_{10}N$, α-H), 3.42 (br, 36H, CH_2, NCH_2) 3.33 (br, 12H, CH_2, C*H*$_2$NH-C_3N_3), 3.07 (br, 24H, C*H*$_2$NHCys), 2.55 (br, 24H, β-H Cysteine), 1.96 (br, 12H, G1-NCH_2CH_2), 1.60 (br, 24H, G2-NCH_2CH_2), 1.59-1.50 (br, 54H, $C_5H_{10}N$, γ-$C_5H_{10}N$, β-H), 1.39 (s, 108H, $C(CH_3)_3$). $^{13}C\{^1H\}$ NMR (75.5 MHz, $CDCl_3$) δ 170.36 (s, Cysteine

Carbonyl), 166.14 (s, C_3N_3), 165.32 (s, C_3N_3), 164.90 (s, C_3N_3), 155.35 (s, C(O)), 144.38 (s, 4° C Trityl) 129.50 (s, β-C Trityl), 127.97 (s, α-C Trityl), 126.75 (s, γ-C Trityl), 80.06 (s, *C*$(CH_3)_3$), 66.88 (s, α-C Cysteine, $C(Ph)_3$), 44.3 (br), 42.2 (br), 37.01 (s, *C*H_2NHCys), 28.31 (s, $C(CH_3)_3$, 27.9 (s, NCH_2CH_2), 25.76 (s, $C_5H_{10}N$, β-C), 24.92 (s, $C_5H_{10}N$, γ-C). MS (MALDI): calc. 8290.86 (M^+); found 8308.35 (broad).

G2-Cys - 0.98g of G2-CysProt was dissolved in 10ml of TFA with 1.5% EtSiH at 0°C. The reaction was left to stir for 48h to ensure complete deprotection. The solution was diluted with 20ml of H_2O and the product was purified by washing with Et_2O (3x20ml) and subsequently lyophilized to give 490mg (98%) of white solid. ^{1}H NMR (300 MHz, D_2O) δ 4.20 (br, 30H, NH), 4.05 (br, 12H, α-H Cysteine), 3.9-3.0 (br, 132H, CH_2 piperazine, $C_5H_{10}N$ α-H, NCH_2, C*H*$_2$NH-C_3N_3, C*H*$_2$NHCys), 2.95 (br, 24H, β-H Cysteine), 1.90-1.10 (br, 90H, G1-NCH_2CH_2, G2-NCH_2CH_2, $C_5H_{10}N$ γ-$C_5H_{10}N$ and β-H). $^{13}C\{^1H\}$ NMR (75.5 MHz, D_2O) δ 167.76 (s, Cysteine Carbonyl), 165.0 (br, C_3N_3), 54.56 (s, α-C Cysteine), 52.59 (br), 45.8 (br), 37.69 (br, *C*H_2NHCys), 27.0 (br, NCH_2CH_2), 25.0 (br, $C_5H_{10}N$ β-C, $C_5H_{10}N$ γ-C). MS (MALDI): calc. 4194 (M^+); found 4191.76 (broad)

CHAPTER III

APPLICATIONS OF CYSTEINE DENDRIMERS BASED ON MELAMINE

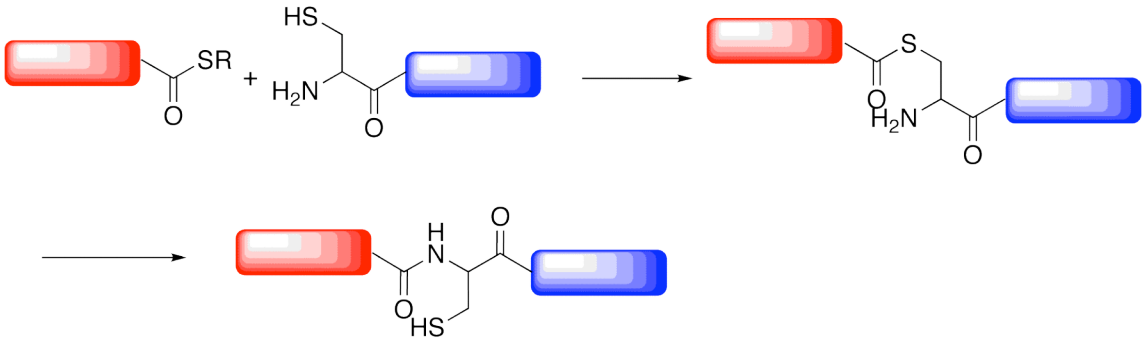
Peptide and Protein Dendrimers

Introduction

Until the advent of various ligation techniques - including thioester, thioether, oxime, directed disulfide, thiazolidine, and peptide bond formation - peptide synthesis was a difficult task limited to approximately 50 amino acids in length.[55] The classic synthesis of peptides involved protected functional groups that had several limitations, including difficult preparation and purification of the protected segments, solubility issues and difficult characterization.

In the early 1990's Kent et al[56] developed a chemoselective strategy involving unprotected peptide segments. The early use of the strategy allowed for the synthesis of both enantiomers of HIV-1 protease, the protein like TASP molecule, analogs of the HIV-1 protease, heterodimers of b/HLH/Z transcription factors, as well as receptor mimetics.[55] In 1994 native chemical ligation (Scheme 3.1) was introduced by Dawson and Kent[57] giving a natural peptide bond at the ligation site.

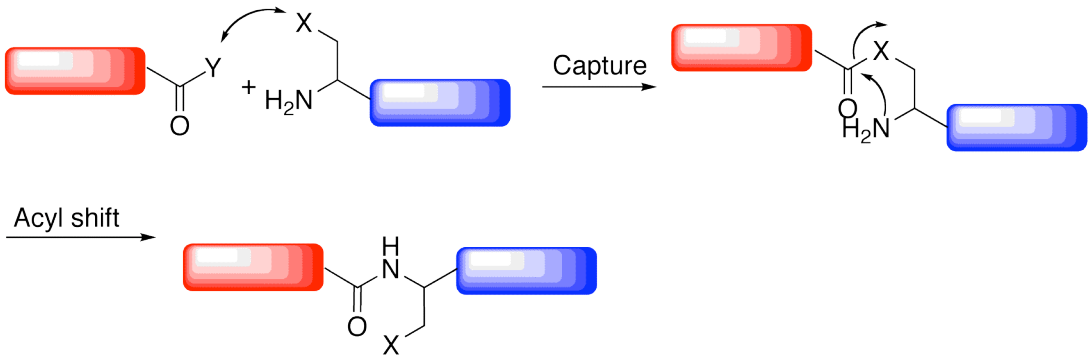
Scheme 3.1. Native chemical ligation of a cysteine terminated peptide with a thiol ether terminated peptide.[57]



This reaction is performed under physiological conditions with a high degree of chemoselectivity. An interesting feature is that the reaction occurs at the N-terminal cysteine residue regardless of other cysteine residues present in either segment. This strategy has been used for the synthesis of natural and non-natural peptides and proteins, and also for the coupling of peptides to constructs such as dendrimers.[58-62]

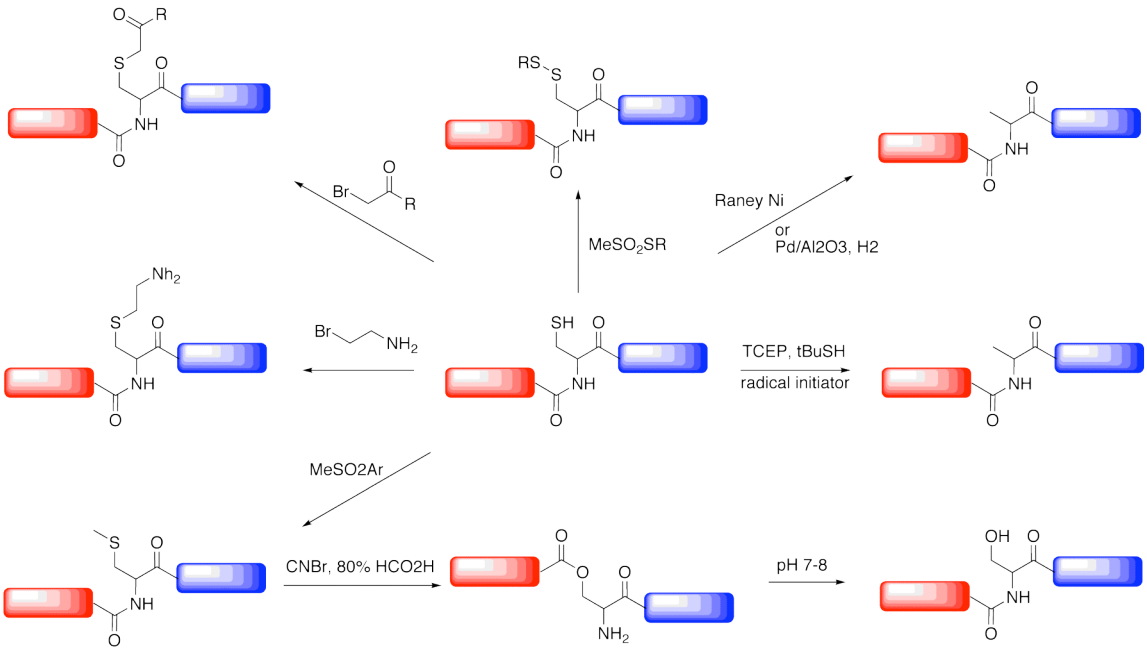
The various ligation strategies[63-65] (Appendix A) for peptides and proteins developed generally follow scheme 3.2.

Scheme 3.2. General mechanistic scheme for peptide ligation.



Several post-modifications to the newly formed internal cysteine residue to give natural as well as non-natural residues are shown in scheme 3.3.[63]

Scheme 3.3 Post chemical modification upon native chemical ligation.



Peptide dendrimers are dendrimeric structures that have covalently attached peptidic frameworks (on the terminus and/or within the structure), or non-covalently attached peptides (encapsulated via hydrogen bonds or ionic interactions). Numerous synthetic routes to construct peptide dendrimers have been reviewed by Sadler,[62] Tam[66] and Crespo et al.[58] The synthetic strategy of the peptide dendrimer described herein takes advantage of native chemical ligation.

Peptide dendrimers have been studied extensively for their potential as delivery vehicles, diagnostic reagents, vaccines and inhibitors.[47,58-62,66-71] Of particular interest to our research is the utility of chemical ligation of cell penetrating peptides (CPP) with cysteine-functionalized dendrimers as a means to gain entry into the cell. Once inside the cell, the peptide terminated dendrimer will act as a cargo delivery vehicle, which can ultimately be varied by attaching different cargo and/or peptides depending on the desired task.

TAT (Figure 3.1) is a CPP of interest because no cell line appears to resist uptake, translocation is achieved within 30 seconds (5 minutes for other CPPs), and it is able to deliver various cargo intracellulary with molecular weights several times greater than its own (mw = 1746).[72]

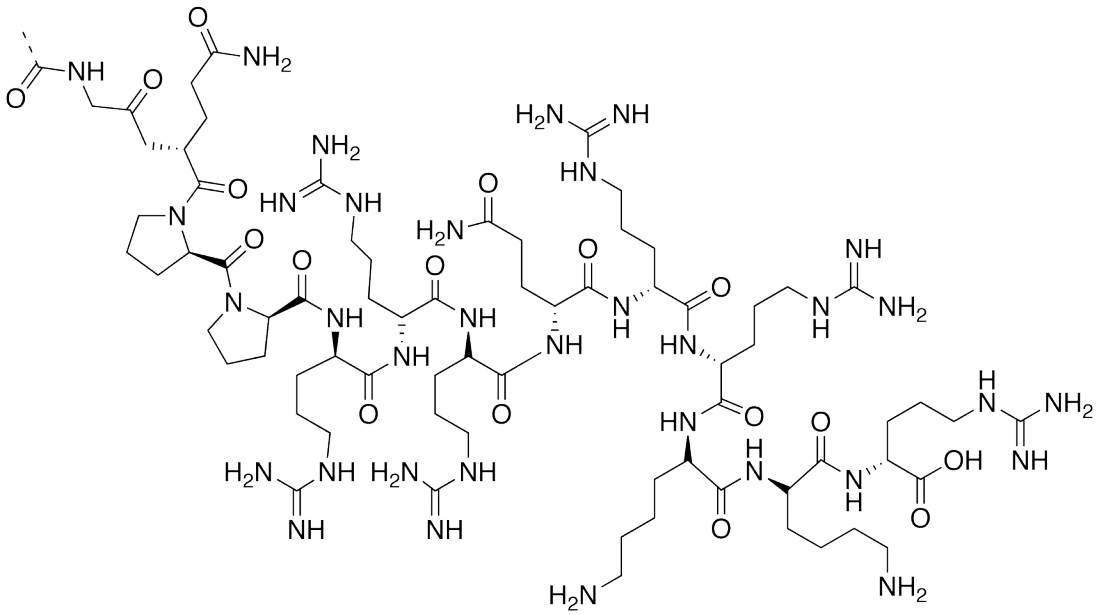


Figure 3.1. Chemical structure of the TAT peptide

Glutathione, located inside the cytosol, is responsible for keeping thiols in bio-molecules in their reduced state. This is of great interest to our strategy since this may allow cargo to be delivered into the cell by attachment of the cysteine derivative through a disulfide bond. Once the desired target molecule (Figure 3.2) has been synthesized, its utility can be determined by its ability to penetrate the cell, enter the cytosol, and release the desired cargo through disulfide bond cleavage.

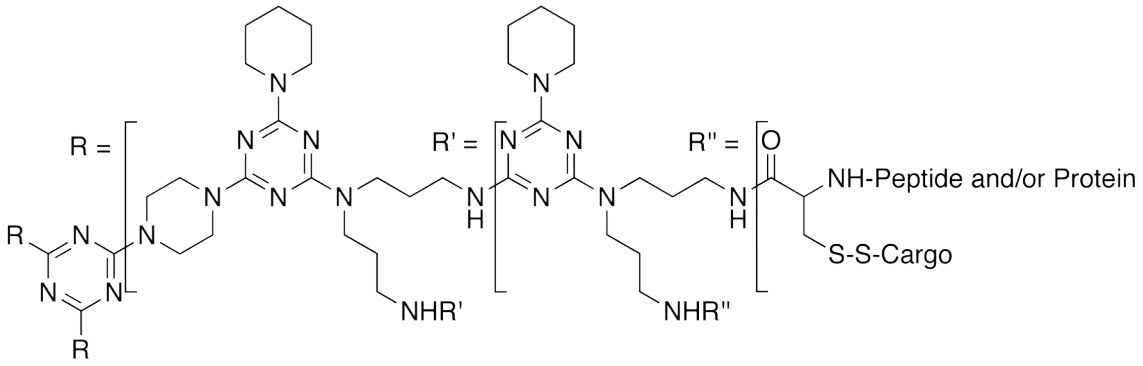


Figure 3.2. Detailed chemical structure of one embodiment of a G2, C-linked cysteine terminated dendrimer with 12 cargo groups.

Results and Discussion

The following content was obtained from Alfredo Angeles-Boza, our collaborator in the preparation of peptide and protein dendrimers.

SYNTHESIS

From figure 3.3 it seems apparent that ligation with the fluorescent protein mCherry to the dendrimer was successful, however we have not been able to repeat these results. The supposed product was a precipitate that formed during the reaction. This precipitate was dissolved in 0.1% Triton X100 detergent, and 10 mM TCEP buffer solution and run in SDS-PAGE gel. The solution phase of the reaction did not contain any product as determined by SDS-PAGE gel. SDS-PAGE gel was used extensively in this study to determine product formation.

To improve the reaction conditions, subsequent reactions were performed in different amounts of detergent and TCEP, to no avail. A precipitate was formed, however it did not contain any of the dendrimer-mCherry conjugate.

Reactions with DMSO and SDS to improve the solubility of the dendrimer, were also performed however no reaction seemed to have occurred. The conditions used for this reaction were standard protocol, however the dendrimer was initially dissolved in DMSO.

Although the dendrimer, at concentrations lower than 50 mM, is soluble under the conditions used in the reaction (25 mM Hepes buffer, 100 mM MesNa, and 50 mM TCEP), it precipitates slowly from the solution (If the concentration is greater, it precipitates almost immediately).

Our efforts were then directed toward synthesis of the TAT-Melamine dendrimer construct by using a 100-fold excess of the peptide. A product that seems to have a mass (10 kda) corresponding to the product between the dendrimer and the TAT peptide (3-4 attachments) is shown in figure 3.4 (TAT = 1746, Dend = 4194). This product has been obtained in two different reactions, but further characterization of the product has been hampered by the presence of DMSO in the sample. Attempts to remove the DMSO using rotary-evaporation failed. Purification by HPLC is hampered because the product is eluted early preventing efficient separation.

CHARACTERIZATION

Our collaborators did not perform full characterization of the products obtained, however SDS-PAGE gels were run (Figure 3.3 and 3.4) and show possible product formation for both the protein and the peptide.

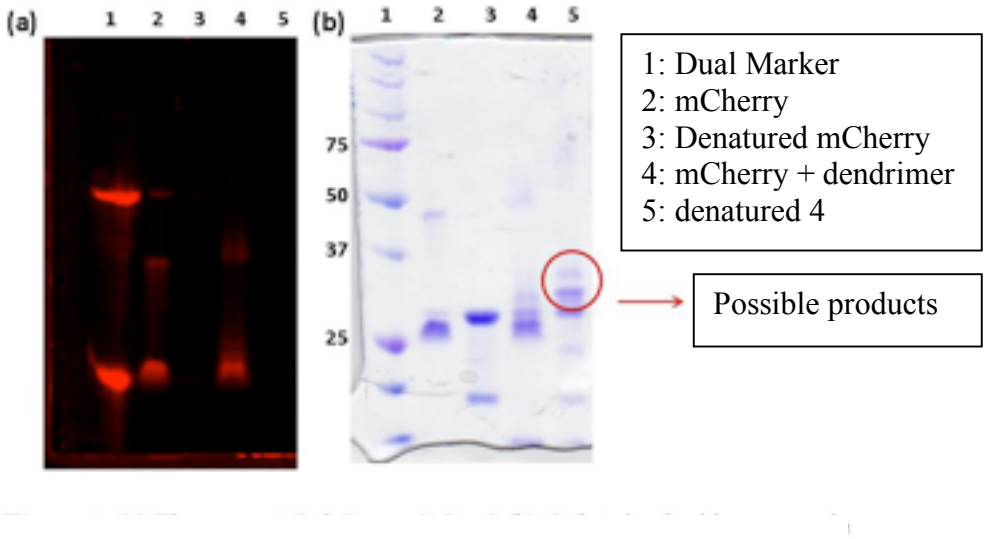


Figure 3.3. Gels run on dendrimer after native chemical ligation with mCherry. (a) Fluorescent Gel (λcm = 610nm), (b) Gel stained with coomassie.

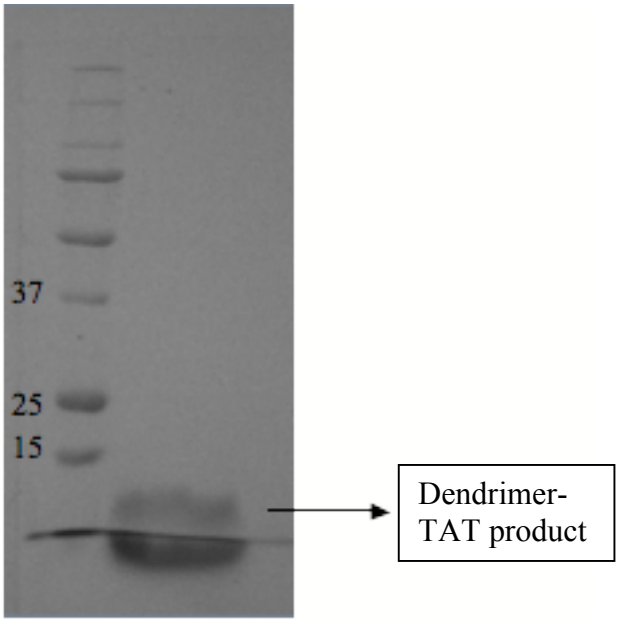


Figure 3.4. Gel run on dendrimer after native chemical ligation with TAT.

Experimental Section

GENERAL

Expressed proteins were routinely analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols. Recombinant mCherry was obtained by sub cloning the mCherry gene in pTXB1-EGFP that was previously cloned. The DNA encoding mCherry was isolated by PCR from the pRSET-B mCherry plasmid (provided by R.Y. Tsien, HHMI investigator, Department of Pharmacology, UCSD, CA) using the 5' primer (5'-GGC GAC CAT ATG GTG AGC AAG GGC GAG GAG-3') and 3' primer (5'-GCC GCC GAA TTC CTT GTA CAG CTC GTC CAT GCC-3'), encoding the NdeI and EcoRI restriction sites, respectively.

The digested PCR product was ligated into the NdeI, EcoRI digested pTXB1-EGFP vector. This cloning procedure results in the insertion of the amino acid sequence EFGKKKRKVG between the C-terminal lysine of mCherry and the N-terminal cysteine of *Mxe* GyrA. The protein was expressed and purified according to previously established protocols.[73]

SYNTHESIS

Using an excess of the dendrimer the ligation reaction was performed at 4°C by adding the dendrimer to the mCherry thioester solution (260 μM, 10 μL) giving a final concentration of 63 μM in a buffer consisting of 25 mM Hepes, 10 mM TCEP, and 0.05% Triton X100 at pH 7.5. Analysis was performed after 24 hours.

Using an excess of mCherry the ligation reaction was performed at 4°C by adding the mCherry thioester solution (260 μM, 90 μL) to the dendrimer giving a final concentration of 7 μM in a buffer consisting of 25 mM Hepes, 10 mM TCEP, and 0.05% Triton X100 at pH 7.5. Analysis was performed after 24 hours.

The ligation reaction between TAT and the dendrimer was performed at 4°C by adding the TAT thioester solution (9 mM, 90 μL) to the dendrimer dissolved in DMSO giving a final concentration of 0.07 mM in a buffer consisting of 25 mM Hepes, 10 mM TCEP at pH 7.5. Analysis was performed after 24 hours.

Drug Delivery

Introduction

Our strategies for drug delivery have been developed with cancer therapy in mind. Cancer is a term for numerous diseases in which abnormal cells divide without control and invade other tissue. Using the blood and lymph systems, cancer is able to spread throughout the body. Under normal conditions old or damaged cells die and are replaced by new cells in a controlled manner. Cancer cells occur when the DNA of the cell becomes damaged producing mutations. These cells not only survive longer than normal cells, but grow and divide ultimately forming a mass of tissue called a tumor.

Many potentially effective drugs, which are effective against cancer, are not useful due to their low solubility under physiological conditions and/or their high toxicity. One way to overcome this difficulty is to design drug delivery vehicles such as dendrimers, polymers and molecular containers. These delivery vehicles not only reduce the toxicity of the drug and increase their solubility, but can also be used to specifically target the cancerous cells. There are two ways in which these delivery systems can single out tumor cells. The first is by attaching antibodies to the vehicle, which target receptor molecules on the cellular surface. The second is through the enhanced permeability and retention effect (EPR) of cancer cells. First discovered by Maeda,[29] larger molecules can easily penetrate the cellular membrane because cancer cells divide far more rapidly than do healthy cells, and their cellular membrane is what is referred to as “leaky,” this is known as the EPR effect. This effect has become the generalized method preferred for drug delivery vehicles.

Cancer therapy can be approached in several ways, most of which inhibit cell proliferation. One method of interest is inhibiting the DNA enzyme topoisomerase I. During cell replication the DNA of the cell is unwound in order to begin the replication process. As the two strands unwind, the DNA ahead of the replication fork becomes over wound. In order to resolve this problem the cell uses enzymatic proteins called topoisomerases. Similar problems occur during transcription, recombination, repair, chromatin assembly, and chromosome segregation processes. Topoisomerase I repairs DNA by first binding to DNA then cleaving one strand of the DNA. A tyrosine residue (Y723) in the enzyme attacks a phosphate group thereby cleaving a DNA strand. The cleaved strand can now rotate around the other strand giving the desired degree of super helical tension. There are four majors domains associated with topoisomerases mode of action: (1) an amine terminal domain, residues 1-200, responsible for targeting; (2) the linker domain, residues 636-712, assist in cleavage and directs the coiling of DNA; (3) the carboxylic acid domain, residues 713-765, contains the catalytic tyrosine responsible for DNA cleavage; and (4) the core domain, residues 201-635, wraps around the DNA allowing Y723 to undergo transesterification with a phosphodiester bond of the DNA backbone, resulting in cleavage of the strand. After the formation of a DNA/enzyme complex the free strand rotates around the stationary strand to relieve topological stress. Once rotation is complete a second transesterification reaction occurs yielding a relaxed DNA coiled structure.[74-76]

Camptothecin (CPT) is a natural product first isolated in 1958 from Camptothecin acuminate and has displayed cytotoxicity by inhibiting topoisomerase

I.[77,78] CPT and its analogs can mimic a DNA base pair, inhibit topoisomerase by intercalating into the site of DNA cleavage after the first transesterification and stabilize the covalent intermediate. Once the base stacking interaction between CPT and the base pairs occur the second transesterification reaction is prevented and thus the release of relaxed DNA, which results in cell cycle arrest and eventual cell death. CPT is too toxic and water-insoluble to be used in clinical settings, but has been loaded into or attached to several systems.[79-92]

A polymeric micelle (thiolated poly(ethylene glycol)-b-poly(glutamic acid) conjugated to CPT through a disulfide linkage was constructed.[89] A 20% (w/w) drug load was achieved and CPT was released slowly under extra-cellular and endosomal conditions, however 90% of the drug was released after 24hrs under cytosol (reductive) conditions. An interesting aspect of this delivery vehicle is the use of photochemical internalization (PCI) to enhance cytotoxicity. PCI allows the micelle to enter the cytosol by damaging the endosome and bypass lysosomal hydrolases. Interestingly, the drug loaded micelle showed no cytotoxicity without PCI.

A CPT bioconjugate consisting of CPT coupled via glycine linker to folic acid conjugated to a 3.4 kDa PEG chain (Figure 3.5) was achieved.[89] Folic acid was used to target the folate receptor and mediate endocytosis because folate receptors are over expressed on the surface of tumor cells. The size of the PEG chain was chosen so as to remove ambiguity of the EPR effect causing tumor cell selectivity. The bioconjugate showed higher efficacy when compared to free CPT, and the delivery system showed enhanced cytotoxicity on cell lines with folate receptors compared to those without said

receptors. Without the PEG chain no enhancement of efficacy was observed when compared to free CPT. The authors attributed this to the greater stability of the system due to steric hindrance so that release of CPT is slow enough that the bioconjugate can be endocytosed and CPT can be released inside the cell.

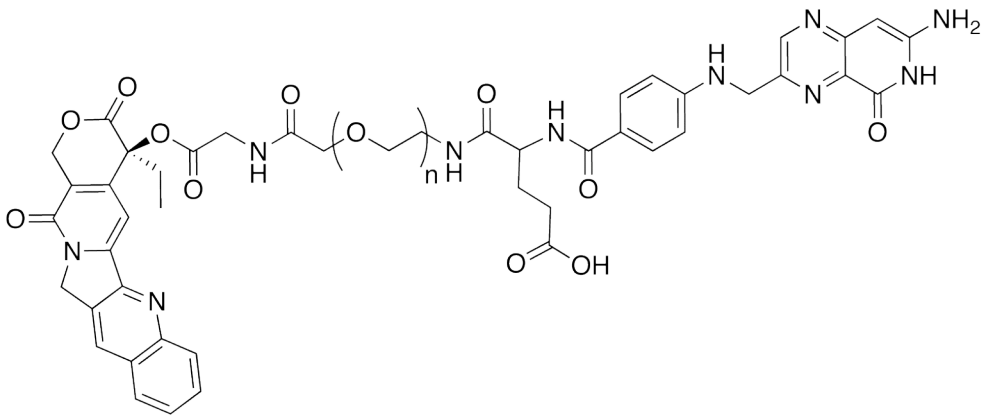


Figure 3.5. Camptothecin/glycine/PEG conjugate.[89]

Research in Mark Davis group has put CPT conjugates in preclinical modes. Complete tumor regression in all animals bearing H1299 tumors and the majority of animals with disperse Ewing's sarcoma tumors was observed with the construct shown in figure 3.6.[79,85,86,91] The conjugate is also effective against tumor cells that have become resistant to irinotecan, an analogue of CPT. This drug conjugate displays antitumor activity against a wide range of tumors and shows higher antitumor activity compared to free camptothecin and irinotecan. The plasma half-life is significantly higher than that of free CPT, and accumulates mostly in tumor tissue rather than the liver, lung, spleen, and heart.

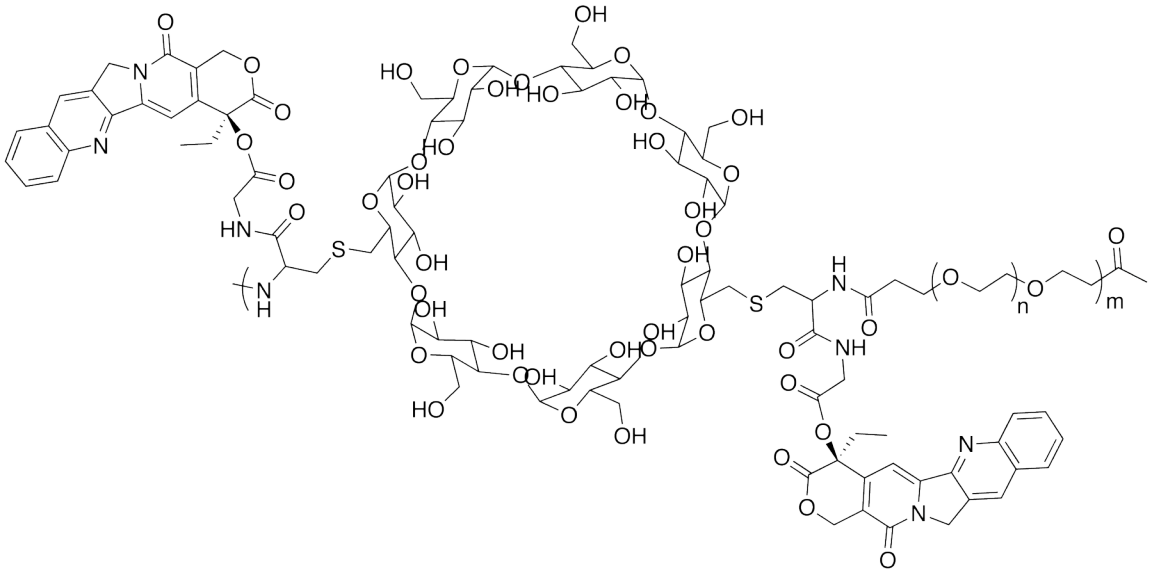
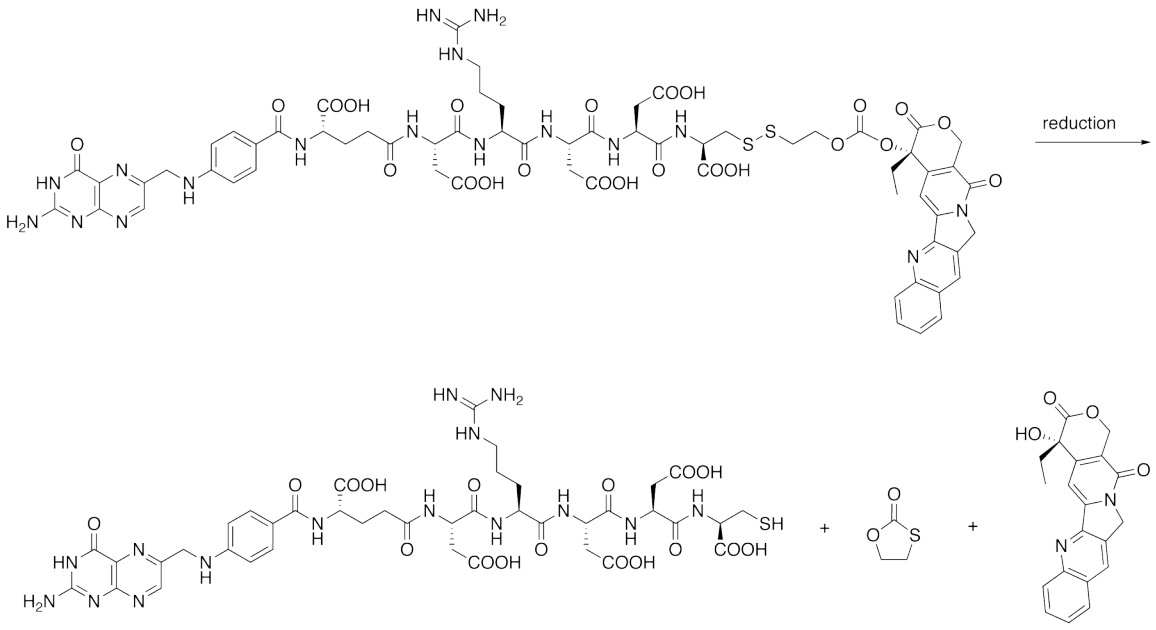


Figure 3.6. Cyclodextrin based polymer conjugated to camptothecin.[79,85,86,91]

A bioconjugate taking advantage of over-expressed folate receptors on tumor cells was prepared by coupling folic acid to a 5 mer peptide with a cysteine residue on the periphery (Scheme 3.4).[84] CPT was coupled to pyridinyldithio butanoic acid and subsequently attached to the folic acid derivative via disulfide exchange. This system inhibited cell proliferation in human KB cells and its activity is highly dependant on the folic acid folate receptor interaction. The dependency was concluded after excess folic acid decreased the activity of the system completely.

Scheme 3.4. Biological mediated delivery of camptothecin by reduction of a disulfide bond.[84]



In order to probe the need for bioconjugates of CPT to exhibit the lactone, Greenwald et al. developed two PEG open lactone tripartate prodrugs (Figure 3.7).[81] It was initially proposed that camptothecin and its derivatives must exhibit the lactone to be effective. The majority of research has been based on derivatizing the 20-OH of CPT due to the supposed need for the closed lactone. They found little *in vitro* cytotoxicity for their systems as expected, however *in vivo* test revealed equivalent cytotoxicity to the closed ring derivative. This indicates that lactonization occurs in the acidic environment of the tumor generating the active form of the drug. This approach is very advantageous in that it provides access to stable nontoxic forms of the drug that become active only after they enter a sufficiently acidic environment.

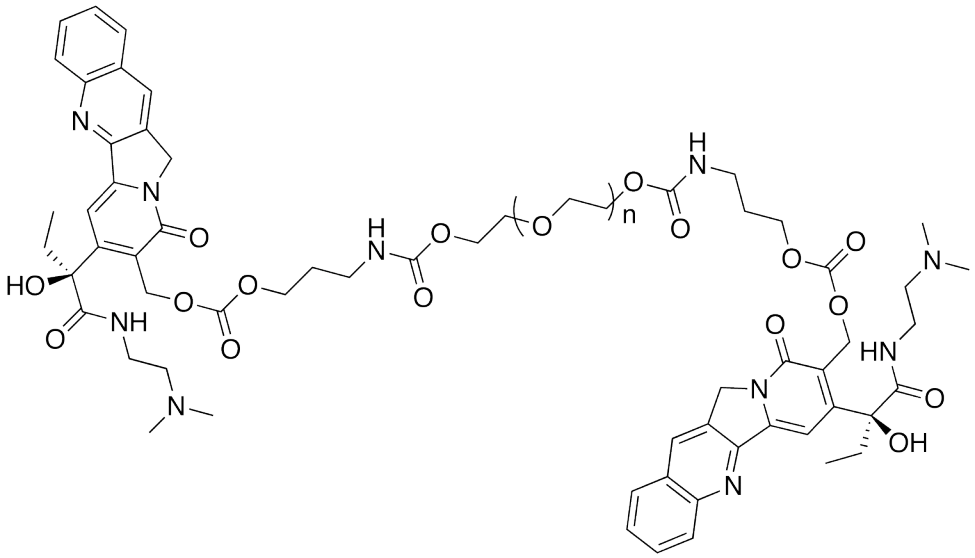


Figure 3.7. Camptothecin/aminopropanol/PEG/aminopropanol/camptothecin conjugate.[81]

Several open-ring lactone conjugates of CPT were prepared with polyamine side chains.[80] The amines were linked through the carboxylic functional group of the lactone to form an amide, and the resulting primary hydroxyl was acylated. The polyamine side chains were chosen to bind electrostatically to the DNA backbone and assist CPT entry into the binding site of topoisomerase I. Three interesting observations came out of this study: 1) the antiproliferative effects of these compounds increased greatly after 72 hours, whereas at 1hr they were less potent than CPT and several of its analogues; 2) the inhibition of tumor growth during long term exposure is directly related to the number of charges on the polyamine substituent. Presumably the polyamine promotes lactone formation. The polyamine may also block binding of topoisomerase to DNA preventing initial cleavage; 3) these compounds may also show activity in the open-ring form.

Several bioconjugates were prepared consisting of a poly-α-(L-glutamic acid) backbone with a library of linkers and varying CPT loading.[88] The linkers comprised of glycine and 4-O-butyryl showed the highest activity. Further, different lengths of the glycine linker (1, 2, and 3 mers) were compared and the 3-mer-glycine linker showed greater activity, but with lower solubility. Solubility was decreased when the average molecular weight of the polymer or CPT loading was increased. The conjugate consisting of a single glycine linker with 30-35wt % CPT was the best due to its high aqueous solubility, stability, and efficacy as well as its synthetic ease. This conjugate showed a 6-fold improvement for exposure of tumor tissue to CPT over free CPT.

Fragmentation of a G2 dendrimer prodrug is activated by a single cleavage at the core of the dendrimer by penicillin-G-amidase, subsequently releasing four molecules of CPT.[93] To enhance solubility and prevent aggregation, PEG chains were attached via click chemistry to alkyne groups on the dendrimer. The toxicity of this conjugate is 100-1000 fold less than free CPT, however the toxicity neared free CPT after incubation with the enzyme. Tumor size decrease by 75% has been observed using the dendrimer-drug conjugate and intratumoral injection of the enzyme.

A CPT pro-drug has been developed based on poly[N-(2-hydroxypropyl) methacrylamide] with a -Gly-Phe-Leu-Gly- or -Gly-6-aminohexanoyl-Gly- linker.[90] Hydrolysis to release CPT occurred during the first 24 hrs with the -Gly-Phe-Leu-Gly- linker however hydrolysis of the -Gly-6-aminohexanoyl-Gly- linker was significantly slower, with tumor growth inhibition lasting 73-88 days. The linkers were chosen so as to release CPT upon proteolytic cleavage resulting in low levels of drug during

circulation and high levels after tumor accumulation. The conjugate comprising 10-wt % CPT with the -Gly-6-aminohexanoyl-Gly- linker showed the best results, by increasing the efficacy of CPT through long exposure compared to higher local concentrations.

Our strategy for creating a drug delivery vehicle relies on a disulfide exchange reaction between the cysteine terminated dendrimer and Camptothecin-(Pyridyl-SS)-Propanoate (Scheme 3.5). Subsequent attachment of a biologically relevant molecule such as PEG is needed to solubilize the dendrimer-drug conjugate and increase the molecular weight to invoke the EPR effect.

Results and Discussion

SYNTHESIS

Synthesis begins by activating the thiol of mercapto propionic acid for disulfide exchange with 2,2'-dithiodipyridine to form 3-(2-pyridyldithio) propionic acid. This procedure is fairly straightforward, however hydrogen peroxide is used to convert the majority of pyridine-2-thione back to the dithiodipyridine starting material, which can be recycled upon purification by column chromatography. The next step is coupling to CPT to form the ester. This step is difficult due to the low reactivity of the tertiary alcohol. Attempts to use the commercially available 3-(2-pyridyldithio) propionic acid NHS ester proved fruitless due to the low reactivity of CPT, where no product formation was observed by TLC at RT. EDCI was attempted however this procedure only gave minimal conversion to the ester. DCC gave higher conversion to the ester and upon purification by column chromatography, 27.8 mg of yellow solid was obtained

(theoretical = 27.4 mg). After running the product next to DCC on TLC, it became apparent upon staining with ninhydrin that the excess impurity was DCC and that both had the same R_f. Precipitation with methanol should afford the pure CPT derivative, however the compound was used without further purification. Due to the large difference in solubility between the CPT derivative and the cysteine dendrimer, the disulfide exchange reaction was performed in CH_2Cl_2 with the hopes that loading with CPT would eventually bring the dendrimer into solution. Though all of the solid did not dissolve, the mass spectra (Figure 3.8) showed up to seven substitutions using three equivalents of the CPT conjugate. There is a clear ladder of peaks with a mass difference of approximately 440 mass units (calc. 435.5) corresponding to a mixture of 1-7 disulfide exchange reactions occurring on the dendrimer.

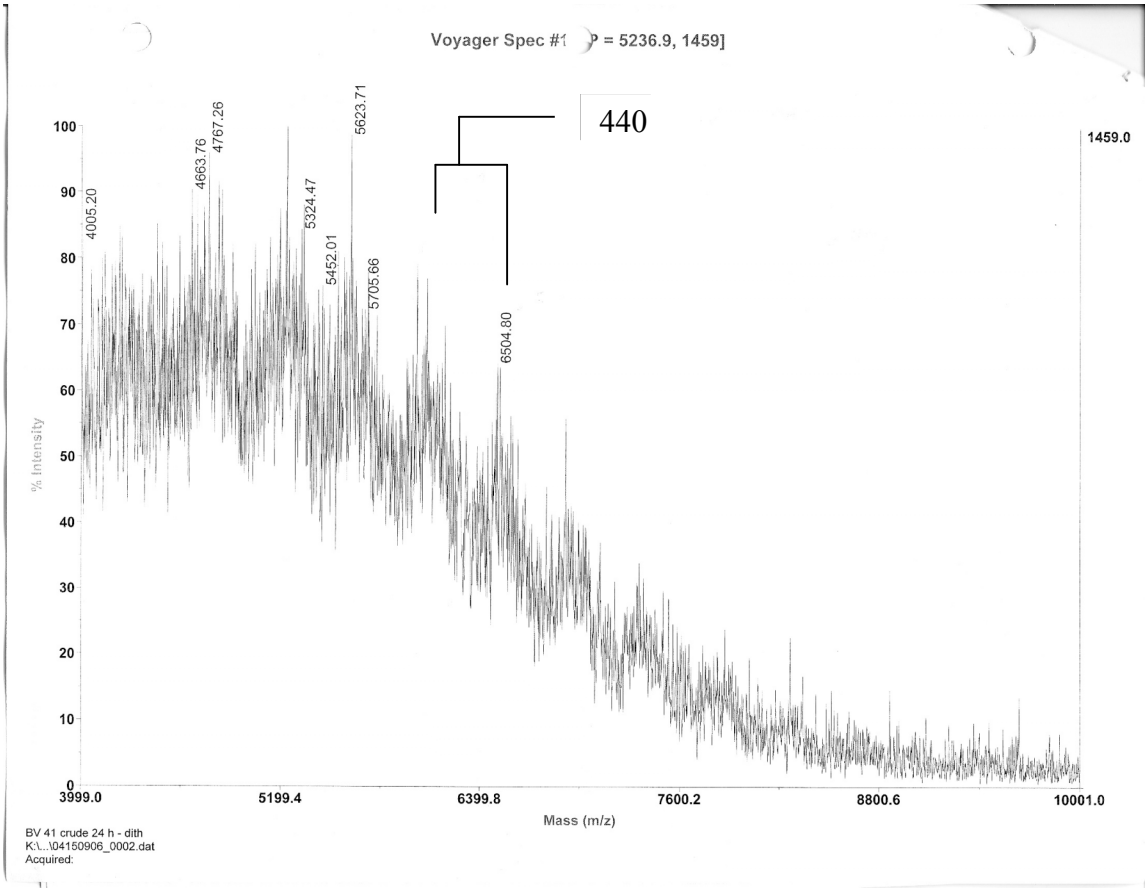
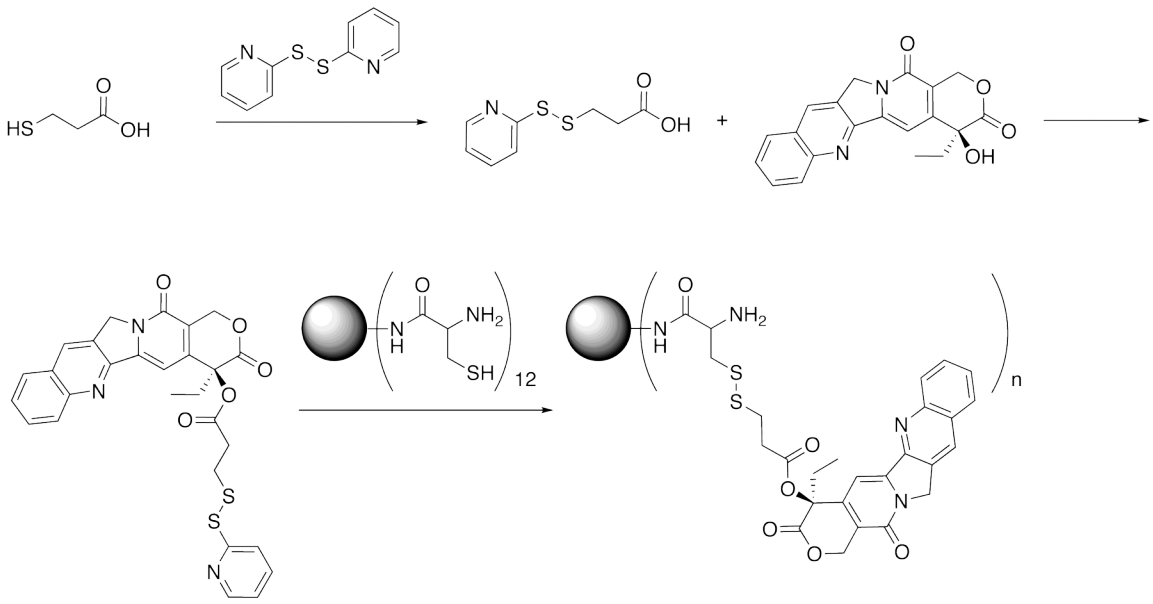


Figure 3.8. Mass Spectrum of G2-(Cys(NH_2)-SS-Camptothecin)$_n$, calc. 9407.64 (M^+); found 4194 – 7240 (mixture of products).

Scheme 3.5. Synthetic strategy for camptothecin loading onto a cysteine terminated melamine dendrimer by attaching mercaptopropionic acid through the 20-OH position.



CHARACTERIZATION

Characterization of the 3-(2-pyridyldithio) propionic acid intermediate is straightforward with peaks at 8.48, 7.71-7.61, and 7.17-7.15 corresponding to the aromatic protons on the pyridyl group. The peaks at 3.08-3.06 and 2.83-2.80 correspond to the β- and α-protons respectively. Integration is also consistent with expected results. ^{13}C NMR shows a peak at 175.16, which corresponds to the carbonyl carbon. Peaks at 159.07, 149.40, 137.45, 121.31, and 120.72 correspond to the carbons on the pyridyl

ring. Peaks at 34.27 and 34.17 correspond to the β- and α-carbons respectively. Mass spectroscopy shows a peak at 216.01 corresponding to M+H.

The camptothecin derivative has a larger R_f than camptothecin and is also fluorescent. This new spot shows all peaks that correspond to camptothecin as well as 3-(2-pyridyldithio) propionic acid in ^{1}H and ^{13}C NMR. Impurities prevent confidence in integration. Mass spectroscopy shows a peak at 546.11 corresponding to M+H.

Mass spec characterization of the camptothecin derivative loaded onto the dendrimer shows a mixture between 1 and 7 substitutions with a possibility of 8 substitutions. NMR is needed to obtain an average number of drug molecules loaded onto the dendrimer.

Experimental Section

GENERAL

All reagents were used without further purification. Pyridyl-SS-propanoic acid was synthesized by a procedure developed by a fellow group member, Jong Doo Lim.

SYNTHESIS

Pyridyl-SS-Propanoicacid - 1.24g of pyridyl disulfide was dissolved in 7ml EtOH. 238μl of mercaptopropionic acid was dissolved in 8ml EtOH and added dropwise through an additional funnel over 45min to the pyridyl disulfide solution. 200μl of acetic acid was subsequently added. The reaction was stirred for 2h upon which time the solvent was removed by roto-evaporation. The slurry was then dissolved in 8ml of

$CHCl_3$ and 8ml of H_2O. The aqueous layer was made pH 8 by addition of 10% Na_2CO_3. 0.5 ml of H_2O_2 was then added dropwise over 10 min or until the yellow solution turned clear. The solution was left to stir for 30min. The aqueous layer was made pH 10 by addition of sat. Na_2CO_3. The product was purified by first extracting with $CHCl_3$ (3 x 8 ml) to remove excess pyridyl disulfide, then addition of 6M HCl until pH 3 followed by extraction with $CHCl_3$. The solvent was removed by roto-evaporation and the product was purified by column chromatography (1:1, hexane:EtOAc) to give 0.326mg (55.35%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.49 (t,d, 1H, NC*H*C_4H_3, pyridyl), 7.71-7.65 (d,t, 1H, NC_2H_2C*H*C_2H, pyridyl), 7.64 (d, 1H, NC_3H_3C*H*C, pyridyl), 7.19-7.15 (m, 1H, NCHC*H*C_3H_2, pyridyl), 3.10-3.06 (t, 2H, β-H), 2.83-2.78 (t, 2H, α-H). ^{13}C{^{1}H} NMR (75.5 MHz, $CDCl_3$) δ 175.16 (s, C(O)), 159.07 (s, 4^o C, pyridyl), 149.40 (s, N*C*HC_4H_3, pyridyl), 137.45 (s, NC_2H_2*C*HC_2H, pyridyl), 121.31 (s, NC_3H_3*C*HC, pyridyl), 120.72 (s, NCH*C*HC_3H_2, pyridyl), 34.27 (s, α-C), 34.17 (s, β-C). MS (MALDI): calc. 215.01 (M^+); found 216.01 (M+H).

Camptothecin-(Pyridyl-SS)-Propanoate - 31.7 mg of Pyridyl-SS-Propanoicacid, 7 mg of DMAP, and 47 mg of DCC was dissolved in 0.5 ml of CH_2Cl_2 and cooled to 0 ^{o}C. After stirring for 30 min. 17.5 mg of camptothecin was added. The reaction was stirred for 1h at 0 ^{o}C then warmed to room temperature and stirred for 24h. The product was purified by column chromatography (1% MeOH in DCM) to give 27.8 mg (101.4%, DCC was observed by TLC). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47-8.44 (m, 1H, NC*H*C_4H_3, pyridyl), 8.45 (d, 1H, A-ring camptothecin), 8.23 (1H, A-ring camptothecin), 8.21 (s, 1H, B-ring camptothecin), 7.96 (m, 1H, A-ring camptothecin), 7.85 (m, 1H, A-

ring camptothecin), 7.69 (m, 2H, $NC_2H_2CHC_2H$ pyridyl, D-ring camptothecin), 7.60 (m, 1H, NC_3H_3CHC, pyridyl), 7.08 (m, 1H, $NCHCHC_3H_2$, pyridyl), 5.74-5.66 (m, 2H, E-ring camptothecin), 5.45-5.39 (m, 2H, C-ring camptothecin), 3.05 (m, 2H, β-H), 2.28 (m, 2H, α-H), 2.19 (m, CH_2CH_3, camptothecin), 0.98 (m, CH_2CH_3, camptothecin). $^{13}C\{^1H\}$ NMR (75.5 MHz, $CDCl_3$) δ 175.6 (s, C(O), ester), 159.07 (s, 4° C, pyridyl), 157.78 (s, C(O), lactone), 157.32 (s, C(O), amide), 153.0 (s, B,C-ring camptothecin), 149.67 (s, D,E-ring camptothecin), 149.56 (s, $NCHC_4H_3$, pyridyl), 148.84 (s, A,B-ring camptothecin), 142.45 (s, C,D-ring camptothecin), 137.18 (s, $NC_2H_2CHC_2H$, pyridyl), 131.16 (s, A-ring camptothecin), 130.82 (s, B-ring camptothecin), 130.6 (s, B,C-ring camptothecin), 129.52 (s, A-ring camptothecin), 128.49 (s, A,B-ring camptothecin), 128.13 (s, A-ring camptothecin), 128.0 (s, A-ring camptothecin), 121.31 (s, NC_3H_3CHC, pyridyl), 120.8 (s, $NCHCHC_3H_2$, pyridyl), 119.52 (s, D,E-ring camptothecin), 96.40 (s, D-ring camptothecin), 73.31 (s, E-ring camptothecin), 67.10 (s, E-ring camptothecin), 51.8 (s, C-ring camptothecin), 34.38 (s, α-C), 33.52 (s, β-C), 31.77 (s, CH_2CH_3), 7.7 (s, CH_2CH_3). MS (MALDI): calc. 545.11 (M^+); found 546.11 (M+H).

G2-(Cys(NH_2)-SS-Camptothecin)$_n$ – 9 mg of Camptothecin-(Pyridyl-SS)-Propanoate was dissolved in the minimal amount of $CHCl_3$. 20mg of G2-Cys was added. 20 μl of acetic acid was subsequently added and the reaction was stirred for 5 days. The reaction was stopped and the solvent was removed by roto-evaporation. ^1H NMR (300 MHz, $CDCl_3$) δ $^{13}C\{^1H\}$ NMR (75.5 MHz, $CDCl_3$) δ MS (MALDI): calc. 9407.64 (M^+); found 4194 – 7240 (mixture of products).

CHAPTER IV

CONCLUSIONS

Synthesis of the cysteine dendrimer is fairly straightforward, however low yields have been obtained presumably due to the purification process. By modifying this process, higher yields for the coupling step could be obtained. Changing our strategy to more closely mimic Meijers (full deprotection is achieved within 2h), having Trityl protecting groups for both the thiol and amine would be more efficient.

Using native chemical ligation to prepare peptide dendrimers has proved difficult with our dendrimer due to solubility issues. The dendrimer seems to precipitate around pH 5. Native chemical ligation is normally performed at pH 7 and is slowed at lower pH. Due to the solubility issues we faced, we proposed that changing the capping group from the hydrophobic piperidene to more hydrophilic groups such as those in figure 4.1 would prove beneficial.

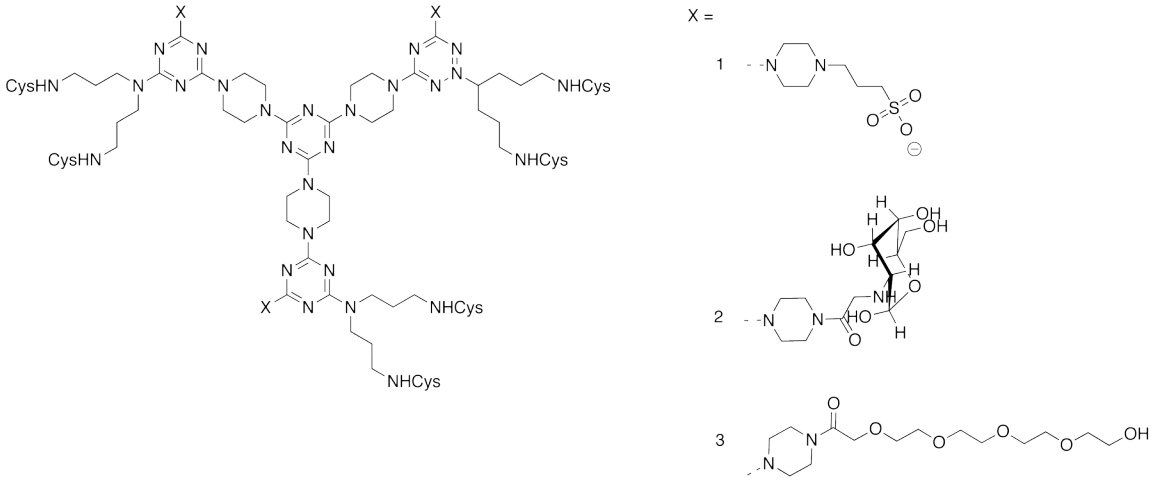
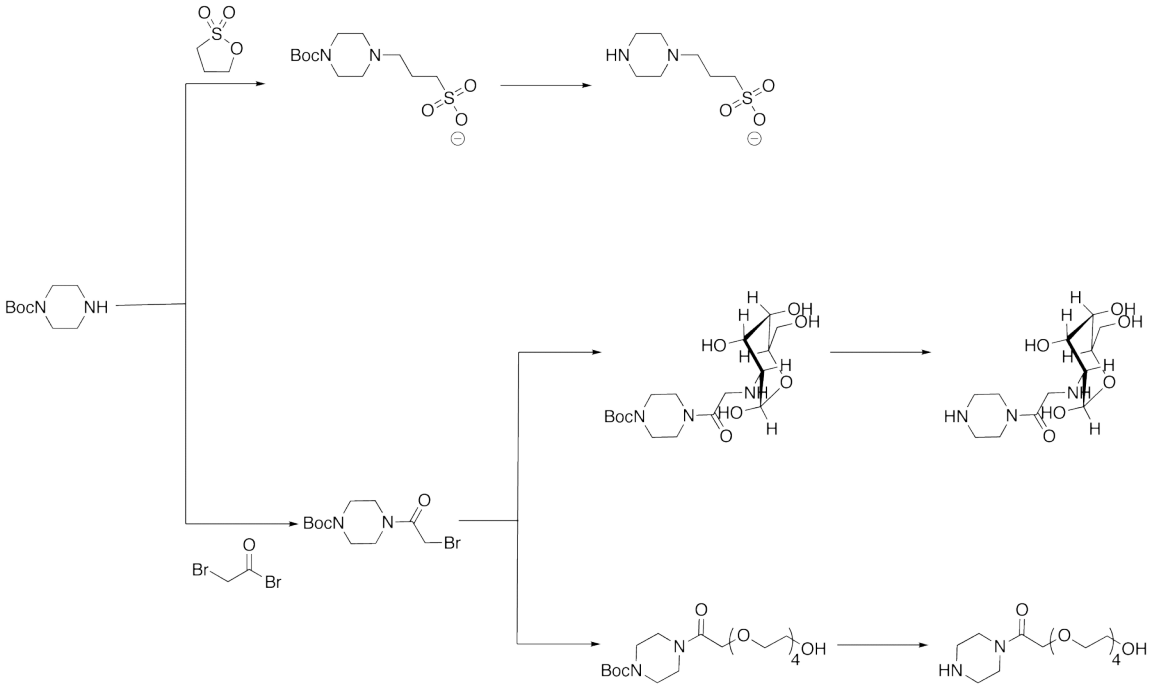


Figure 4.1. Potential capping groups to enhance the water solubility of a cysteine dendrimer based on melamine.

Piperazine was chosen because cyclic secondary amines are preferential for the third substitution of cyanuric chloride while the other amine can be used to conjugate to water-soluble molecules (Scheme 4.1).

Scheme 4.1. Synthetic strategy for water-solubility enhancing capping groups.



The next step would be to attach a fluorophore through the thiol to obtain biodistribution data. If the dendrimer were found to localize within tumor cells, attaching an anticancer drug such as camptothecin via a disulfide bond (Figure 4.2) would follow.

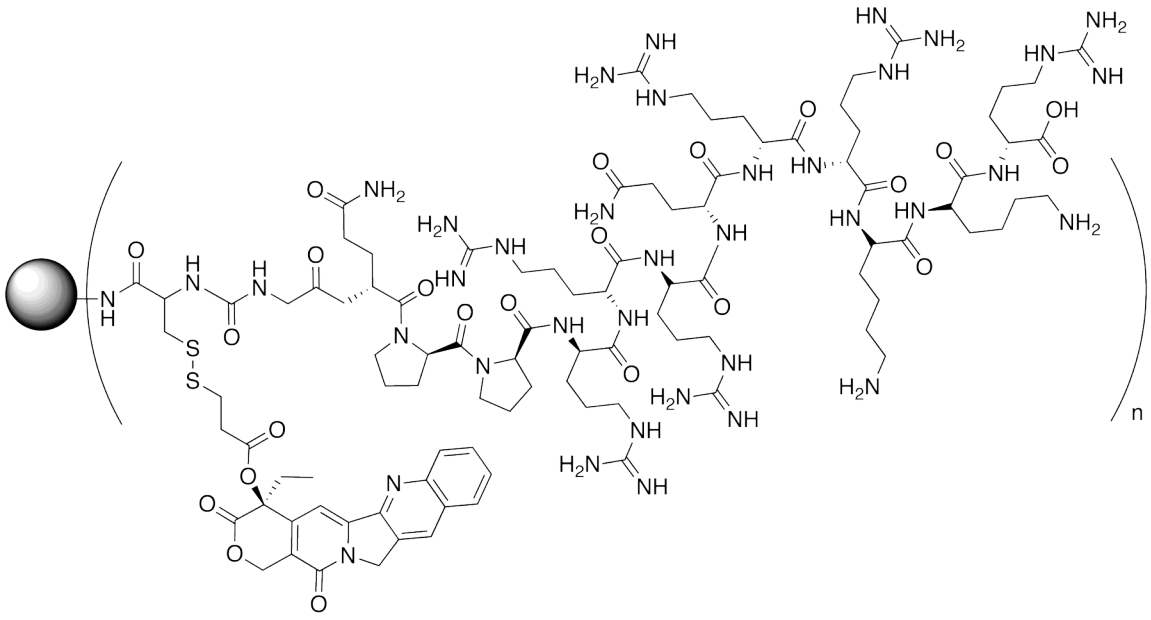
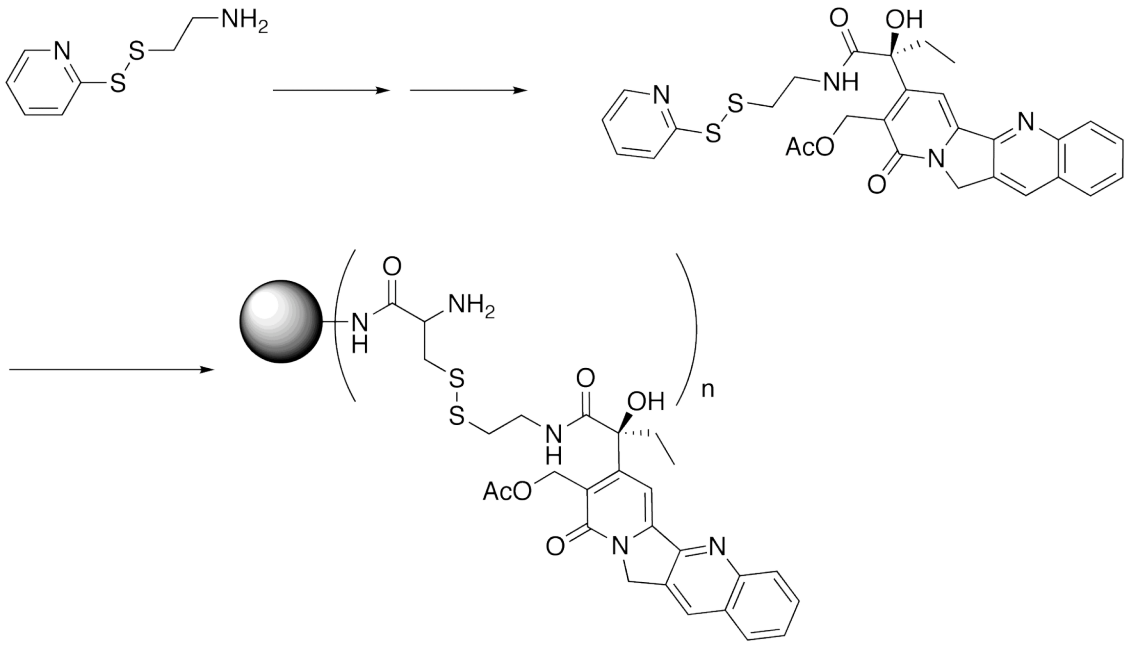


Figure 4.2. Potential end target for cysteine dendrimers based on melamine with a TAT-peptide to aid cell penetration and a bio-labile link to camptothecin for cancer therapy.

Synthesis of the CPT derivative has proved rather difficult due to the low reactivity of the hydroxyl group. Making the acid chloride of pyridyl-SS-propanoic acid may allow for higher conversion to the ester. Another route of interest may be an open lactone CPT conjugate. These conjugates have been shown to be cytotoxic only *in-vivo* revealing the high stability of these conjugates compared to their ester counterparts. I propose using 3-(2-pyridyldithio) ethylamine to form an amide with the carboxyl group of the lactone and selectively acylate the free primary hydroxyl to increase stability (Scheme 4.2). This approach may prove to be highly advantageous over the other due to not only the expected stability of the final product but also the ease of synthesis and expected higher yields.

Scheme 4.2. Potential synthetic strategy for camptothecin loading onto a cysteine terminated melamine dendrimer by attaching 2-aminoethane thiol through the lactone carbonyl group.



It may prove advantageous to first incubate the dendrimer in immobilized TCEP solution to remove any inter- and intra-molecular disulfides before performing the disulfide exchange reaction. A different solvent, co-solvent, or phase transfer catalyst is needed to create a homogeneous solution and maximize the number of substituents.

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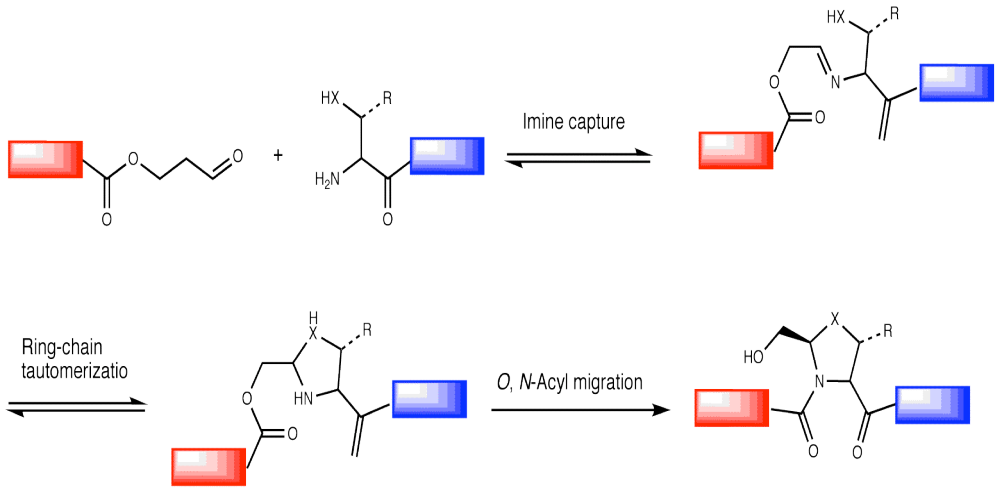
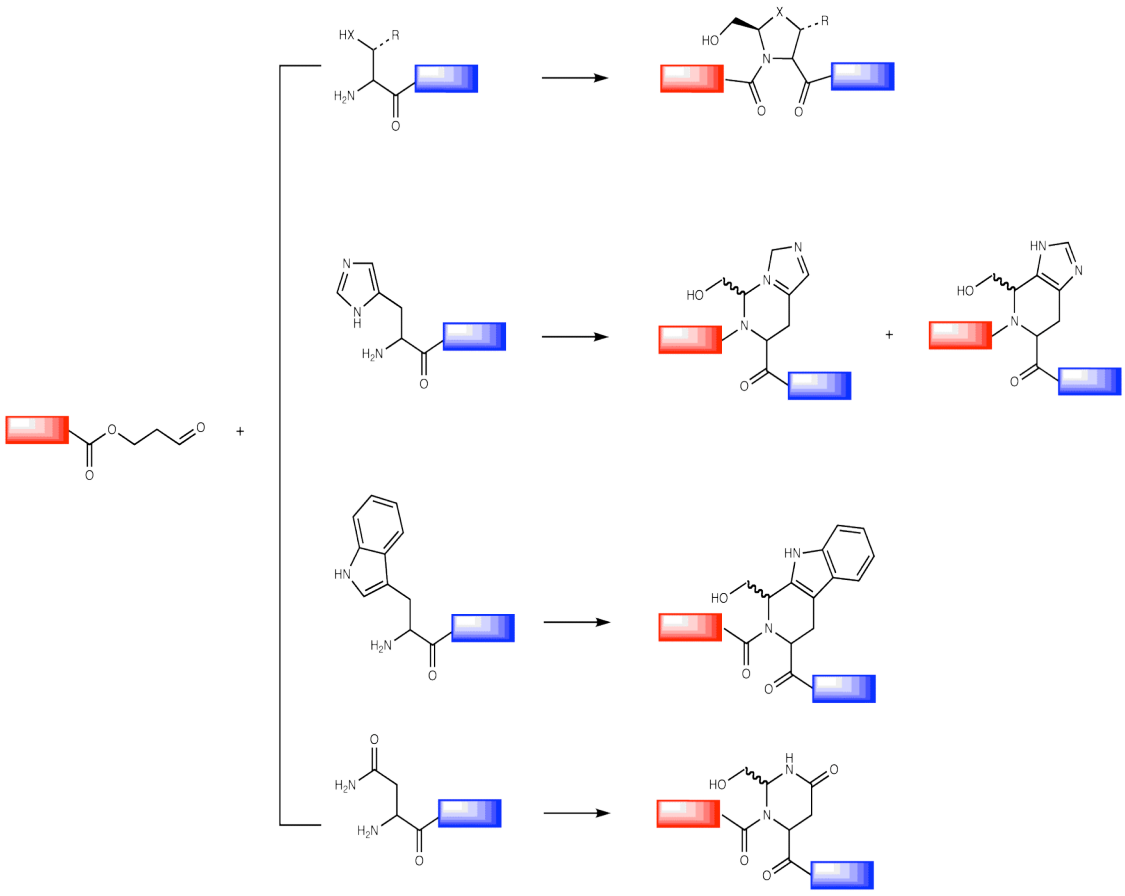
APPENDIX A

PEPTIDE LIGATION STRATEGIES

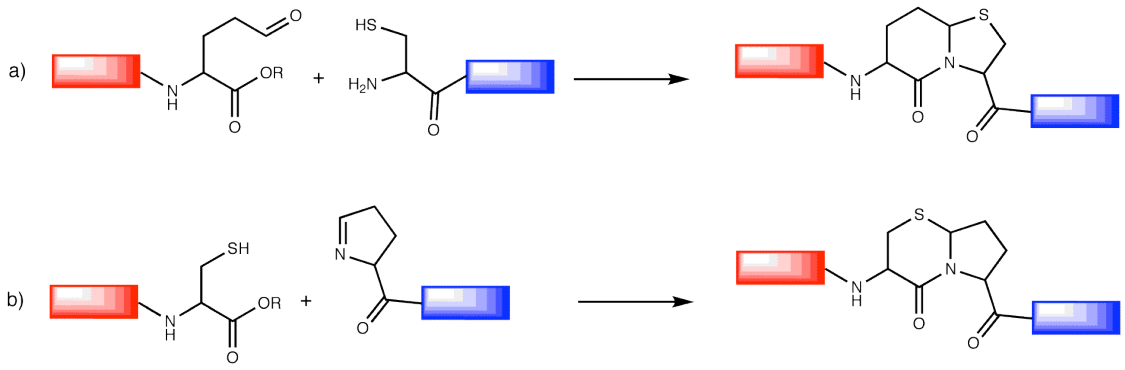
Scheme A.1. Imine ligation.



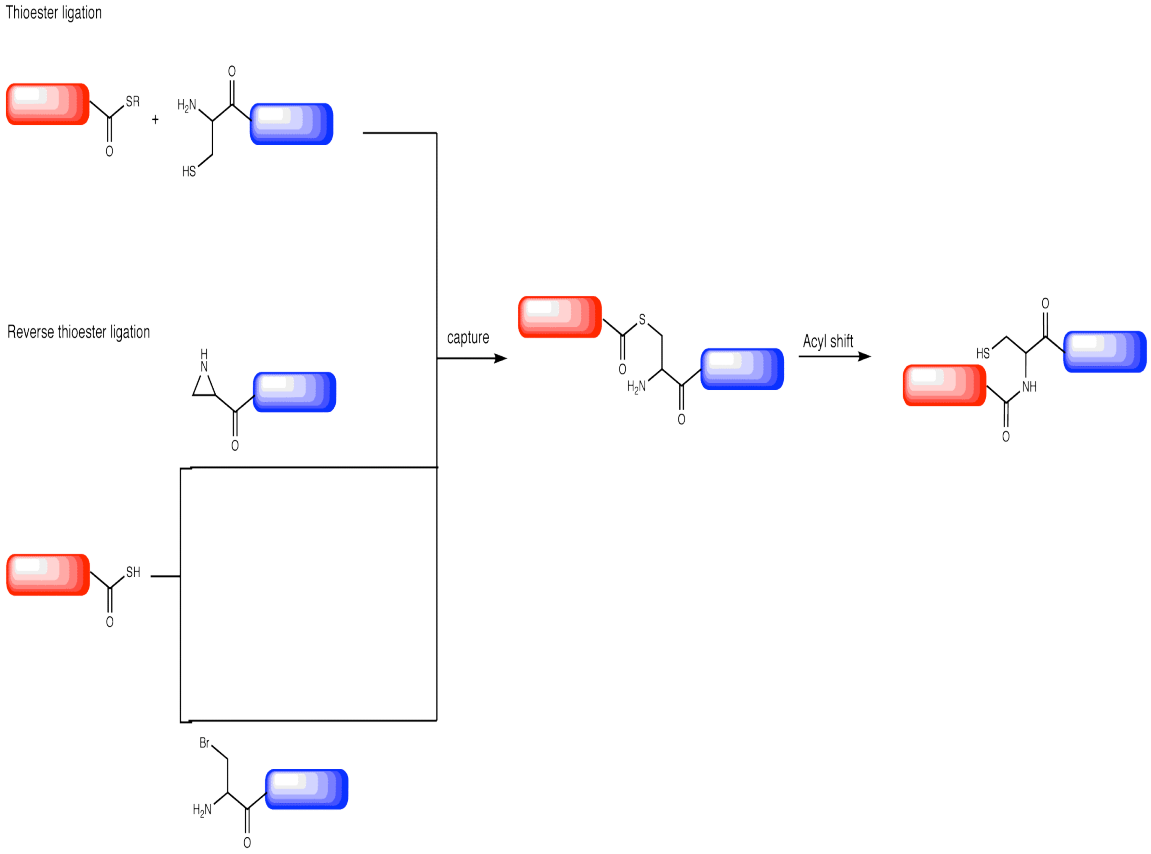
Scheme A.2. Imine ligation of N-terminal amino acids to form pseudoproline and oxazolidine rings.



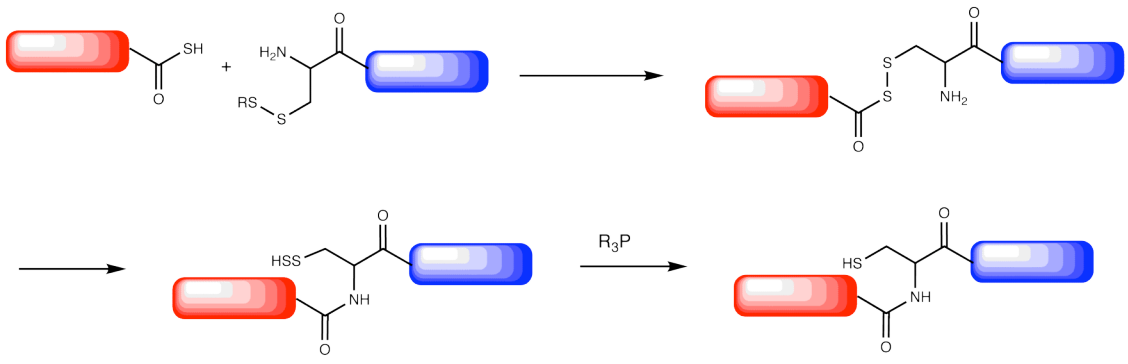
Scheme A.3. Imine ligation to form heterocycles with N-terminal amino acids.



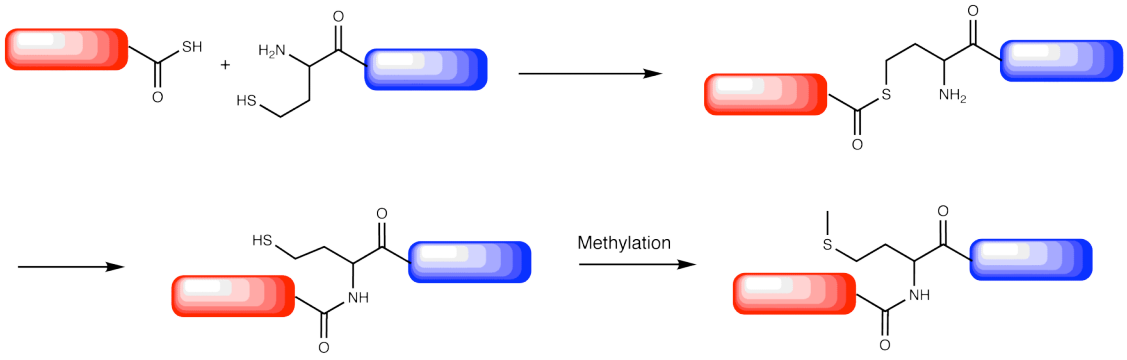
Scheme A.4. Thioester ligation.



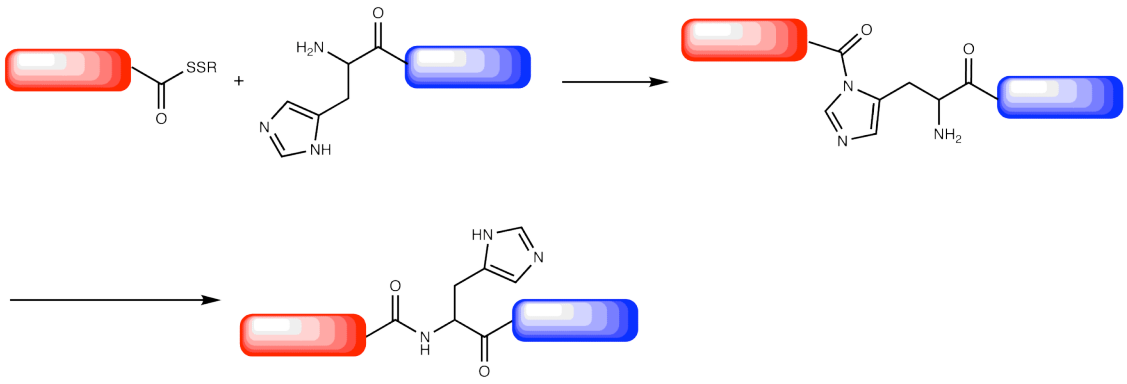
Scheme A.5. Cys-perthioester ligation.



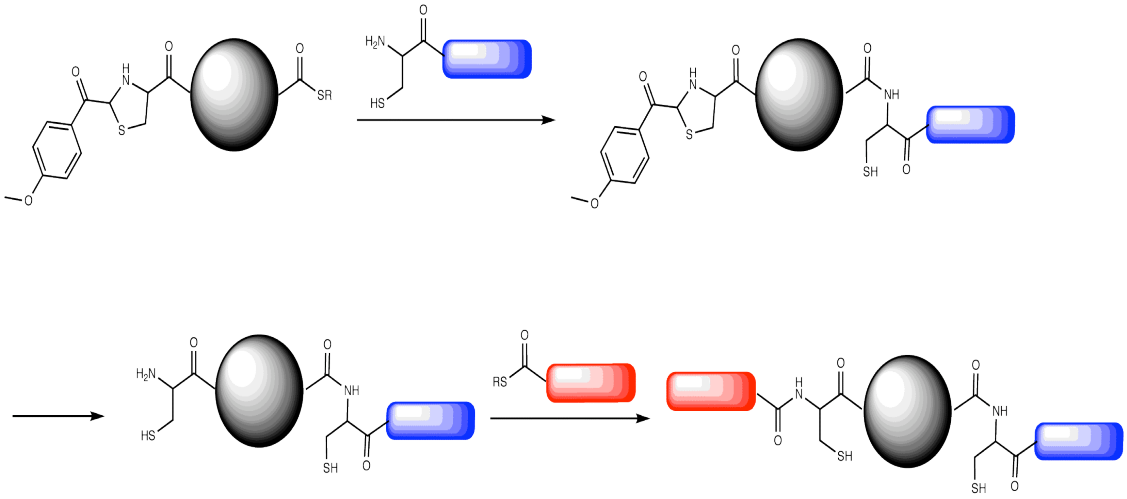
Scheme A.6. Methionine ligation.



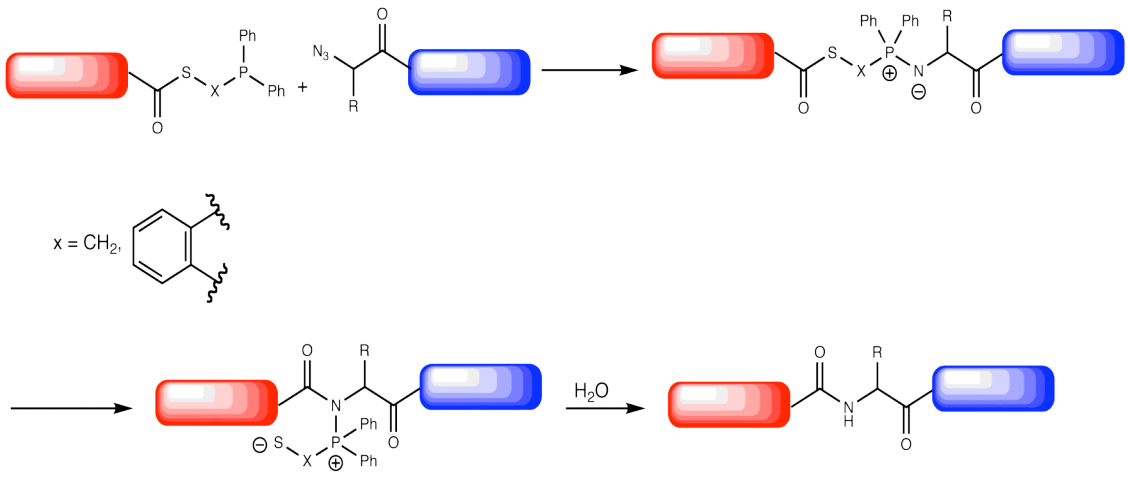
Scheme A.7. Histidine ligation.



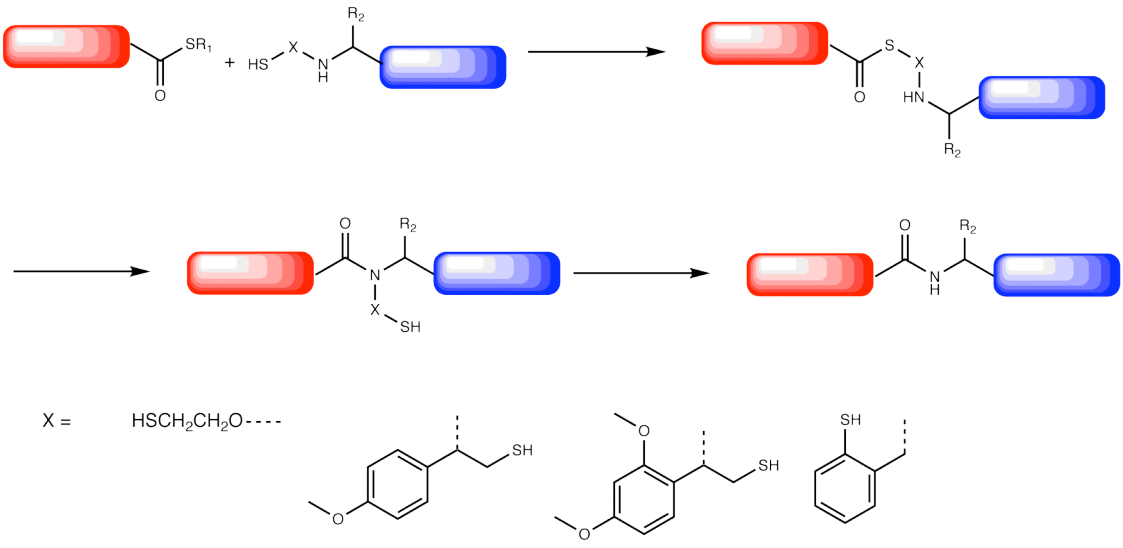
Scheme A.8. Sequential orthogonal ligation.



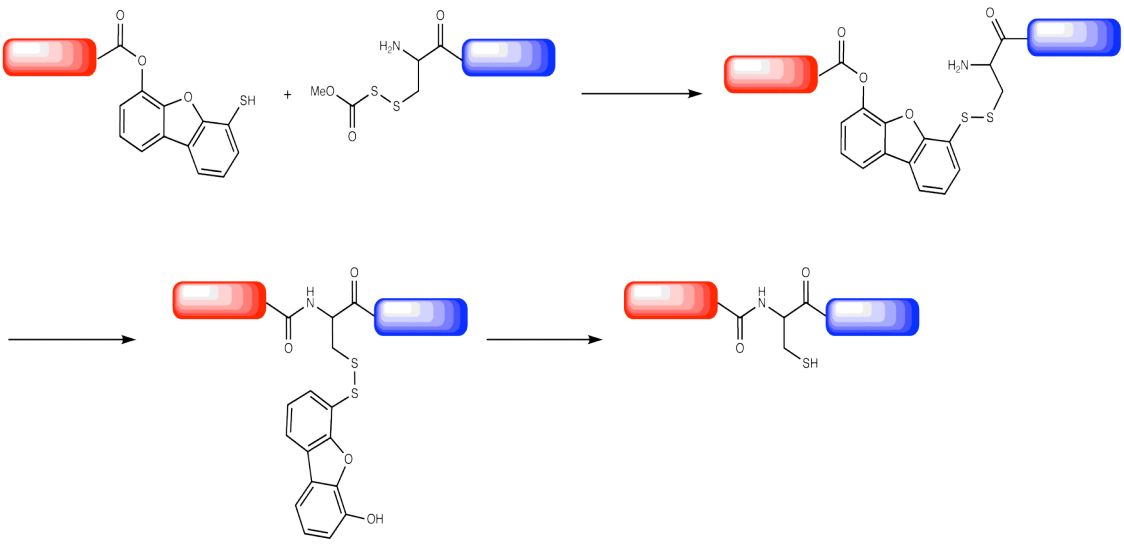
Scheme A.9. Staudinger ligation.



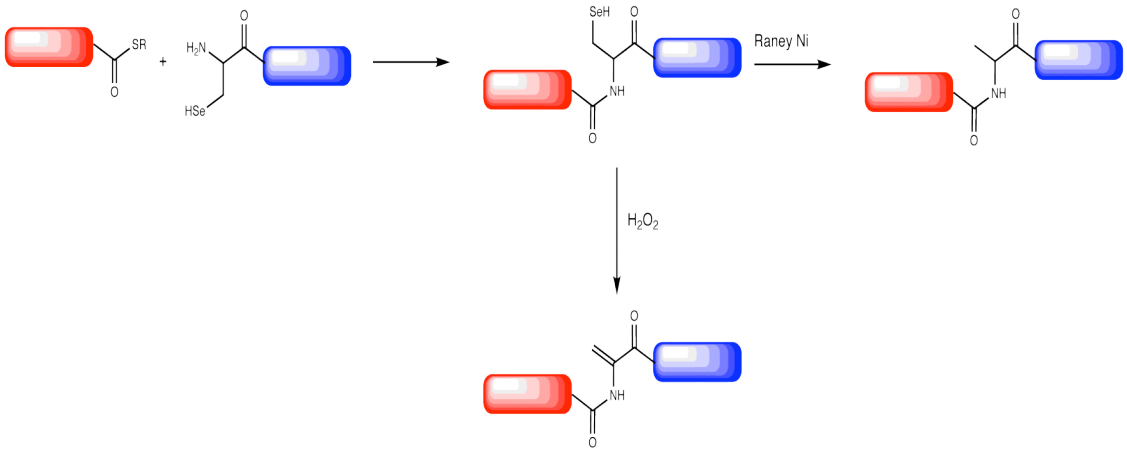
Scheme A.10. Cysteine Mimetic Ligation.



Scheme A.11. Prior thiol ligation.



Scheme A.12. Sec Ligation.



APPENDIX B

^{1}H NMR, ^{13}C NMR, AND MASS SPECTRA FOR COMPOUNDS

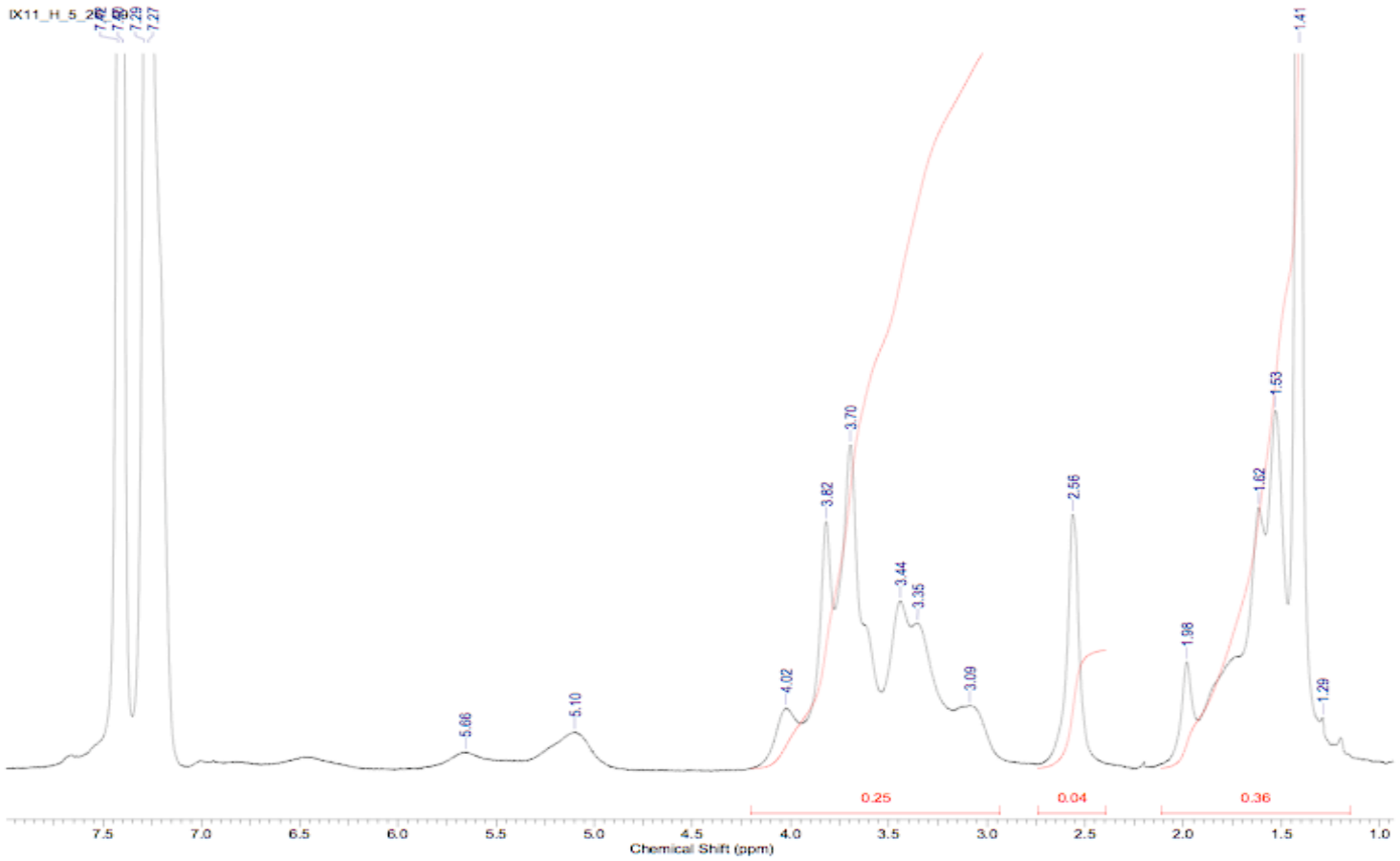


Figure B.1. G2-CysProt ^{1}H NMR

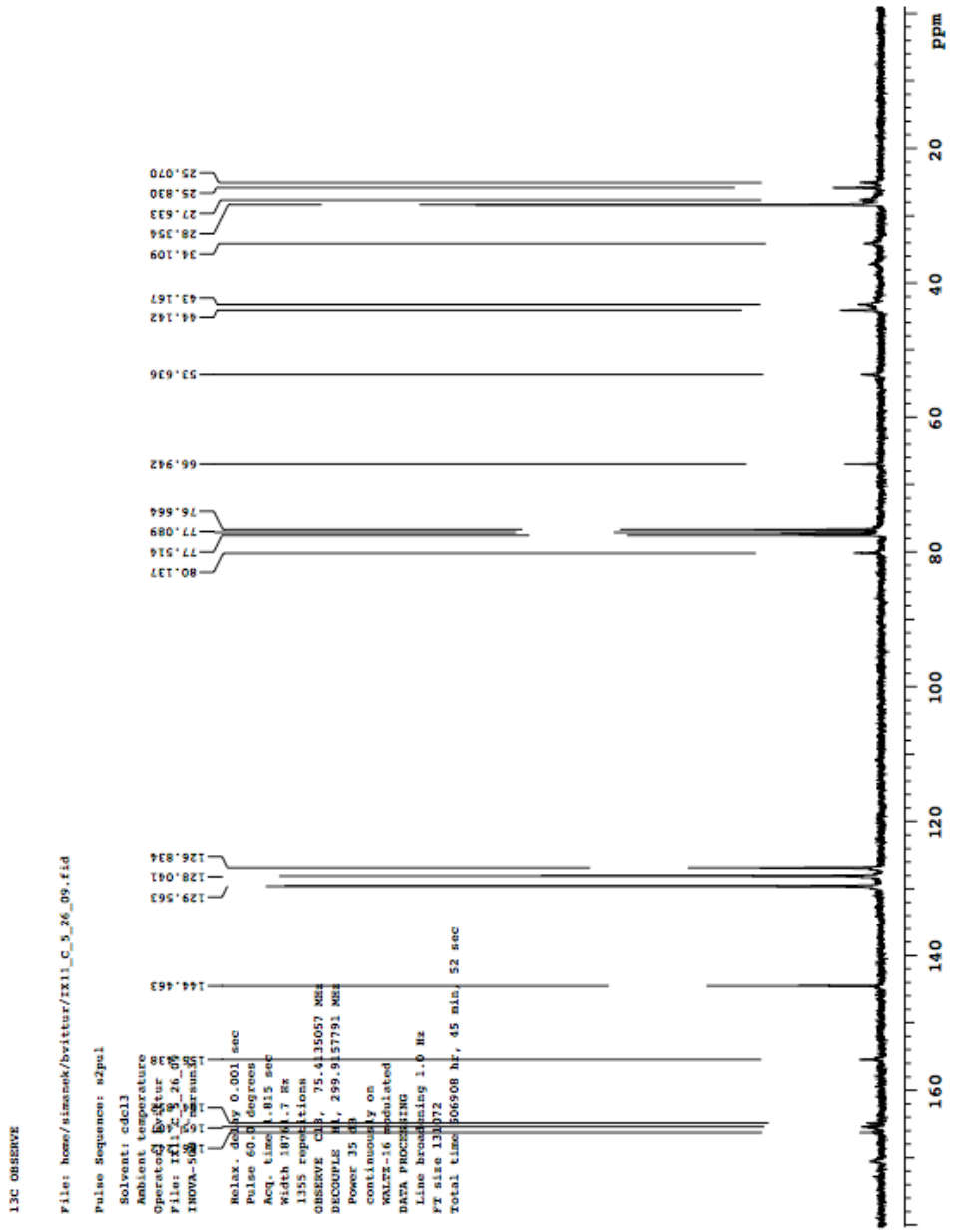


Figure B.2. G2-CysProt ^{13}C NMR

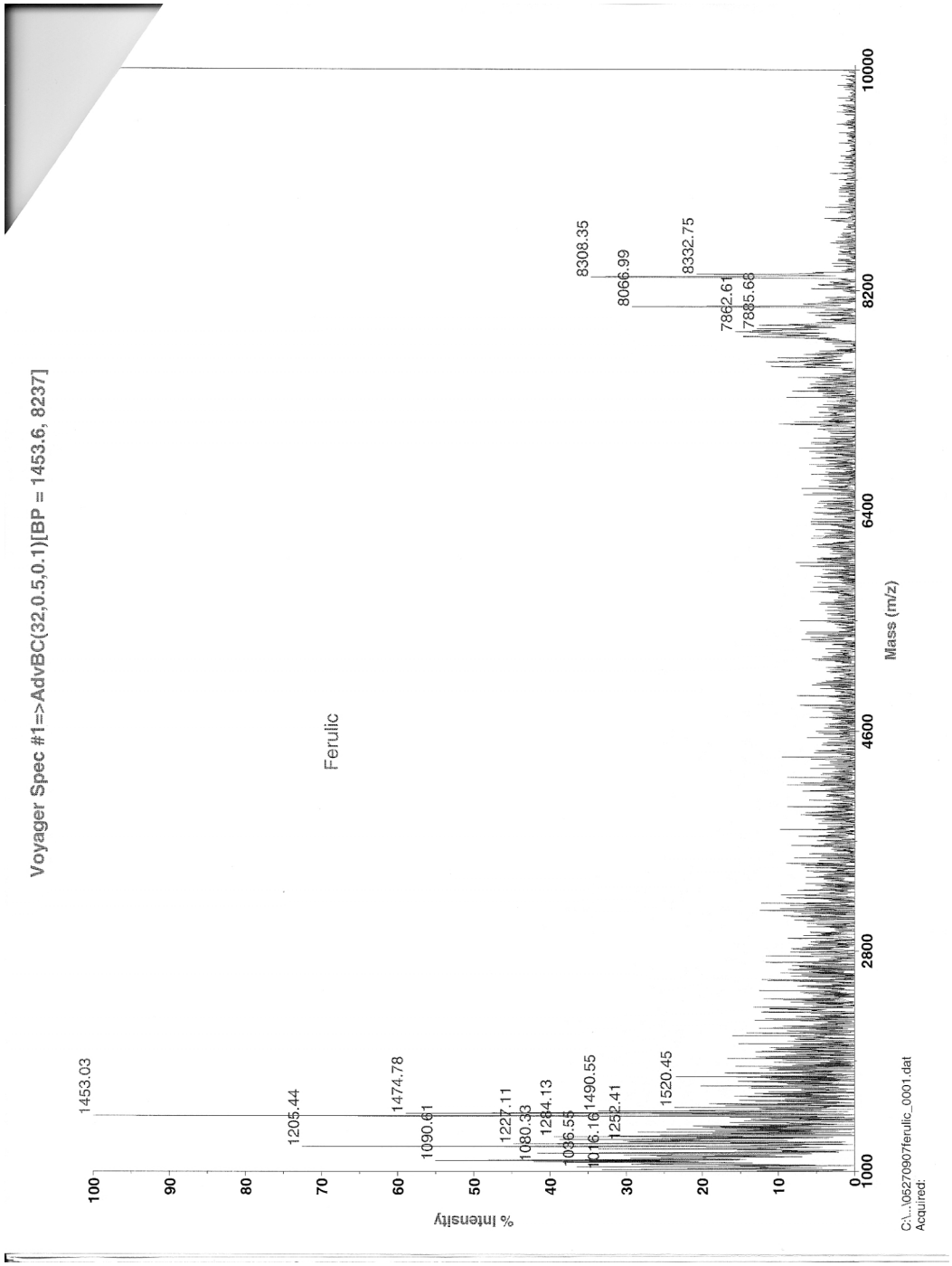


Figure B.3. G2-CysProt Mass Spectra

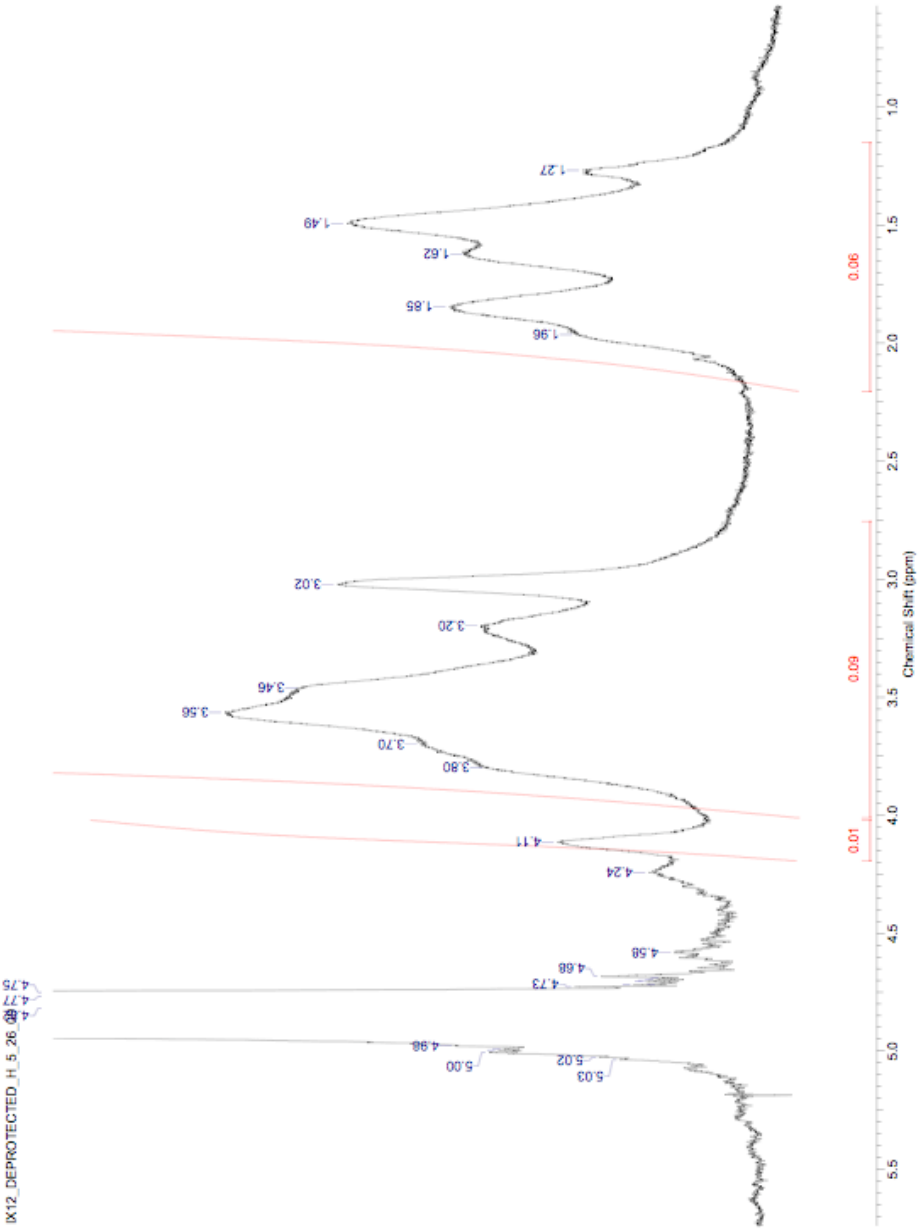


Figure B.4. G2-Cys ^{1}H NMR

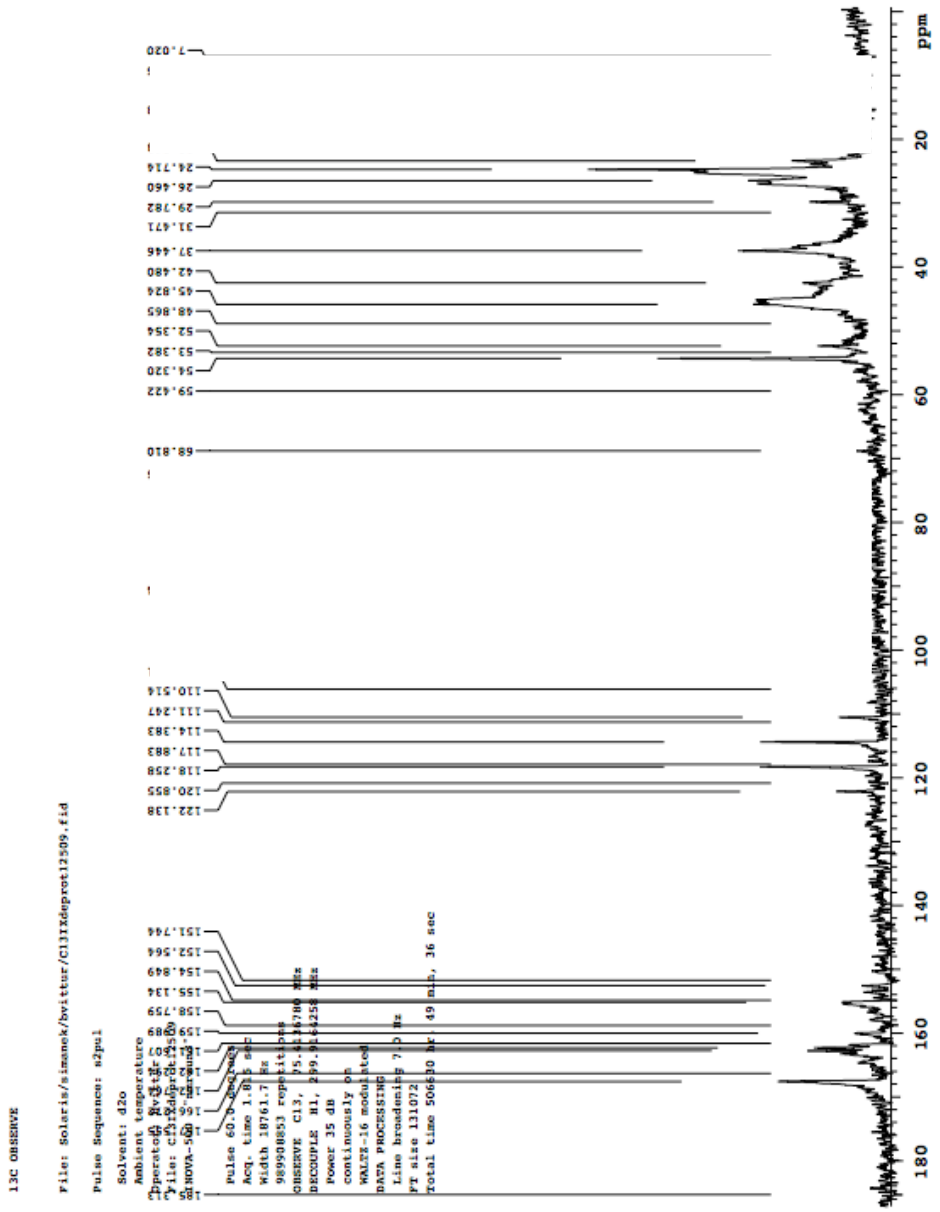


Figure B.5. G2-Cys ^{13}C NMR

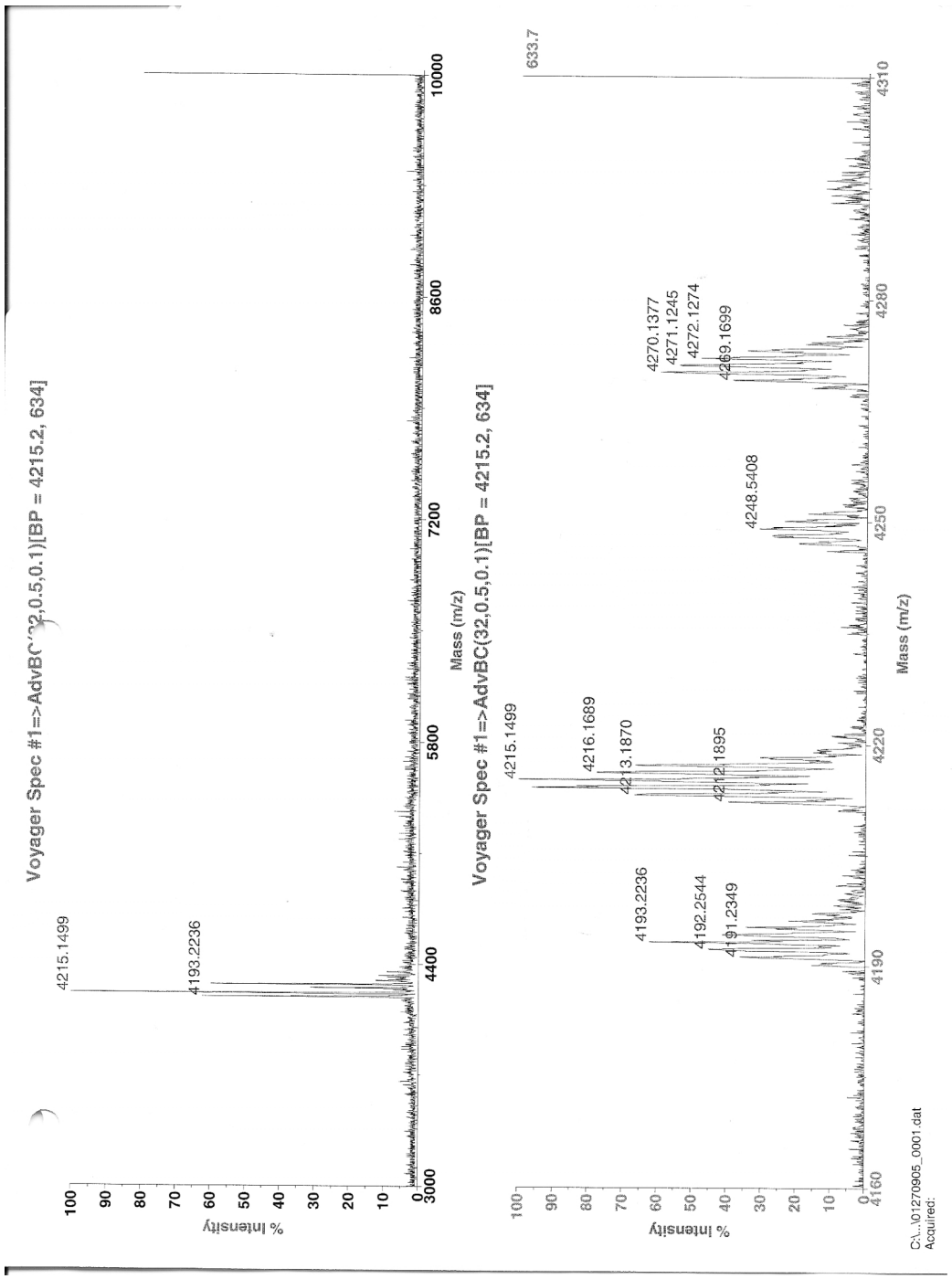


Figure B.6. G2-Cys Mass Spectra

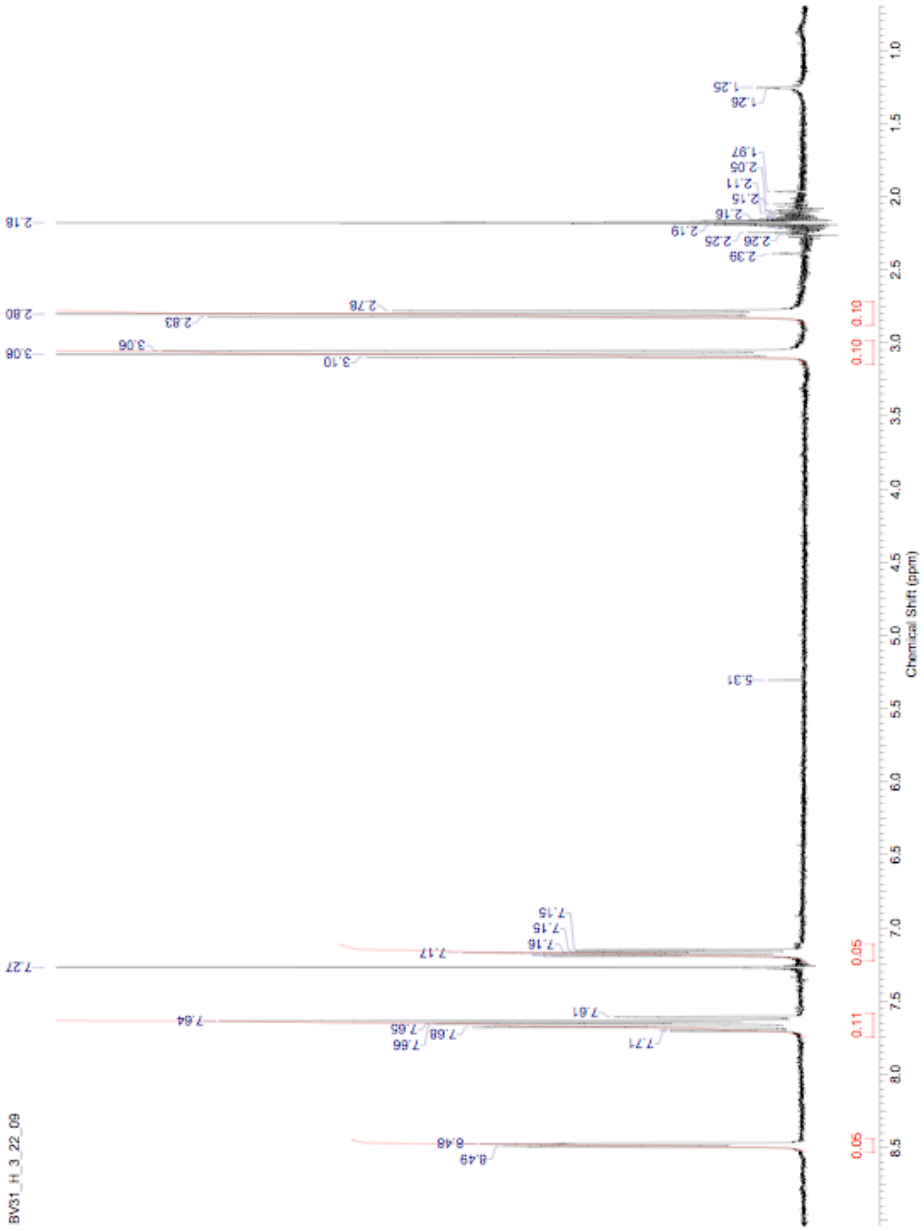


Figure B.7. Pyridyl-SS-Propanoicacid ^{1}H NMR

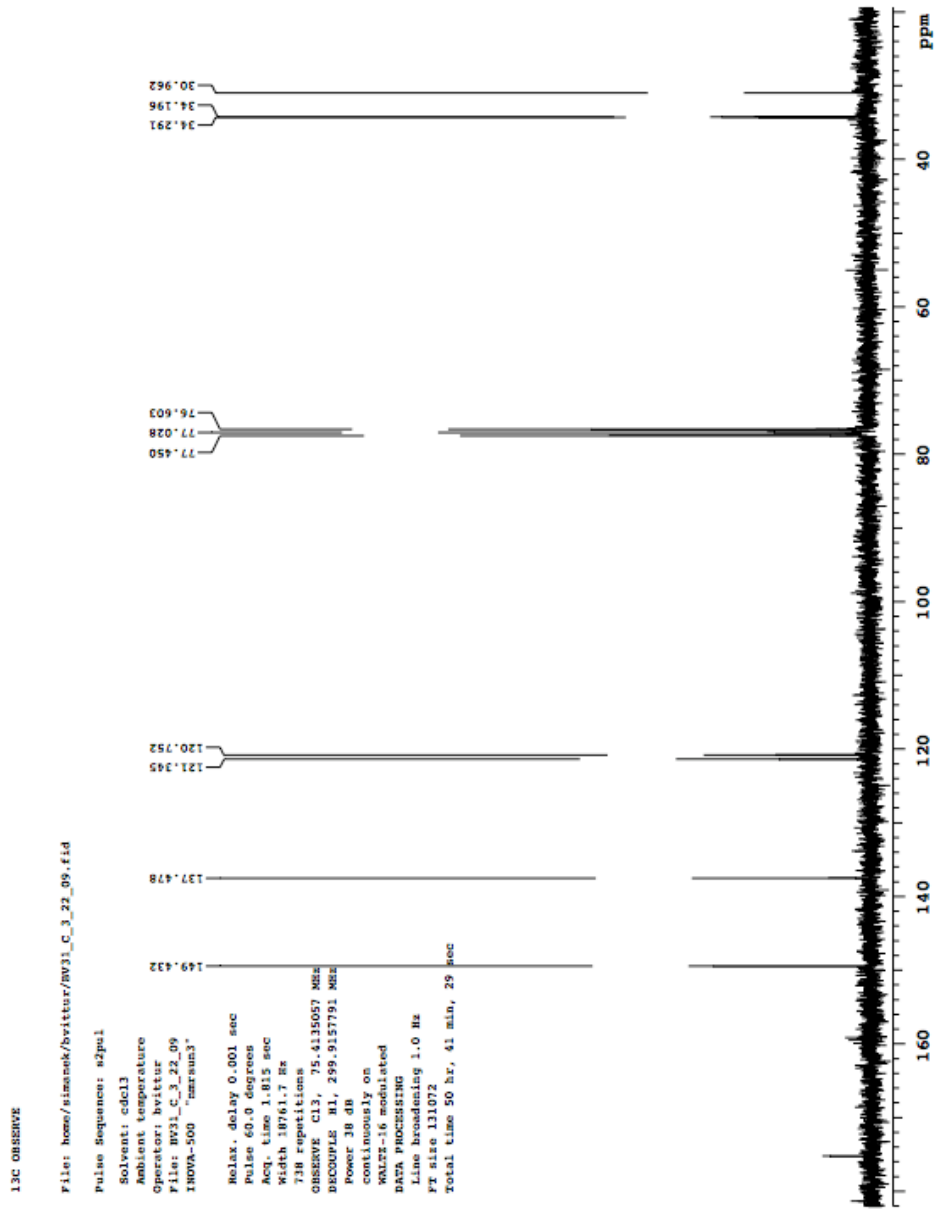


Figure B.8. Pyridyl-SS-Propanoicacid ^{13}C NMR

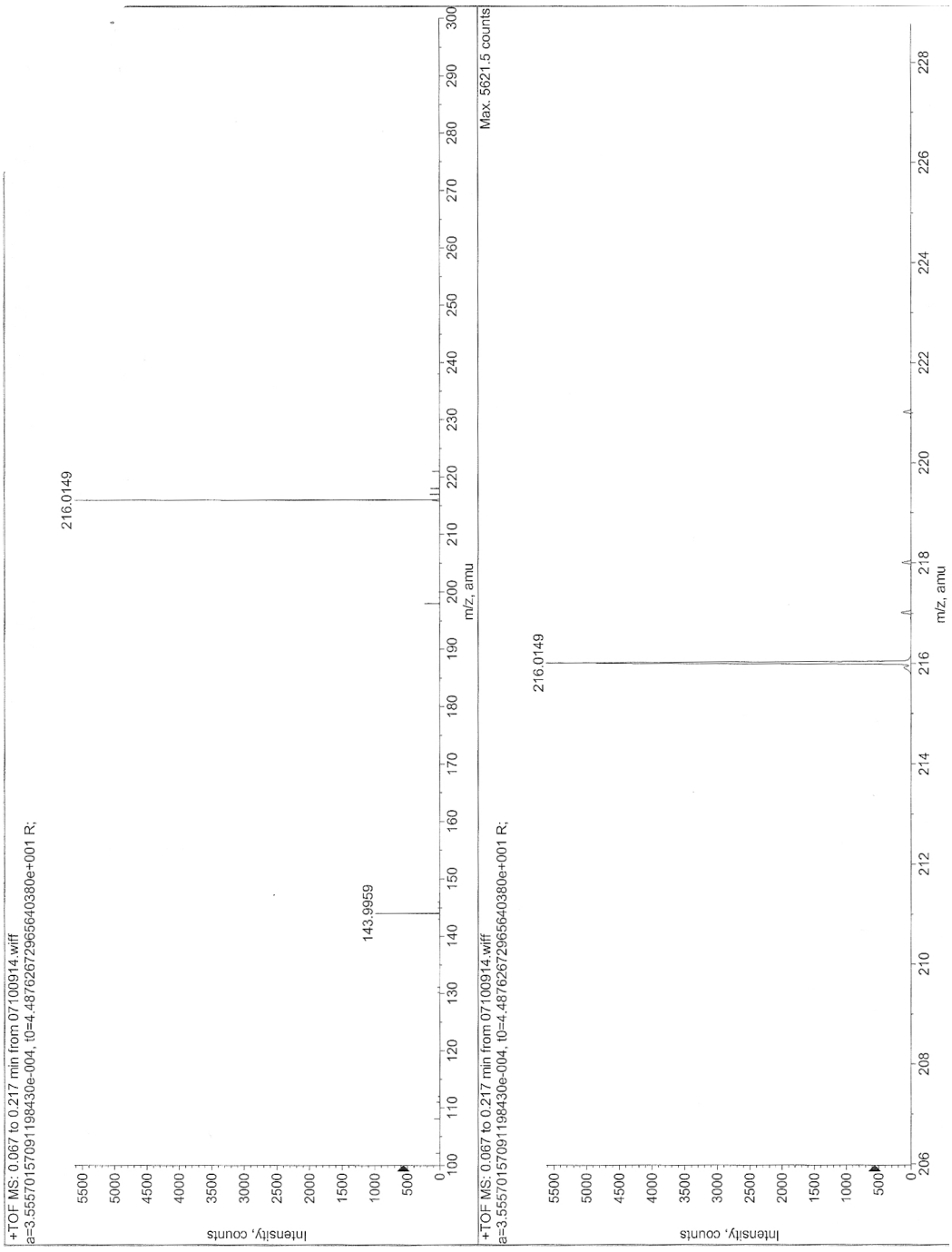


Figure B.9. Pyridyl-SS-Propanoicacid Mass Spectra

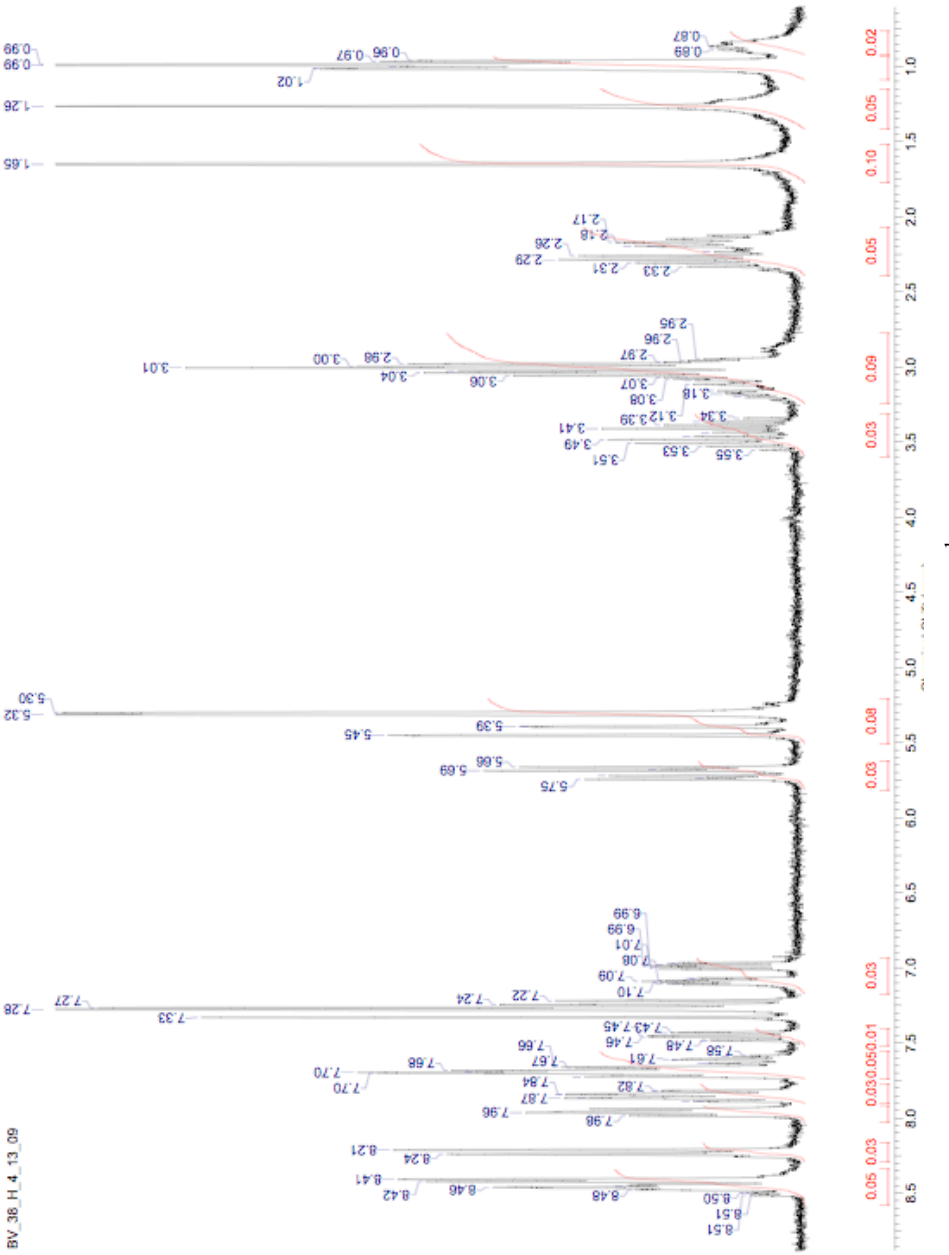


Figure B.10. Camptothecin-(Pyridyl-SS)-Propanoate ^{1}H NMR

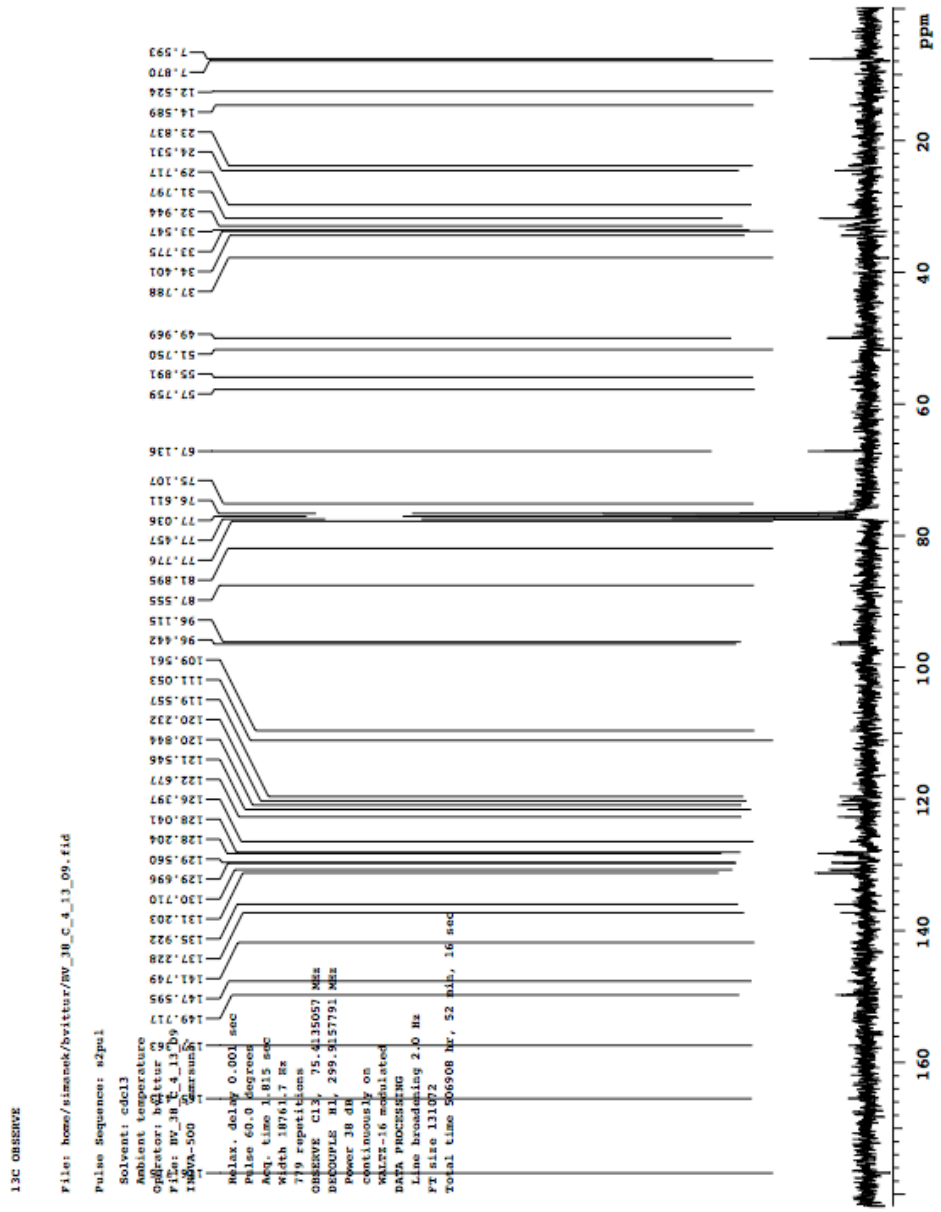


Figure B.11. Camptothecin-(Pyridyl-SS)-Propanoate ^{13}C NMR

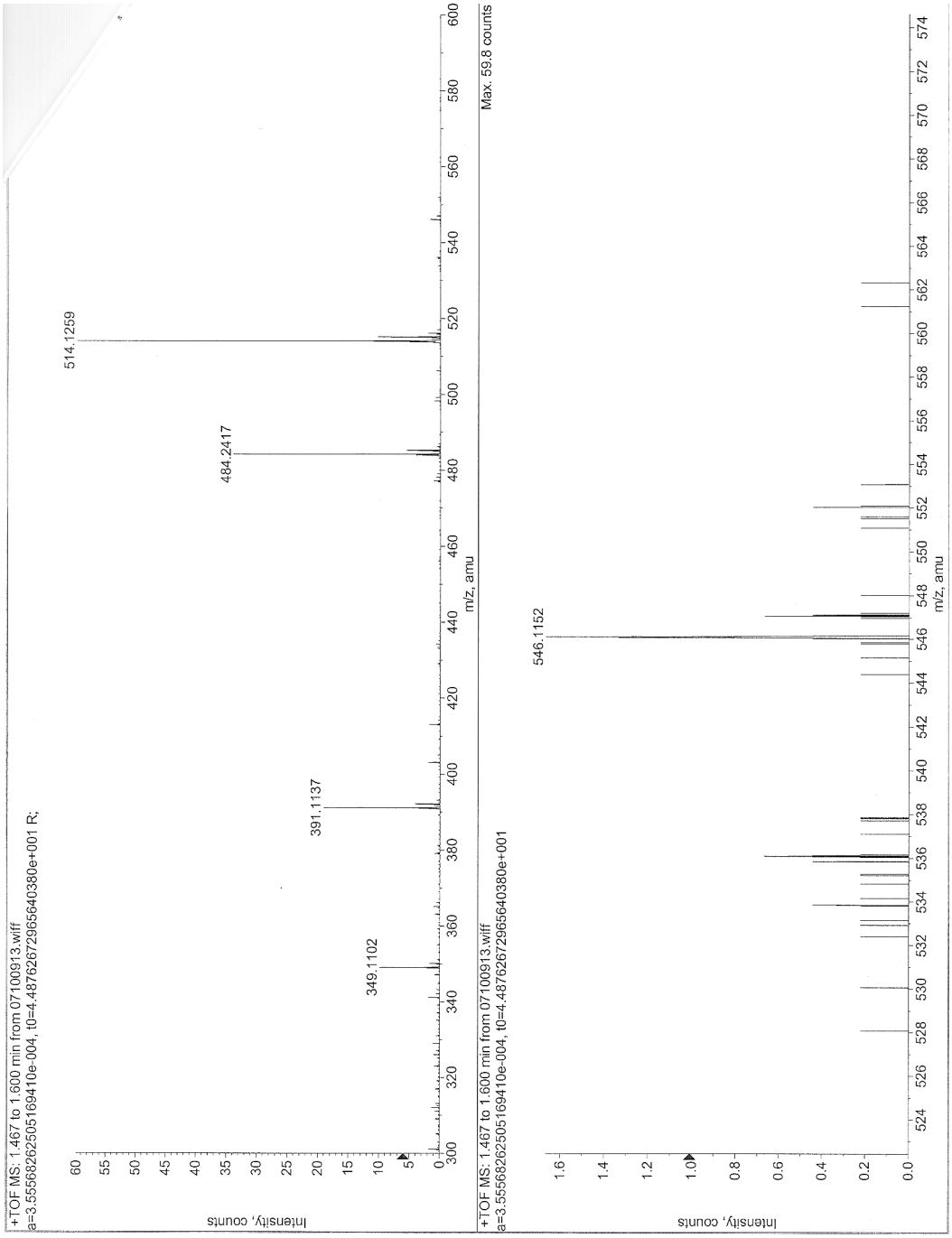


Figure B.12. Camptothecin-(Pyridyl-SS)-Propanoate Mass Spectra

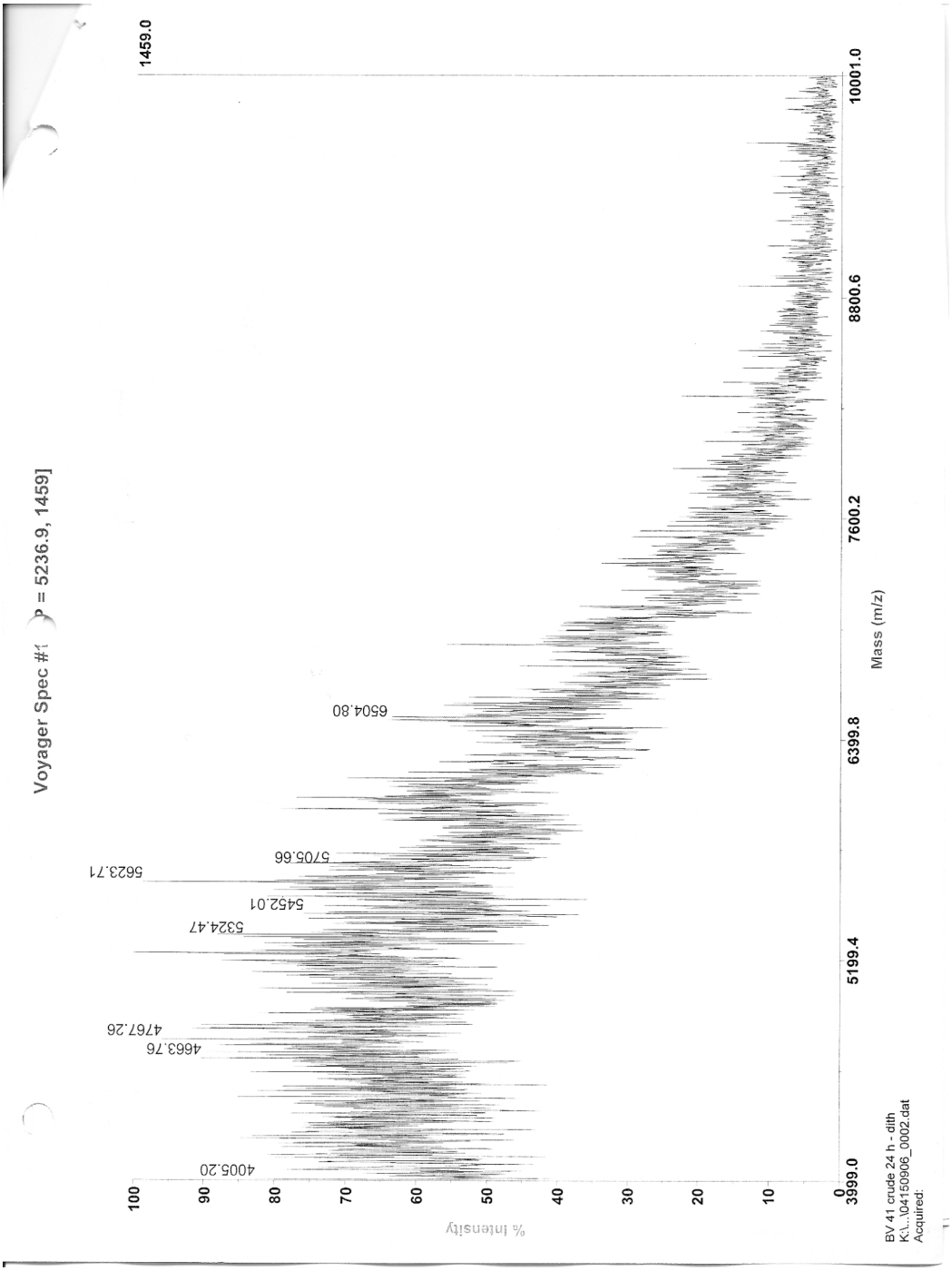


Figure B.13. G2-(Cys(NH_2)-SS-Camptothecin)$_n$ Mass Spectra

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