DISSERTATION

PANNING PEPTIDE LIBRARIES ON FILAMENTOUS PHAGE

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Ft. Collins, Colorado

Spring 1996

COLORADO STATE UNIVERSITY

August 5, 1996

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JENNIFER A. TRAVERS ENTITLED PANNING PEPTIDE LIBRARIES ON FILAMENTOUS PHAGE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PANNING PEPTIDE LIBRARIES ON FILAMENTOUS PHAGE

This Ph.D. project involved using the filamentous phage as a tool to express peptide libraries on its external appendage called the pIII protein. The peptide libraries were designed based on a motif of the honeybee toxin Apamin. Apamin is expressed on the end of the pIII protein and a portion of the apamin section is randomized to produce all possible combinations of amino acids to give a peptide library.

The library phage are then panned on a derivatized solid support to determine if any library members have an affinity to a target ligand. The target chosen is a portion of the peptidoglycan layer in bacterial cells called L-lysine-D-alanine-D-alanine. This is the site for binding of the antibiotic vancomycin. Library members that bind to this site should have vancomycin-like activity.

This project entailed preparing the libraries, synthesizing the ligand, and derivatizing a variety of solid supports for panning. Many different panning experiments were performed on several libraries and the results are described herein.

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ACKNOWLEDGMENT

I would like to thank several other scientists who were involved in this project. Dr. Mary Doubleday, with her boundless energy and enthusiasm was a true inspiration throughout her tenure in the group. Trained in classical organic chemistry, Mary plunged into biochemistry without a moments hesitation. A consummate scientist, Mary deserves the credit for getting this project rolling.

Dr. Matt Peterson also contributed to the research by developing the second library. His diligence and attention to detail are greatly appreciated. Dr. Monica Baloga assisted as well, by performing several panning series on the second library.

Renee Gallegos was a continuous help throughout the project. An experienced microbiologist, she helped all of the chemists in developing their biological technique. Exploring new procedures, equipment and novel procedures, she was always there to guide us. Her help on this project was invaluable.

Lastly, I would like to thank my advisor, Dr. Robert M. Williams. Besides scientific guidance, Bob has provided me with continual support through all the growing pains of graduate school. He has helped me achieve my dream of becoming a teacher by providing me with the opportunity to be his assistant in his undergraduate organic chemistry lectures. Bob gave me increasing responsibilities in these classes, and eventually I obtained a fellowship to teach my own lecture. Bob's continual enthusiasm and fascination with biological chemistry has always been inspirational. I hope that I too, will continue to feel as passionate about chemistry for the rest of my career.

DEDICATION

I would like to dedicate this dissertation to my husband Stephen P. Kelly. With Steve's unwavering support, I found the strength to persevere in the face of adversity.

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INTRODUCTION

I. PHAGE DISPLAY BACKGROUND

A. Morphology and Life Cycle of Filamentous Bacteriophage

A bacteriophage or phage is a virus which infects a bacterial host, utilizing much of the host's enzymes for replication. It consists of a single strand of circular closed DNA (called the (+) strand), surrounded by a protein coat.

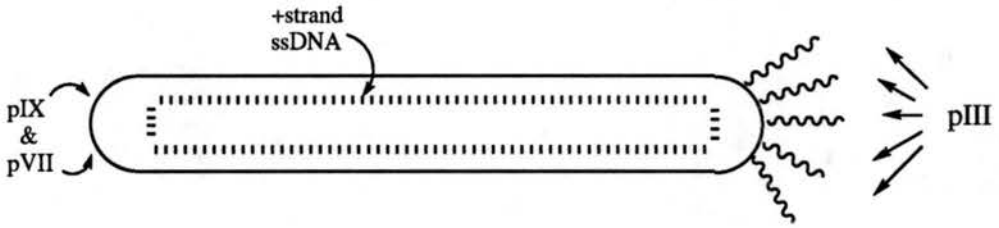


Figure 1: Diagram of a filamentous bacteriophage.

The filamentous phage is long and stringy, approximately 900nm long and 9nm wide. The walls of the protein tube consist of thousands of copies of one small protein, called pVIII. At one end of the phage are 5 copies of the gene III protein called pIII. On the other end of the phage are five copies each of the proteins pVII and pIX.[1]

The pIII protein is essential for the filamentous phage to infect a bacterium. This protein is approximately 42,000 mw. The C-terminus is internal to the phage , while the N-terminus (approximately two-thirds of the protein) is present as a knob or tail extending out from the surface of the phage. This portion of the pIII protein is believed to be responsible for recognition and binding to the bacterial host.

The fd, fl and M13 strains of filamentous phage infect *E.coli* bacteria. They will only infect *E. coli* which express the F' pilus, which is an appendage that branches off of the bacterial cell, extending into the surrounding medium. The phage recognizes and binds to the F' pilus and inserts its DNA, leaving the protein coat outside. The exact manner in which the phage DNA enters the host is still not known, but the strongest evidence suggests that the pilus retracts upon phage adsorption, drawing the phage to the cell surface.[2] Several host proteins then assist the entry of phage DNA to the interior of the cell. When the ssDNA, (the plus strand), enters the cell , the *E.coli* enzymes make a copy of the complimentary minus strand and use it as a template for making many copies of the plus strand for assembly into new phage particles. The phage coat proteins are synthesized at the inner surface of the bacterial cell, and the plus strand is then encapsulated by these proteins as it is extruded from the cell as a new filamentous phage (See Figure 2).

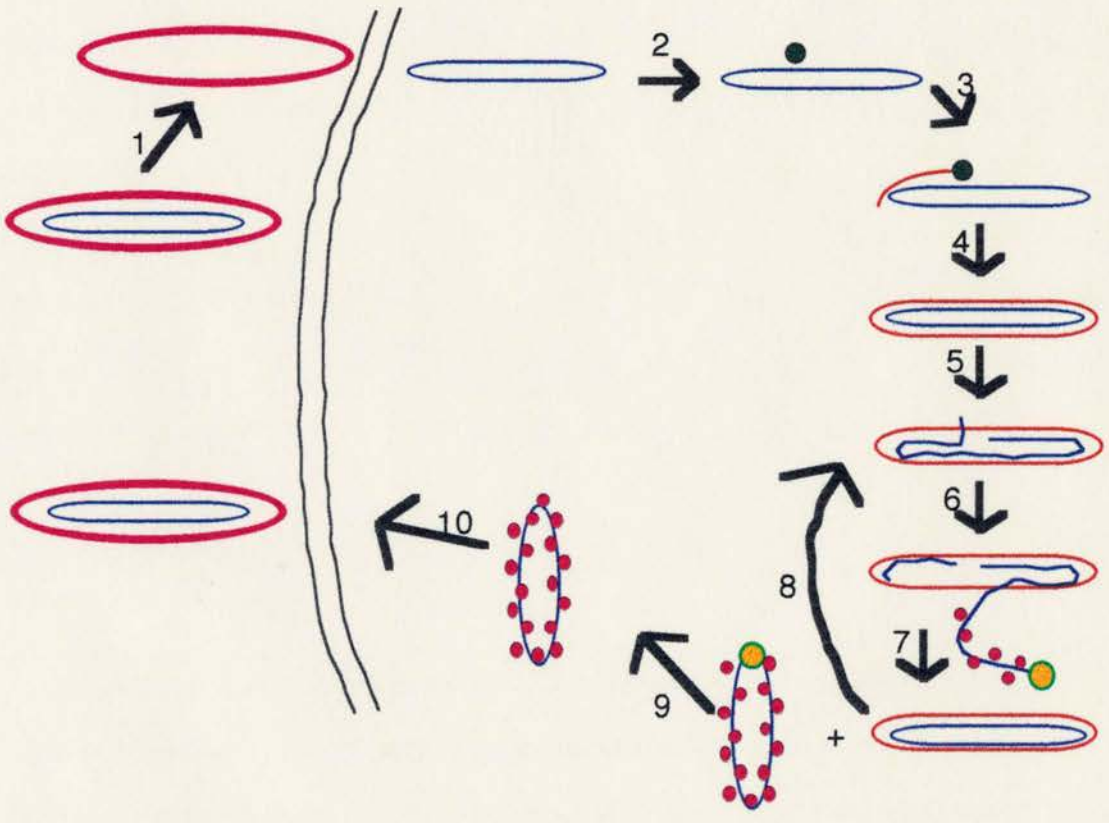


Figure 2. Life cycle of the filamentous phage. Step 1. After the phage is brought to the cell surface by the retraction of the F' pilus, the ssDNA(blue) enters the cell, leaving the protein coat outside. This DNA

B. Creation of Fusion Phage

In 1982 Zinder and Boeke described the utility of filamentous phage as cloning vectors. DNA inserts were cloned into a noncoding intergenic region on the phage genome. They were able to insert fairly large (up to 10kb) fragments of foreign DNA without destroying phage viability. They noted, however, that deletion mutants were common, and plaques of phage with inserts often slowed the growth or killed the bacterial host. One of the suggestions they made was to choose a different site for placing the inserts.[3]

In 1985 George Smith described the placement of DNA inserts into the pIII gene between the internal carboxyl end of the protein and the external amino end. A foreign sequence thus placed between the two domains gives a "fusion phage" that expresses a new protein on the surface of the phage. He demonstrated its usefulness by cloning in fragments of the restriction enzyme Eco R1 and showing that the fusion phage created were able to reversibly bind to Eco RI antibody.[4] He described the first "panning" experiment in which Eco RI fused phage are combined with other mutant phage and the mixture is incubated on an Eco RI-Antibody coated plate. After successive washes with buffer, the Eco RI fusion phage were enriched 3000 fold. Smith suggested that antibody panning might be effective in isolating desired clones from a library of random inserts.

C. Evolution of Fd/tet Phage Vector by G. P. Smith

In 1988 George Smith developed the fd/tet phage. It was prepared by taking the fd strain of filamentous phage and splicing the tetracycline resistance determinant of Tn10 into the origin of minus strand synthesis.[5] He later reported moving the cloning site to the N-terminus of the pIII gene without disrupting infectivity. These phage were treated with

biotinylated antibody directed against the target gene product, then panned on a streptavidin coated plate. Yields of the target phage from a single panning were 1.8-4% while the yield of background phage were only 10^{-5}% corresponding to a 10^4-10^5 enrichment. He discussed prospects for placing an epitope library into this cloning site. The epitope library would contain a synthetic random coding sequence that could be panned for antibody selectivity. [6] This idea of an epitope library was inspired by the synthetic mimotope strategy of Geysen et. al.[7] Soon after, the Smith group reported the successful panning of a small epitope library expressed on the fd/tet phage surface. [8]

An epitope library of fusion proteins contains a randomized region, such that each member of the library expresses a **different** amino acid sequence in the randomized region, and the complete library expresses all possible combinations of amino acids. The epitope library is constructed by ligating a degenerate oligonucleotide into the cloning site. A hexapeptide library is degenerate at six codons or 18 nucleotides. When synthesizing this oligonucleotide, single nucleotides are placed at positions encoding the fixed amino acid codons. For the degenerate codons, equimolar mixtures of all four nucleotides (N) are coupled at the first two sites of the codon and equimolar mixtures of G and T (K) are placed at the third site in the codon. The NNK codon represents 32 (4x4x2) codons, giving rise to all 20 amino acids and one stop codon. If a degenerate codon was synthesized as NNN, 64 codons would be expressed including 3 stop codons. Thus the degeneracy of the genetic code allows us to minimize the number of stop codons while still expressing all 20 amino acids.

In 1990, three different groups reported the construction of epitope libraries expressed on the pIII protein of the fd/tet phage. All three groups used a different approach to prepare the randomized insert that was ligated onto the phage's pIII gene.

Dower *et al* reported the construction of a hexapeptide epitope library.[9] The insert was prepared by synthesizing a 49 bp oligonucleotide (ON-49) which contained six consecutive randomized codons as shown below. Two half-site oligonucleotides were

prepared which were complimentary to the two termini of ON-49. When the half site oligonucleotides are annealed to ON-49, two "sticky ends" are created which are complimentary to the Bst X1 restriction sites on the pIII gene of the phage vector. The insert is then ligated to the vector which has been cleaved with Bst X1.

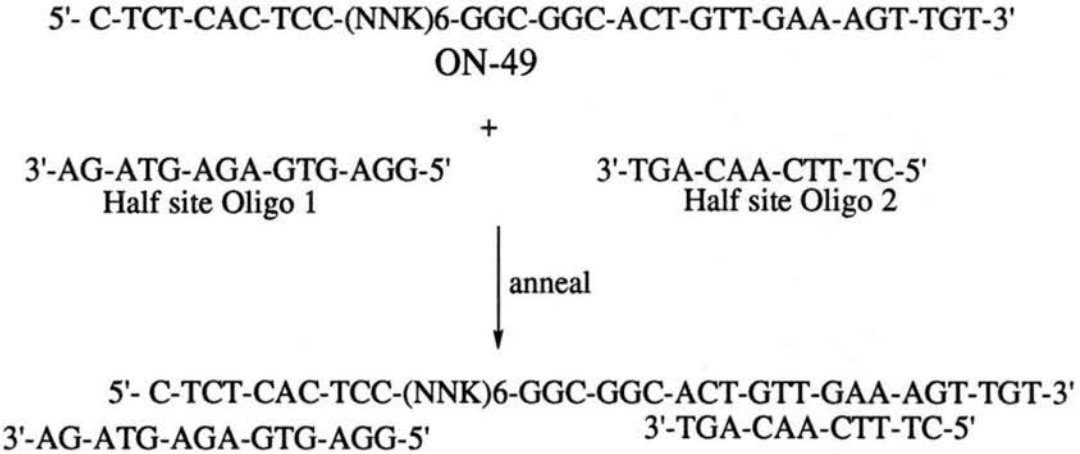


Figure 3: Preparation of randomized insert using the half site oligonucleotide method.

The DNA is transformed into E. Coli by electroporation, and the transformed cells are amplified to give the phage library. This library was panned against a monoclonal antibody specific to the opioid receptor Tyr-Gly-Gly-Phe. After three rounds of panning, 51 high affinity clones were chosen and sequenced individually. All 51 clones contained tyrosine as the N-terminal residue, 48 contained Gly as the second residue.

Smith's group also reported the panning of hexapeptide library that is placed 4 amino acids upstream from the N-terminus.[10] The Smith group used the PCR method to prepare the insert as shown below in figure 4. A 70 base pair degenerate oligonucleotide was prepared on the DNA synthesizer and used as a template for PCR with two 5' biotinylated primers corresponding to the 20 residues at the 5' ends of both strands. The double stranded DNA is digested with Bgl I, and the biotinylated terminal fragments are removed with streptavidin-agarose.

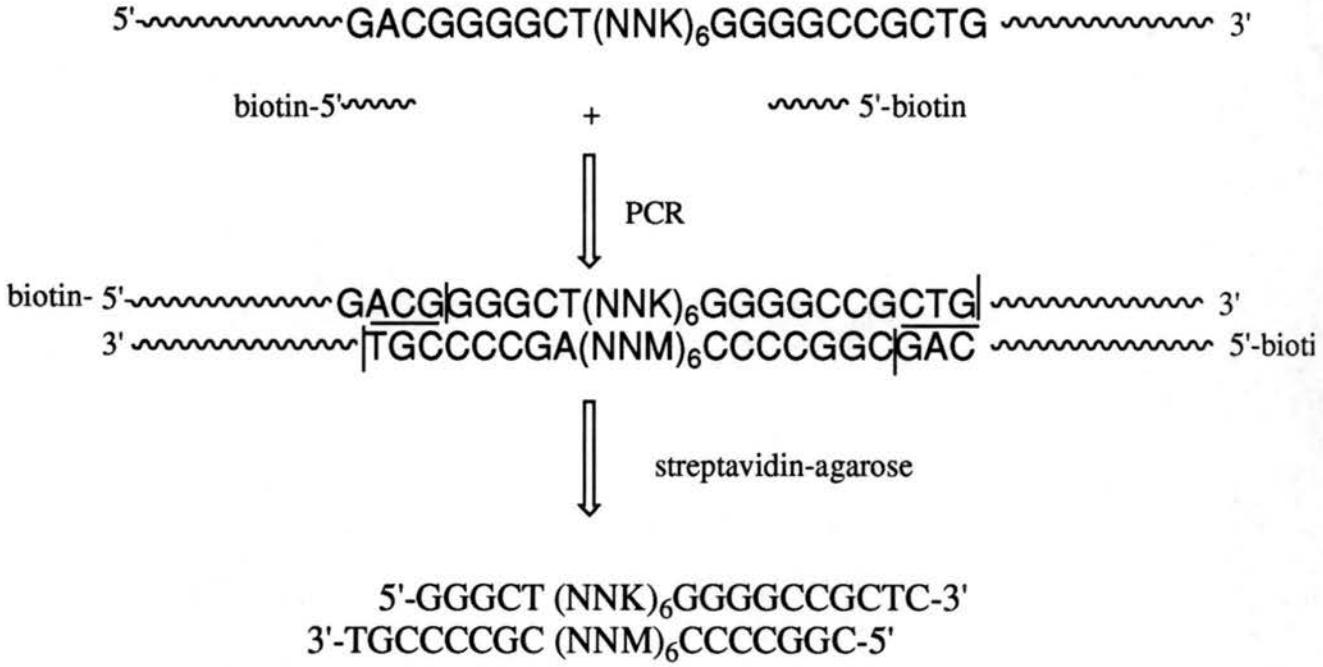


Figure 4: Preparation of randomized insert using the PCR method

The insert was then ligated into the pIII gene of Fd/tet. The DNA was electroporated into E. Coli cells and amplified to produce library phage.

Devlin *et al* used a simple approach for preparing the insert using inosine.[11] Inosine is a nucleotide known for its promiscuous base pairing ability. It can base pair with all four nucleotides. They prepared the plus strand on the DNA synthesizer with 15 adjacent randomized codons. They then prepared the complement by placing an inosine at each degenerate site. Thus this single oligonucleotide has the capability to base pair with any and all of the (4^{45}) 1.2×10^{27} different plus strands generated!

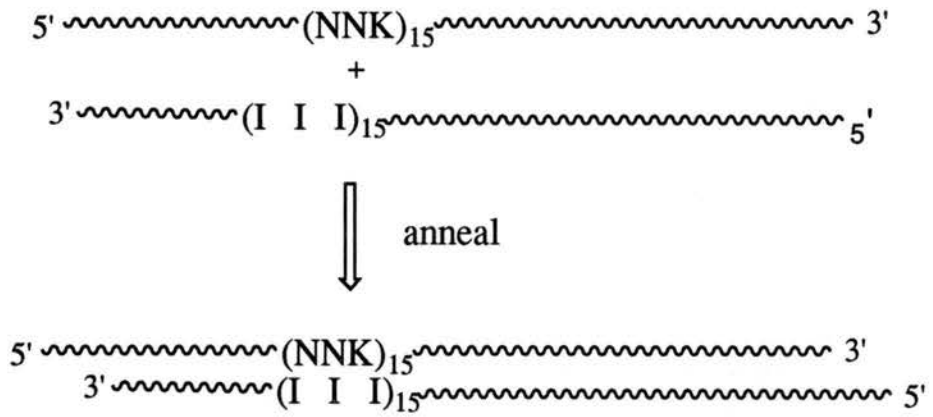


Figure 5: Preparation of a randomized insert using inosine.

The insert was then ligated into a phage vector which had been cleaved with a restriction enzyme. As with Smith and Dower, the phage produced are panned against a monoclonal antibody. Smith suggested broadening the applicability of epitope libraries to find peptide mimics of non-proteinaceous ligands such as carbohydrates, DNA and drugs.[10] Since then, the only report of a non-proteinaceous target ligand has been described. This work was done by a Swedish group in 1994, panning a phage library against a heptanucleotide ligand consisting of seven cytosines.[12]

This research project involves using the phage display library technique to search for peptides with vancomycin-like binding properties.

II. VANCOMYCIN BACKGROUND

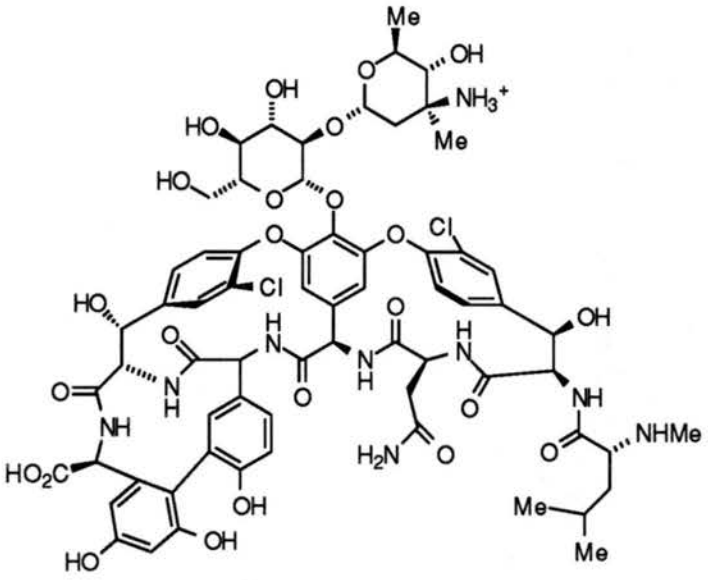


Figure 6: Vancomycin

Vancomycin is an antibiotic which was first isolated in 1956 from a soil microorganism on the island of Borneo. It inhibits bacterial cell wall synthesis by disrupting the synthesis of the peptidoglycan layer. In gram positive bacteria, the peptidoglycan layer is the outermost layer. In gram negative bacteria, there is an outer membrane surrounding the peptidoglycan layer which vancomycin cannot penetrate. Thus vancomycin is only active against Gram positive strains of bacteria.

Vancomycin's clinical use is mainly for treating nosocomial infections. These are hospital-acquired infections of bacteria which have very high resistance to first generation antibiotics. Vancomycin is often a last line of defense for patients with life threatening Gram positive bacterial infections. Most notable is its effectiveness against methicillin-resistant *Staphyloccocus aureus* (MRSA), a particularly devastating organism for the immunocompromised patient.

The peptidoglycan layer of the bacterial cell wall is essential to maintaining the cell's structure, it provides the rigidity needed to resist internal osmotic pressure.

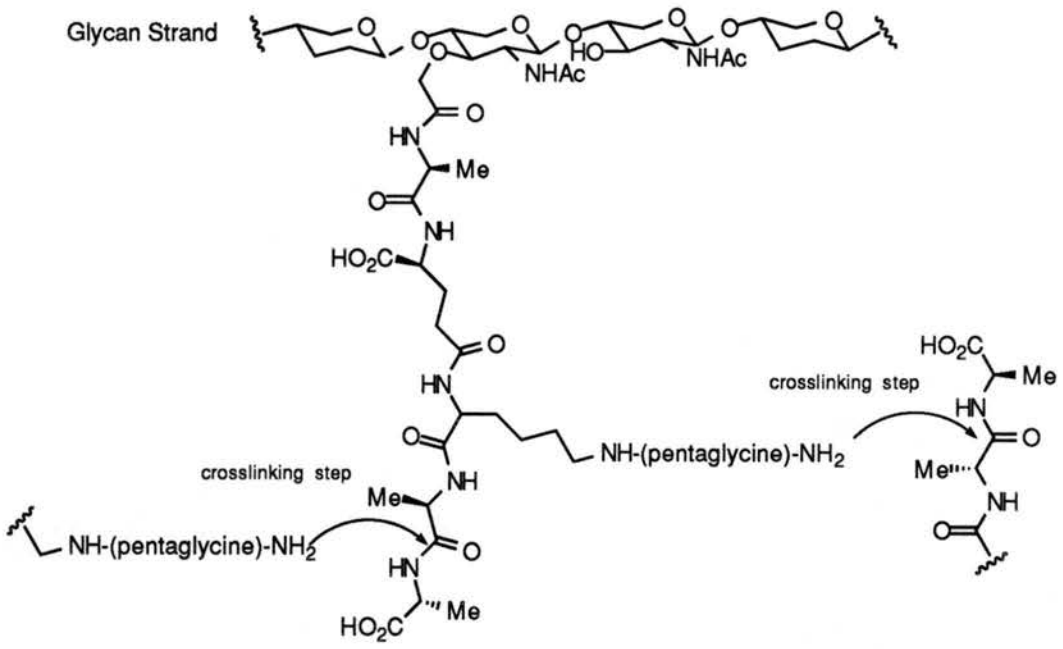


Figure 7: Crosslinking of the peptidoglycan layaer of a typical Gram positive microbe.

Peptidoglycan, as its name implies, consists of both sugars and amino acids (figure 7). The glycan strand contains alternating 1,4-β-linked N-acetylglucosamine(NAG) and N-acetylmuramic acid(NAM). The peptide portion branches off the 3 position of each NAM saccharide. There are 5 amino acids attached to this branch. At the third position is a lysine residue, which has a pentaglycine chain attached to the ε-amine. It is at the amine terminus of the pentaglycine chain where the peptidoglycan strand cross links with another strand. The cross linking step is a transpeptidation reaction where the amine attacks the carbonyl of the penultimate D-alanine of an adjacent strand, replacing the terminal D-alanine.

Vancomycin tightly binds to the end of the peptidoglycan D-ala-D-ala, and prevents the cross linking reaction from occurring. The binding interaction of vancomycin with this ligand has been extensively studied by several groups.[13-16] The binding constant is on the order of 10^{-6} M. This high binding constant is due to four hydrogen bonds and one electrostatic interaction as shown in figure 8.

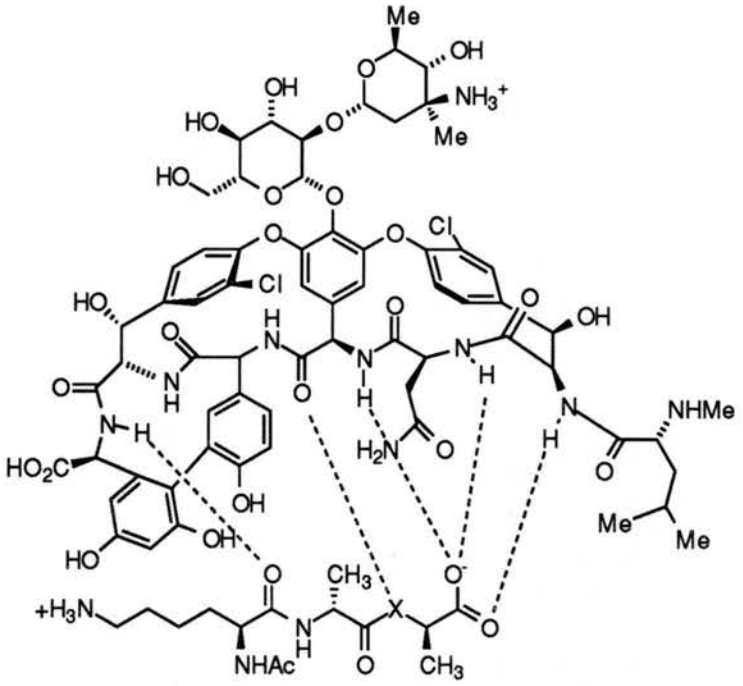


Figure 8: Vancomycin binding complex with the peptidoglycan terminus. When X=NH, the binding constant is 10^{-6}M. When X=O, the internal hydrogen bond does not occur and the binding constant is 10^{-3} M.

III. VANCOMYCIN RESISTANCE

In the past ten years, there has been a dramatic increase in the appearance of vancomycin-resistant Gram positive bacteria. The most highly resistant strains occur in the *Enteroccocus* genus: *Enteroccocus faecalis* and *Enteroccocus faecium*.. The mechanism by which these species become resistant is via a modification of the biosynthesis of the peptidoglycan terminus. Nine genes are involved in this mechanism which ultimately results in the conversion of the terminal D-alanine-D-alanine to D-alanine-D-lactate.[17,18] By effectively replacing NH with O, the central hydrogen bond donor to vancomycin is removed (see figure 8). The altered peptidoglycan doesn't affect the structural integrity of the cell wall, but it substantially reduces its affinity for Vancomycin. In vitro binding studies show that the affinity of vancomycin for D-ala-D-lac is 1000 times less than that for

D-ala-D-ala, which parallels the observed 1000 fold reduced sensitivity of vancomycin-resistant bacteria to drug.

The purpose of this research project is to identify peptides which exhibit vancomycin-like activity. Vancomycin's natural target is the peptide terminus of newly formed peptidoglycan monomers, therefore our target ligand will be L-lysine-D-alanine-D-alanine.

IV. APAMIN BACKGROUND

Apamin is an 18 amino acid peptide neurotoxin from honey bee venom (figure 9). It is known for its stability with respect to pH, temperature, and denaturants. It has been used as a model for protein folding[19,20], and its 3D structure has been intensively studied by NMR 21,22.

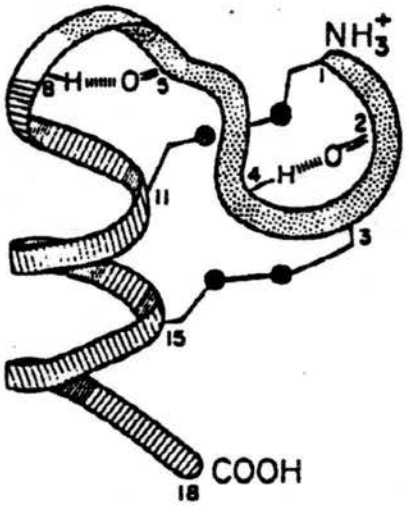


Figure 9: Tertiary structure of apamin.

The features of apamin are a beta turn loop between residues 2 and 5, and an alpha helix from residues 9 to 17. The two disulfide bonds, between cysteines 1 and 11, and 3 and 15, give apamin the tertiary structure shown below and contribute to its stability.

Apamin was chosen as the structural motif for expressing the library because the size of the turn from positions 3- 9 approximates the shape of the binding pocket of vancomycin, which is comprised of a heptapeptide backbone. The libraries generated in this project all contain the general 18 amino acid apamin motif expressed at the terminus of the pIII protein of the filamentous phage. The randomized regions are located in the beta turn loop.

DISCUSSION

I. GENERATION OF THE FIRST LIBRARY

A. Isolation of Fd/tet Phage

The filamentous phage vector used as the template for the first library was a gift from Peter Schultz's lab at the University of California-Berkeley in the form of infected host cells. This vector is designated fd/tet and contains a tetracycline resistance gene. The host cells, called K91, contain the F pilus and are tetracycline sensitive. The infected K91 cells were amplified in LB broth with tetracycline to ensure only phage infected cells grew. Whole phage was isolated by PEG precipitation from the supernatant. The replicative (double stranded) form of phage DNA was isolated from the cells and purified by low melt gel electrophoresis by Dr. Mary Doubleday.

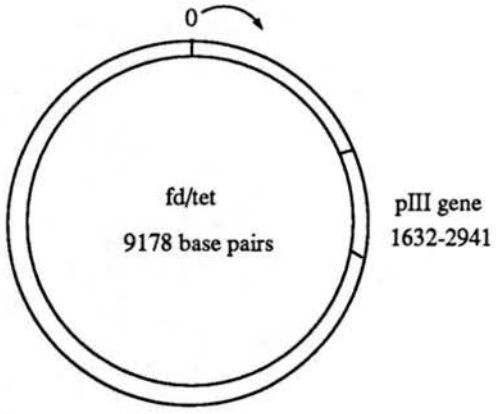


Figure 10: Map of fd/tet genome.

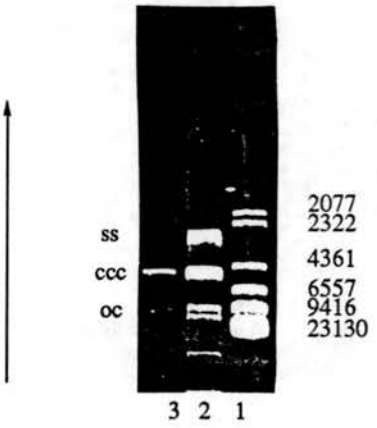


Figure 11: Agarose gel of fd/tetDNA. lane 1-mw marker, lane 2- fd/tet DNA, lane 3-low melt purified fd/tet DNA. ss= single stranded, oc= open circular, ccc= covalently closed circular

The phage has the following sequence at the N-terminus of the pIII gene:

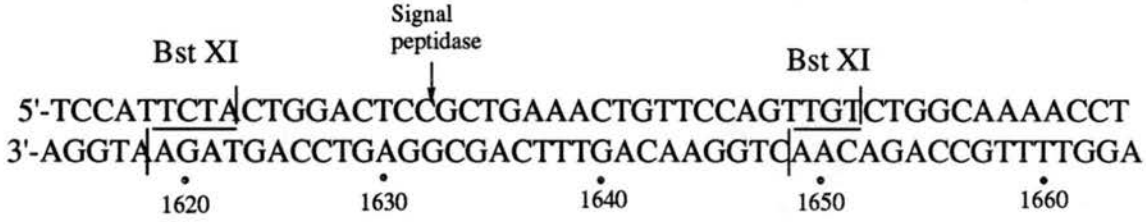


Figure 12: Sequence of fd/tet phage in pIII gene region.

The arrow in figure 12 designates where the signal peptide is cleaved from the nascent pIII protein after it has been assembled with the the other surface proteins to complete the virion. The N-terminus of the pIII begins at position 1634 of fd/tet.

Several primer oligonucleotides: A2, A3, and A6 were prepared on the DNA synthesizer, and purified using polyacrylamide gel electrophoresis (PAGE).

Oligo	Sequence	location
A2	5'-ACAAACCACAACGCCT-3'	1842-1857 (-) strand
A3	5'-CGATCTAAAGTTTTGTCGTCT-3'	1786-1805 (-) strand
A6	5'-CAACTTTCAACGGTACCA-3'	1722-1739 (-) strand

Single stranded DNA was isolated from the phage particles by removing the protein coat with a phenol, phenol/chloroform extraction, and ethanol precipitating the DNA. The ssDNA was sequenced using the Sanger sequencing method and primer A3 to ensure that the correct sequence was still present in the region of interest. The gel shown in figure 10 is read from top to bottom. It confirms the sequence corresponding to residues 1588 to 1692 on the fd/tet vector which contains the N-terminus of the pIII gene including the two Bst X1 restriction sites.

Figure 13: Sanger sequencing gel of fd/tet in pIII gene region corresponding to residues 1558-1692.

B. Preparation and Ligation of the Library Insert

The insert was prepared and ligated by Dr. Doubleday. Two 56mers were prepared on the DNA synthesizer. When the two oligomers were annealed, they formed a 16 base pair overlap. The remainder of the duplex was filled in by using DNA polymerase. The blunt ended duplex was then cleaved with Not I and Sfi I restriction enzymes to give the non-complimentary sticky ended insert as shown in figure 14.

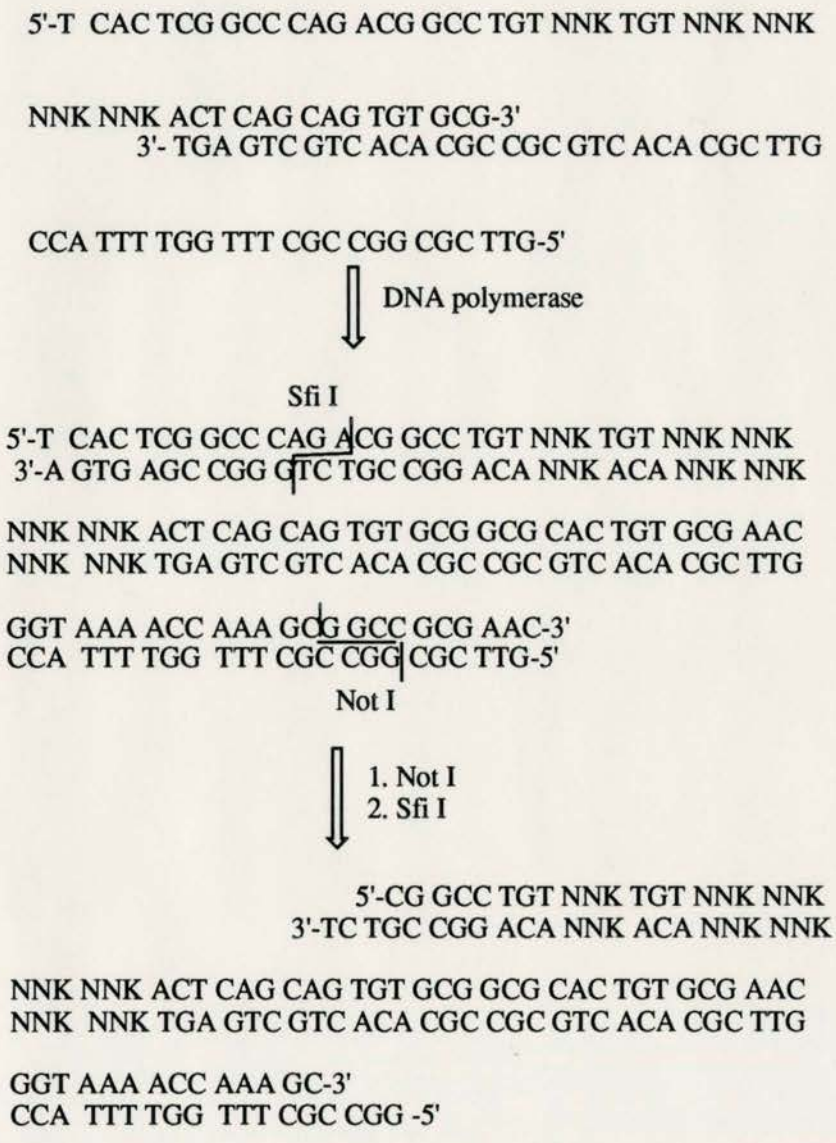


Figure 14: Preparation of library insert.

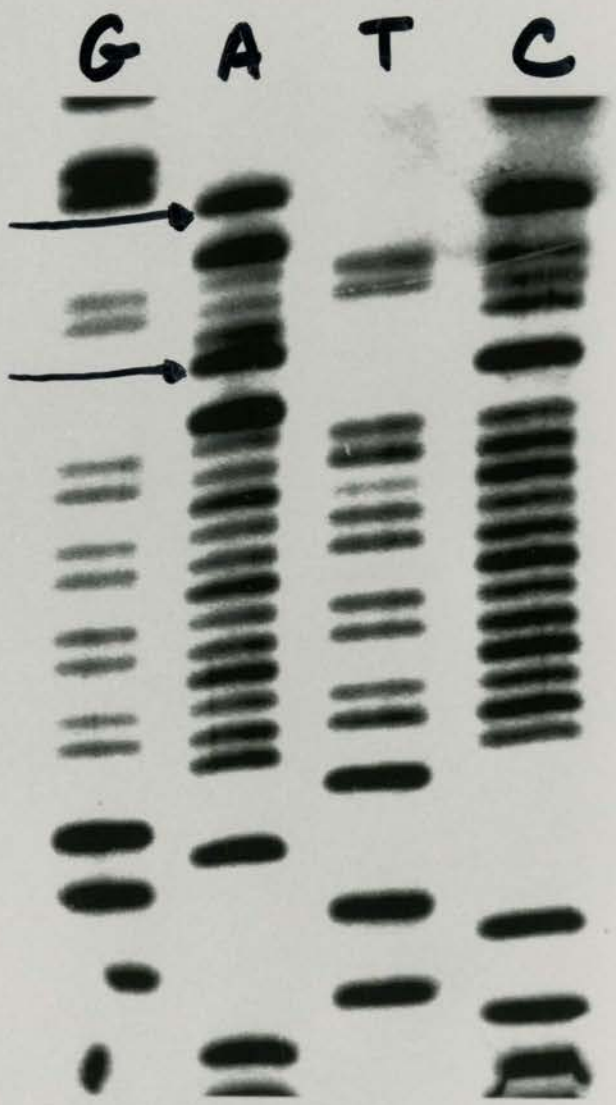


Figure 15: Sanger sequencing gel of fd/tet apamin library. The arrows indicate the two conserved ACA triplets coding for cysteine.

The insert was purified by gel electrophoresis and 5' dephosphorylated. It was then ligated into linearized fd/tet which had been cleaved with Not I and Sfi I. The DNA was then electroporated into MC1061 *E.coli* cells and amplified to give the fd/tet-apamin library. Restriction digests and Sanger sequencing were performed to show the presence and fidelity of the insert with corresponding randomized regions. The randomized regions can be clearly seen in the photograph of the gel on the previous page (figure 15). A band appears in all four nucleotide lanes for N and in the A and C lanes for K.

II. PANNING OF THE FD/TET APAMIN LIBRARY

The process of panning or biopanning involves incubating a phage library on a solid support that is derivatized with the target ligand (figure 16). This methodology is normally used to pan against very large ligands such as antibodies, enzymes and DNA . These large molecules are usually coupled to biotin, and bound to streptavidin coated plates using the very strong affinity of biotin for streptavidin to bind the ligand to solid support [9,11,20,21]. In our case, this strategy was not appealing because the relative size of the streptavidin-biotin conjugate would dwarf the ligand, and the presentation to the media of the tripeptide would likely be masked by the large protein molecules surrounding it. We therefore chose to covalently bind our ligand directly to a solid support using small linkers.

A large variety of solid supports are commercially available. Most common are the carbohydrate sepharose or agarose gel suspensions. These supports can be derivatized with covalent linker attachments. Covalent linkers can also be attached to plastic polymers such as polystyrene and polyethylene.

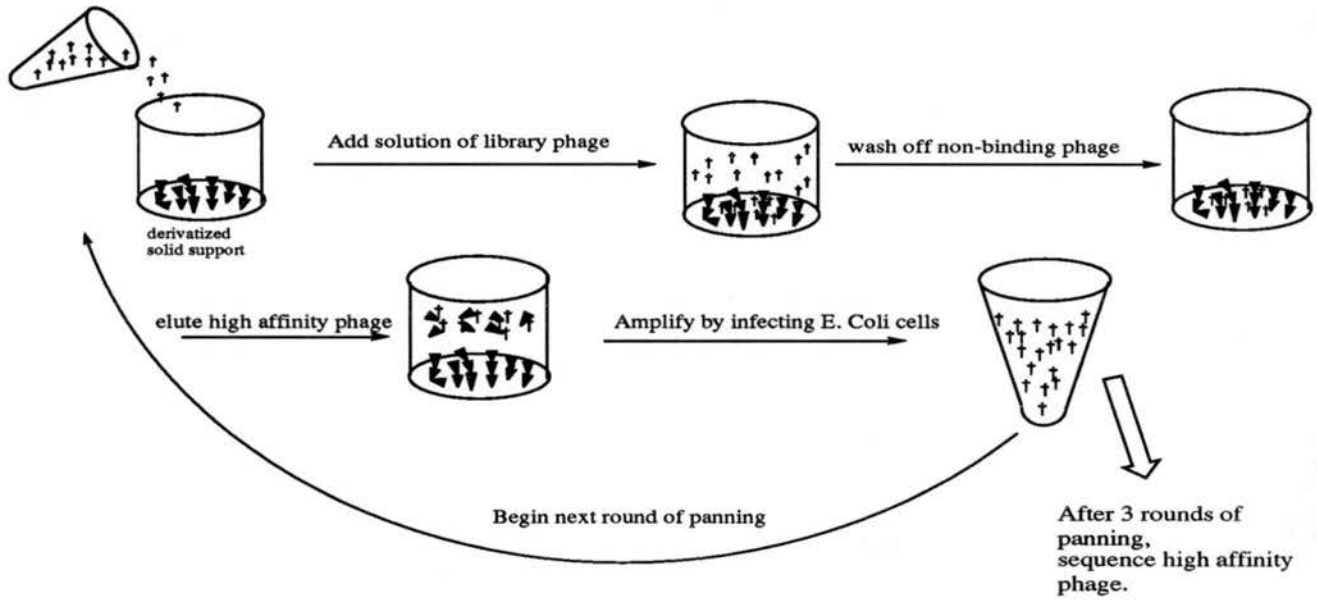
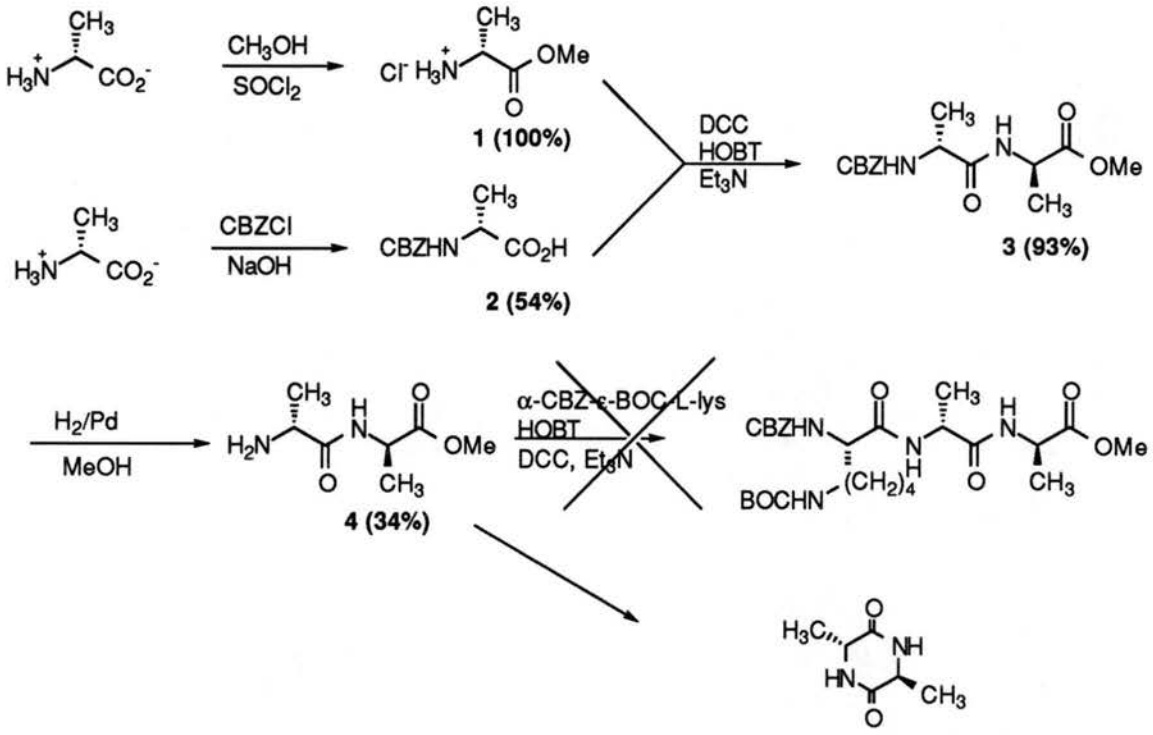


Figure 16: Panning of a phage library.

The first task was to synthesize the tripeptide ligand that vancomycin binds to: L-lysine-D-alanine-D-alanine. The tripeptide can then be attached to the solid support with the ε-amine of lysine. A protecting group is placed at the alpha position to mimic its linkage to the rest of the peptidoglycan strand.

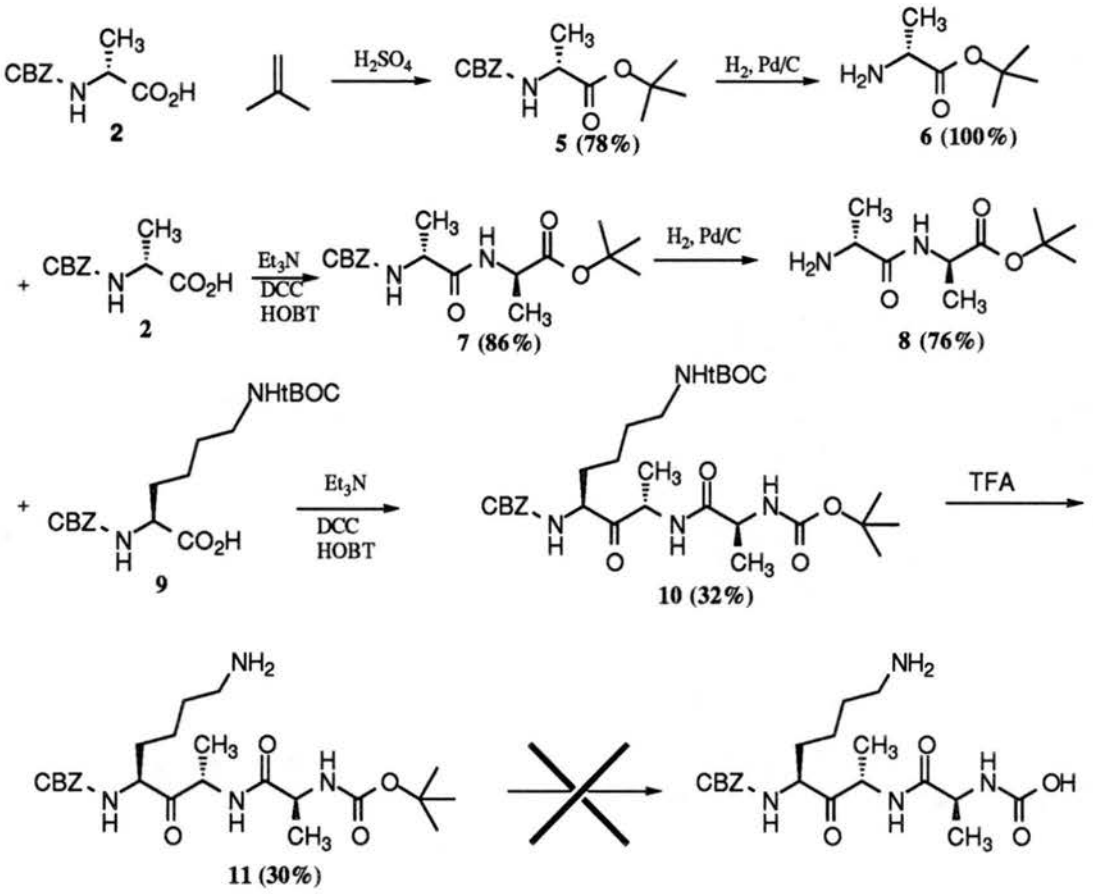
A. Synthesis of α-CBZ-L-lys-D-ala-D-ala

The first approach to synthesizing this tripeptide, illustrated in Scheme I, was to start from the carboxyl end, making the D-alanine methyl ester(**1**) and carbobenzoxy-D-alanine(**2**), both from commercially available D-alanine. **1** and **2** were coupled using the reagent dicyclohexylcarbodiimide(DCC) to give the CBZ-D-ala-D-ala dipeptide methyl ester **3**, and the CBZ group was then removed to give **4**. However, when **4** was subjected to coupling conditions, it cyclized to form a diketopiperazine(DKP) instead of forming the desired tripeptide.



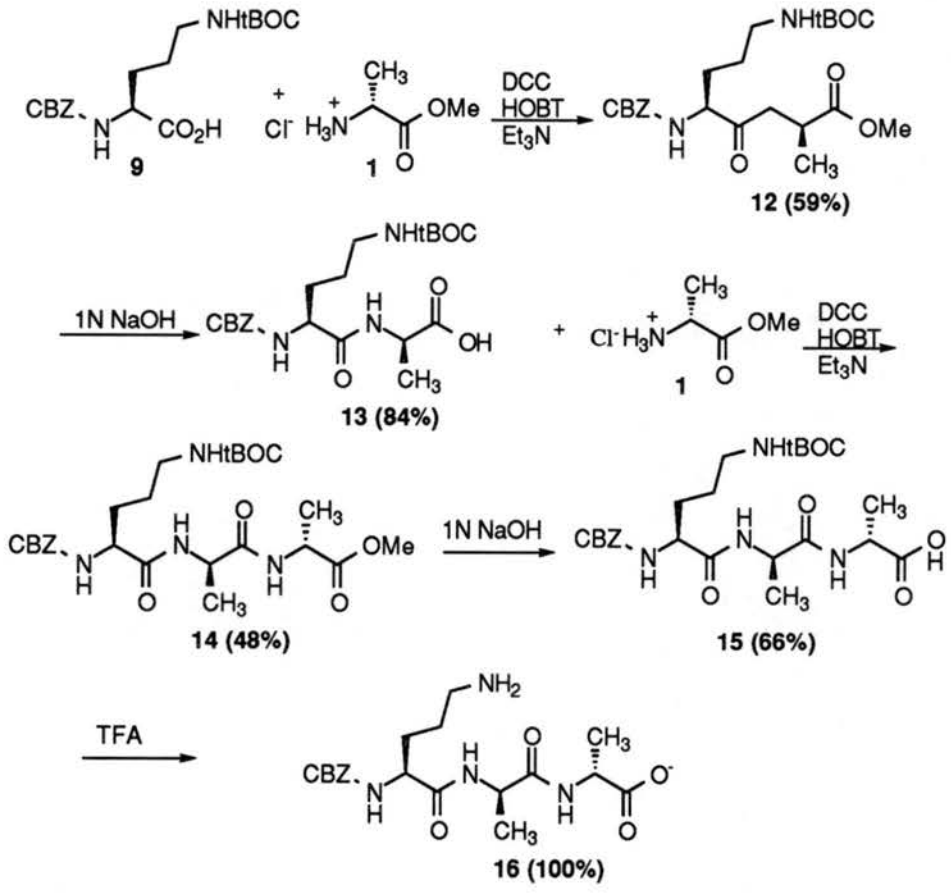
Scheme I

The next plan (Scheme II) to circumvent the problem of DKP formation, was to form the t-butyl ester of the dipeptide under the premise that it would be less likely to cyclize due to steric hindrance. Thus **2** was treated with isobutene to give the isobutyl ester **5**. The CBZ group was removed from **5** by hydrogenation to give **6**, then coupled with **2** to give the dipeptide **7**. The dipeptide was deprotected and coupled with the diprotected lysine acid **9** to give the tripeptide **10**. Treatment with TFA removed the tBOC group from the lysine amine, but the t-butyl ester remained intact under these conditions. In the meantime, a parallel synthesis was being conducted which proved to be more fruitful.



Scheme II

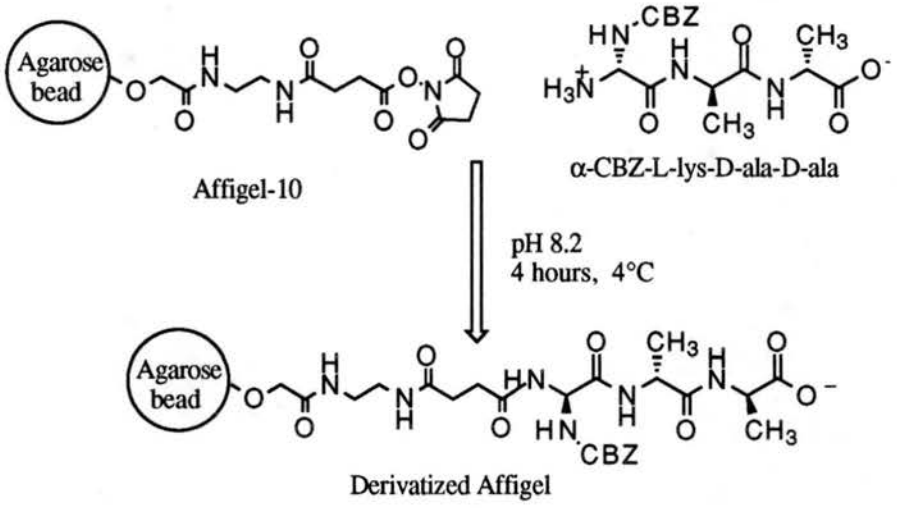
The third approach (Scheme III) was much more successful. By starting from the amino end, **9** was coupled to **1** to give the dipeptide methyl ester **12**. This ester was saponified and coupled to another equivalent of **1** to give the tripeptide methyl ester **14**. **14** was saponified, and the tBOC group was removed with TFA to give the desired product **16** in 5 steps with a 16% overall yield. By building from the amino end, there was no problem with the L-lys-D-ala dipeptide cyclizing to form a diketopiperazine. It was later discovered that the dipeptide **4** will couple without DKP formation when it is in the form of the TFA ammonium salt (see Scheme XIV).


NHtBOC
CBZ
CO2H
9
Cl- H3N
OMe
1
DCC
HOBT
Et3N
12 (59%)
1N NaOH
13 (84%)
14 (48%)
15 (66%)
TFA
NH2
16 (100%)

Scheme III

B. Derivatization of α-CBZ-L-lys-D-ala-D-ala(**16**) to Affigel

The first solid support chosen was Affigel-10 from Biorad. Affigel-10 is a cross linked agarose gel bead derivatized with a neutral 10 atom spacer arm and an N-hydroxysuccinimide ester terminus which reacts with free amino groups at pH 8.2. CBZ-L-lys-D-ala-D-ala(**16**) was covalently coupled to the Affigel by incubating in an excess of the peptide solution to the gel slurry and agitating for four hours at 4°C (Scheme IV).



Scheme IV

. The coupling was monitored by OD_{280} readings. The concentration of the peptide solution was 4.9 mg/mL and the OD reading was measured at .575 units. This corresponds to 9.8 mg of peptide added to the slurry.

solution	OD_{280}	conc.	amount of peptide
2 mL of peptide solution added to gel	.575	4.9 mg/mL	9.8mg added
supernatant #1 (2mL)	.176	1.49 mg/mL	-2.98mg removed
supernatant #2 (3mL)	.045	.38mg/mL	-1.14 mg removed
supernatant #3	.006	-	negligible
			= 5.68 mg bound

Table 1: Monitoring of peptide coupling to Affigel by OD_{280} readings.

After four hours the supernatant solution was removed and the OD_{280} reading was .176 units which corresponds to 2.98 mg that had not bound to the gel. 3 mL more of buffer was added and agitated with the gel. After one hour this supernatant was removed and the OD reading was measured at .045 units. This corresponds to 1.14 mg of peptide removed from the mixture. The following additions of buffer gave negligible OD readings. The total peptide bound was then calculated to be 5.68mg, or 10.6umol.

Reversible binding of vancomycin to the derivatized gel was measured by HPLC and used to determine the extent of derivatization of the beads. First, increasing concentrations of vancomycin were injected on an HPLC to establish a gradient. Vancomycin was then added to 1 mL of Affigel until a saturation point was reached. Using the HPLC concentration gradient, it was calculated that 12 µmol of vancomycin had reversibly bound to the gel. The vancomycin was eluted off the gel using millimolar concentrations of free tripeptide. By combining the results from the OD readings above and

the HPLC study, it was concluded that approximately 10-12μmol of sites are available for binding per milliliter of Affigel.

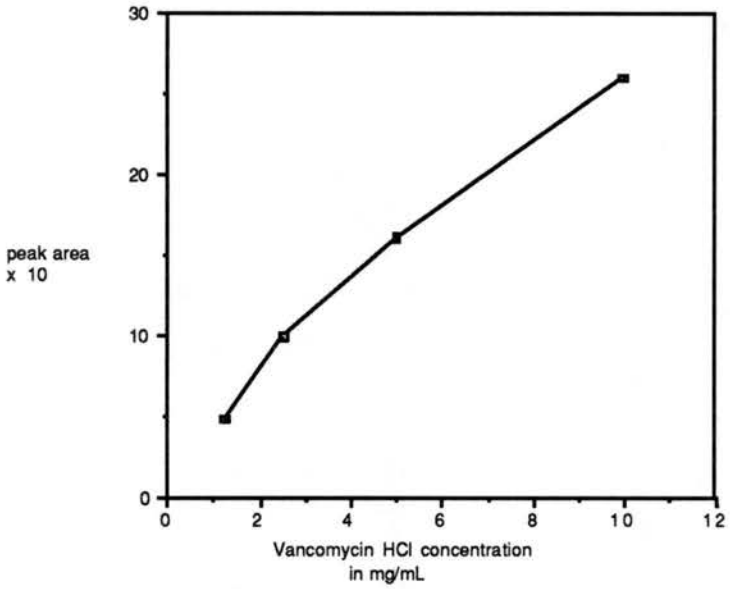


Figure 17: Vancomycin concentration gradient

C. Panning of the Fd/tet-Apamin Library on Derivatized Affigel

The fd/tet-apamin library was split into two aliquots; one was treated with O_2 for one hour, the other was not. The oxidation step is taken to ensure that the two disulfide bridges in the apamin fusion peptide form. The two fd/tet apamin libraries(oxygenated and nonoxygenated) were panned on the derivatized Affigel by incubating with the gel, removing the supernatant, and washing the gel, then removing the bound phage with free α-CBZ-L-lys-D-ala-D-ala(**16**). The eluted phage were amplified and subjected to three more rounds of panning to give a pool of high affinity phage. The O_2 treated phage gave no enrichment, whereas the phage that were not treated with oxygen gave an enrichment of at least 1200-fold. Sequencing of all four rounds of panned phage showed the emergence of deletion mutants.

From the high affinity panned phage, 120 single clones were selected and amplified to give 120 individual phage by Dr. Mary Doubleday. Each clone was subjected to an ELISA assay to identify whether it truly had an affinity for L-lys-D-ala-D-ala. Covalink Nunc plates are 96 well polystyrene plates with a secondary amine functionality attached to the surface. The plates were derivatized with CBZ-L-lys-D-ala-D-ala(**16**) using disuccinimidyl suberate as a linker (figure 18). To determine a "hit", each individual clone was incubated on both derivatized Nunc plates, and underivatized Nunc plates as a blank or background. ELISA assays were performed, and phage from wells which showed absorbance higher for the derivatized vs. underivatized were labeled as hits.

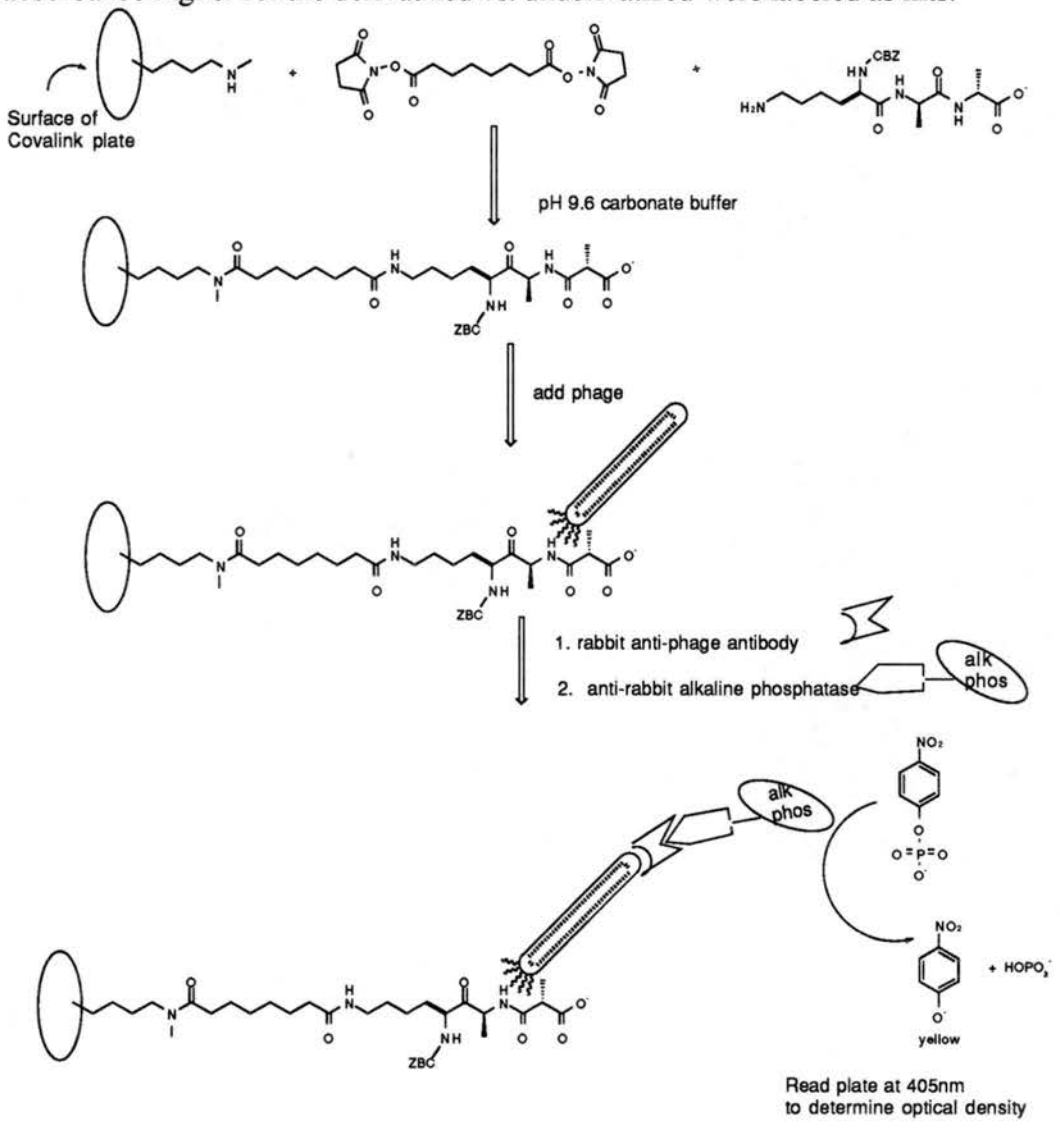


Figure 18: ELISA Assay of clones

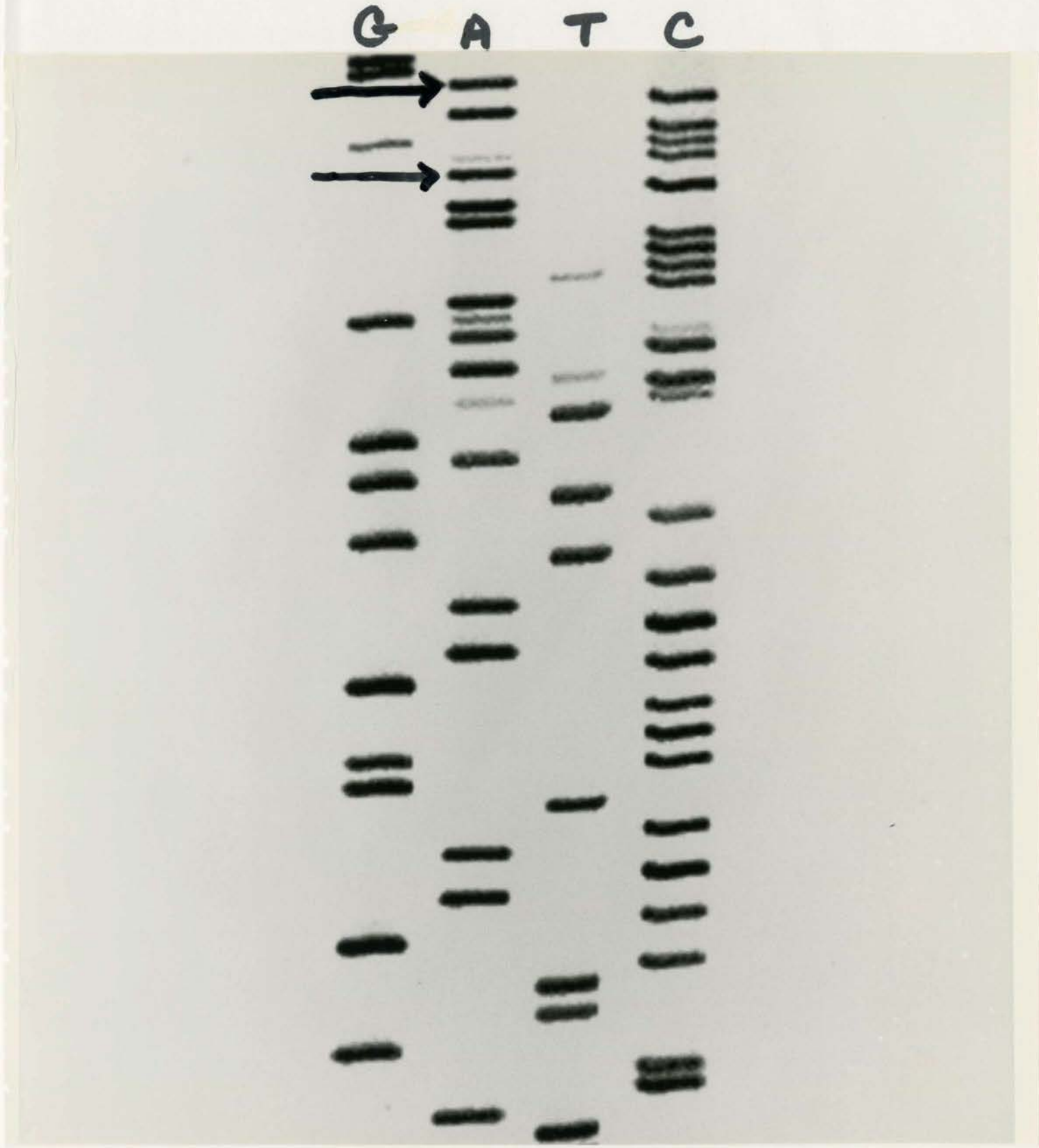


Figure 19: Sequence of clone 12, 22 and 45. The arrows indicate the two cysteine residues.

Of the 120 clones isolated, four hits were obtained, numbers 12, 22, 36, and 45. The four hits were sequenced and three were the same: 12, 22, and 45, while 36 was found to be a deletion mutant. The clone is shown in figure 19. The corresponding amino acid sequence of the clones in the randomized region of apamin:

```
      *     *  *  *  *
1  2  3  4  5  6  7
cys-gly-cys-trp-gly-leu-trp
```

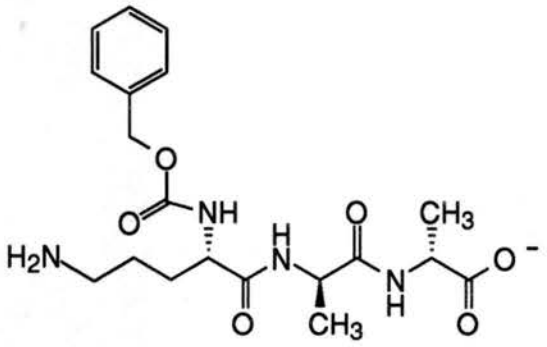
* = randomized site

Dr. Doubleday then ran competition experiments on the four clones. The first experiment(I) involved preincubating the derivatized plate with Vancomycin. It had been shown that Vancomycin binds to this ligand on Affigel, and so should block the binding sites for the phage, thus an ELISA assay should show reduced absorbance compared with no pretreatment with Vancomycin. In the second competition experiment(II), the phage are pretreated with the tripeptide ligand **16**. Presumably, **16** should bind to the phage on the pIII protein and prevent the phage from binding to the plate. Again, absorbance should be diminished compared to no pretreatment with tripeptide. Results show that both competition experiments do somewhat inhibit the binding of phage(Table 2).

Sample	Blank[a]	Control[b]	Phage Only[c]	I	II
12	0.520	0.580	2.360	0.315	1.450
22	0.480	0.470	2.200	0.960	1.590
45	0.470	0.460	0.820	0.627	0.673
36	0.540	0.700	0.310	0.110	0.210

Table 2: Results from competition experiments I and II. Values given are OD_{405} readings. Experiment I: Preincubation of plate with Vancomycin. Experiment II: Pretreatment of phage with tripeptide. a) The blank is an underivatized ELISA plate. b) The control is a derivatized plate treated with phage and eluted with **16**. c) In the phage only experiment, the phage are not eluted from the plate with **16**.

There was some concern about the carbobenzoxy protecting group on the ligand. Compared to the rest of the molecule, the CBZ group is fairly large. The question arose as to whether the CBZ group was contributing to the binding of the high affinity clone. In fact, while drawing the structure of the ligand, abbreviating this group as CBZ is deceptive because it visually implies the group is small. It is almost as long as the tripeptide itself.



16

In order to determine whether the CBZ group had anything to do with the binding, and to find out if in fact the phage were binding to the D-ala-D-ala portion of the ligand, the CBZ group was replaced with the smallest possible protecting group: an acetyl group. A small amount of this α-acetyl-L-lys-D-ala-D-ala was prepared by Dr. Weixu Zhai. Dr. Doubleday then derivatized the Covalink plate with this ligand and performed the same previously described competition experiments I and II.

Sample	Blank[a]	Control[b]	Phage Only[c]	I	II
12	0.510	0.930	1.035	1.100	1.130
22	0.480	1.070	1.440	1.490	1.650
45	0.470	0.730	0.850	0.980	0.697
36	0.540	0.182	0.242	0.190	0.198

Table 3: Results from competition experiments I and II on α-Ac-L-lys-D-ala-D-ala derivatized plates. Values given are OD_{405} readings. Experiment I: Preincubation of plate with Vancomycin. Experiment II: Pretreatment of phage with tripeptide. a) The blank is an underivatized ELISA plate. b) The control is a derivatized plate treated with phage and eluted with α-Ac-L-lys-D-ala-D-ala . c) In the phage only experiment, the phage are not eluted from the plate.

The data from Table 3 seems to indicate that the CBZ group is necessary for these clones to bind to the derivatized solid support, and that perhaps the clones have no affinity for the D-ala-D-ala portion of the ligand.

III. PREPARATION OF THE SECOND LIBRARY

The second library-called Fd/tet-Apamin II, was prepared by Dr. Matthew Peterson. It was designed to again utilize Apamin as the library motif, retaining the cysteines in positions 1, 3, 11 and 15, with a tyrosine fixed at position 2, and positions 4 through 9 randomized. The tyrosine was placed at position two to mimic the aromatic residues found in Vancomycin. The strategy employed for the insert design was adapted from Devlin et al.[22] The two oligomers shown in figure 16 were prepared on the DNA synthesizer. The minus strand contains the randomized region, whereas the plus strand has inosines at each randomized location. Inosine is known for its "promiscuous" base pairing; it can pair with all four bases, so this single plus strand can base pair with all possible permutations of the randomized minus strand. In the bacterial cell, it is the minus strand that is used as a template to synthesize large quantities of plus strand to be incorporated into phage. Another feature of this insert is that the apamin fusion peptide is located at the absolute terminus of the pIII gene, adjacent to the signal peptidase site. In the first library, the apamin insert was several codons downstream from the signal peptidase placing the apamin several amino acids from the end of the pIII gene.

The two oligomers were annealed and ligated into the parent Fd/tet vector that had been linearized by treatment with Bst X1. The DNA was then electroporated into MC1061 *E. coli* cells and amplified to produce the phage library.

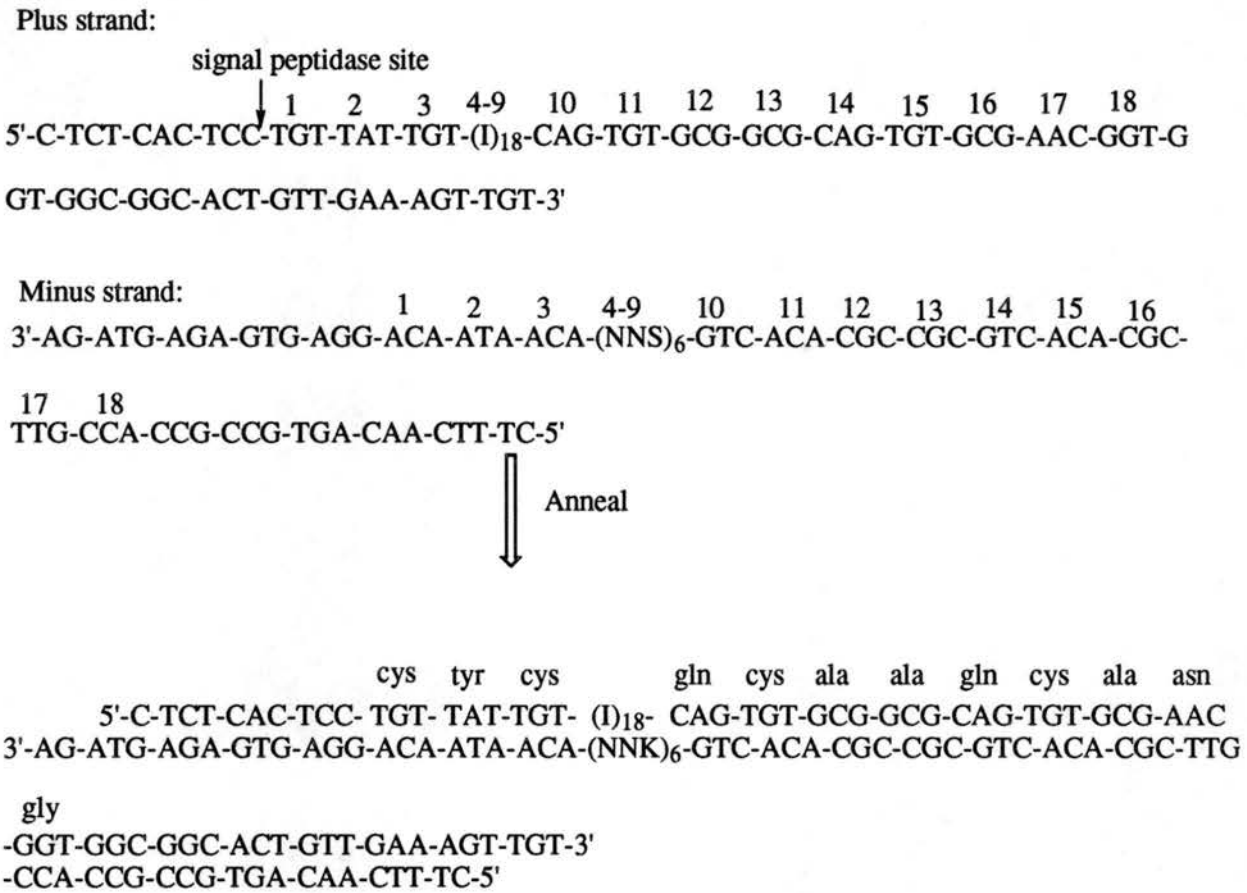


Figure 20: Insert design for Fd/tet- apamin II library. N= equimolar amounts of A, G, C, T. K= equimolar amounts of C and A.

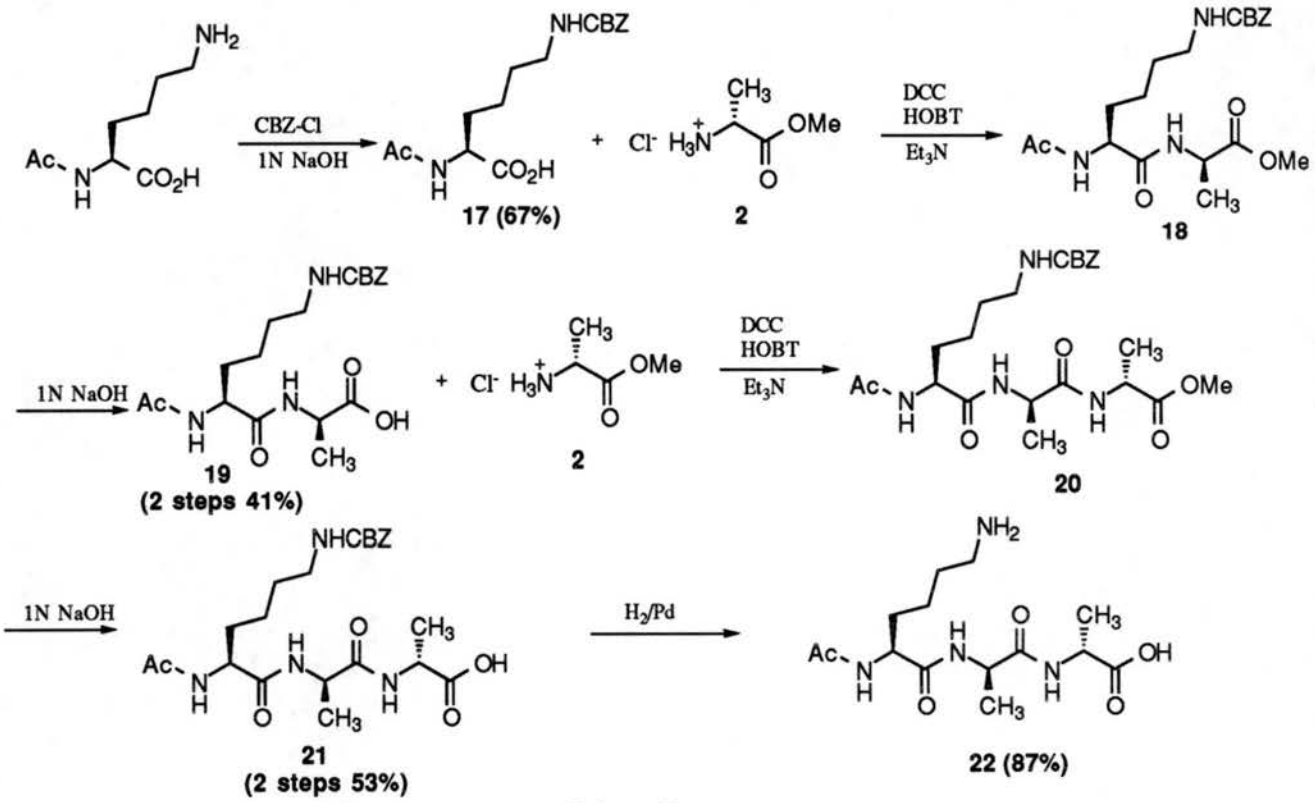
IV. DERIVATIZATION OF SOLID SUPPORTS

A. Synthesis of α-acetyl-L-lys-D-ala-D-ala

Because of the apparent affinity of the clones from the previous library for the CBZ group, an acetyl group would be preferable in the alpha position of lysine. The original material from Weixu Zhai had been depleted, so it was necessary to synthesize a large amount for derivatization of solid supports.

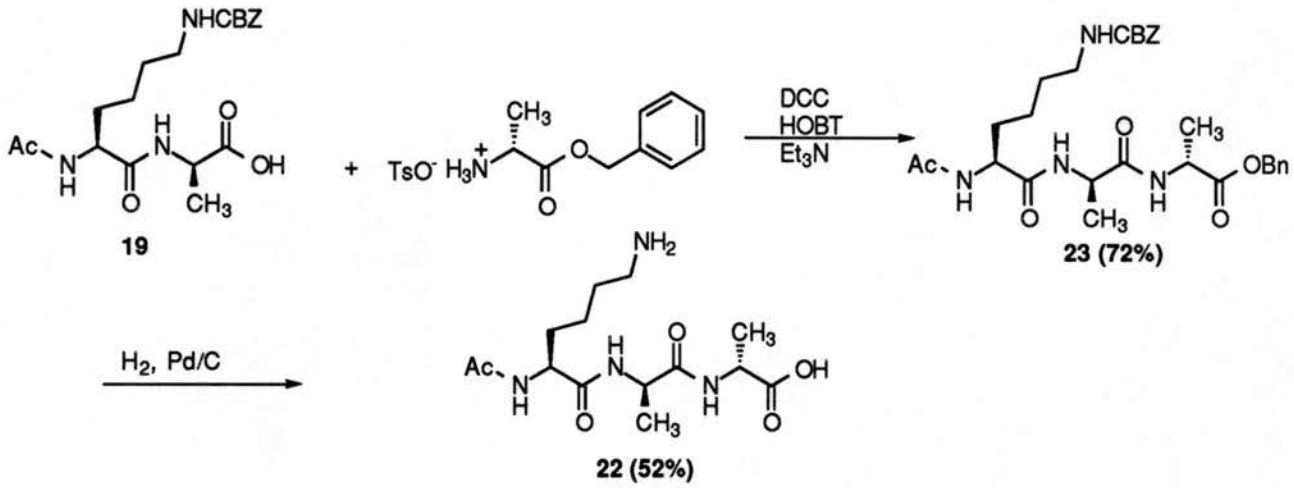
Three different approaches were employed to synthesize the monoacetylated tripeptide. The first method involved building from the amino end (Scheme V). The ε-

amine of commercially available α-acetyl-L-lysine was protected by carbobenzoylation and coupled with **1** to give dipeptide **18**. This ester was saponified and coupled with another equivalent of **1** to give the tripeptide methyl ester **20** which was saponified to give acid **21**. The CBZ group was removed by hydrogenation to give the desired product **22** in 6 steps with a 13% overall yield.



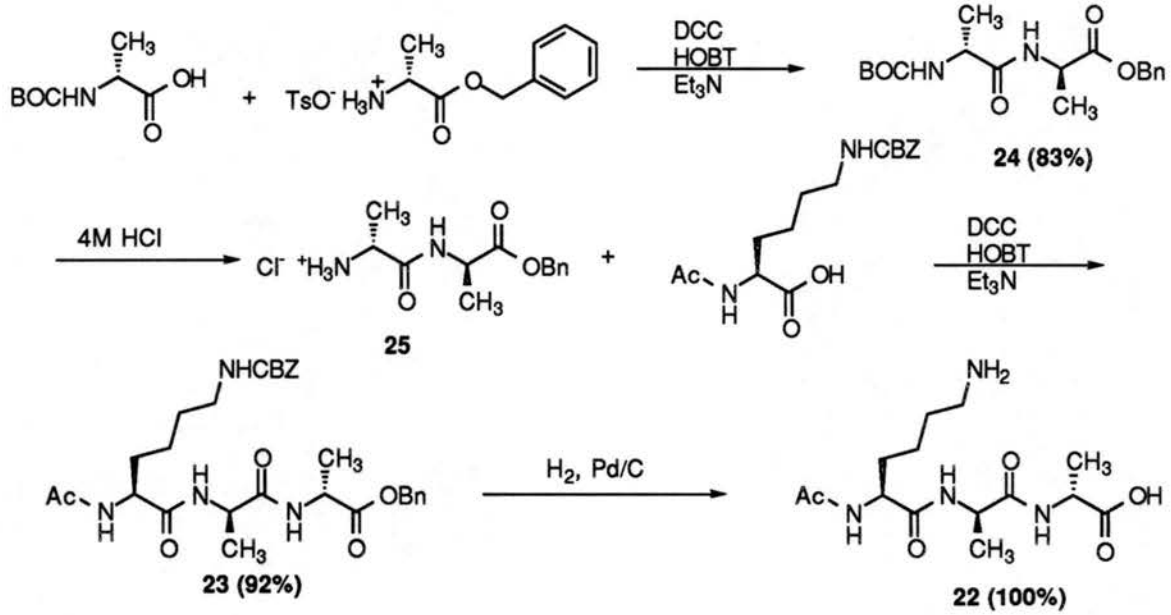
Scheme V

The second method employs coupling the dipeptide acid **19** (from Scheme V above) with the commercially available tosic acid salt of D-alanine benzyl ester. The tripeptide **23** is then deprotected in one step to give the desired tripeptide **22** (Scheme VI). This 5 step sequence had an overall yield of 10%.



Scheme VI

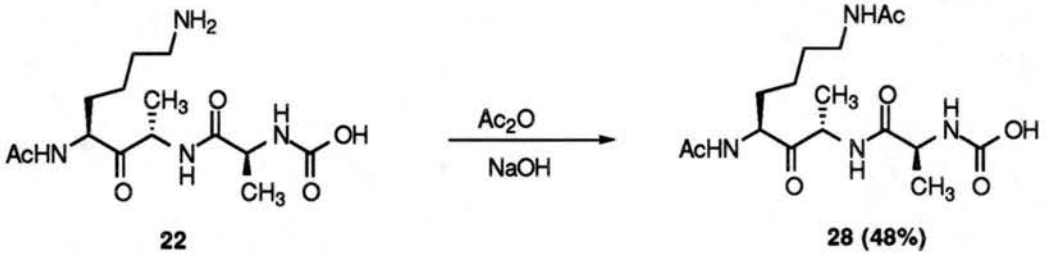
In the third method, tBOC-D-alanine and D-alanine benzyl ester (both available from Sigma) were coupled to give the dipeptide **24**. The tBOC group was removed by treatment with HCl, and the dipeptide was coupled to **17** to give the tripeptide **23**. Deprotection afforded **22** in 4 steps with a 76% yield (Scheme VII).



Scheme VII

B. Synthesis of Ac_2-L-lys-D-ala-D-ala

The diacetyl tripeptide is used for eluting phage off of the derivatized solid supports. It was originally prepared as illustrated in Scheme VIII below. ε-Acetyl-L-lysine(from Sigma) was acetylated in the alpha position by treating with acetic anhydride and sodium hydroxide to give diacetylated lysine **26**. This was coupled to the dipeptide salt to give tripeptide **27** which was deprotected to give the desired product **28**. The diacetylated tripeptide was also prepared by acetylating the monacetylated tripeptide **22** prepared in Scheme VII(Scheme IX).



22 **28 (48%)**

Scheme IX

C. Derivatization of Affigel with α-Acetyl-L-lysine-D-ala-D-ala

Affigel-10 was derivatized with the monoacetylated tripeptide **22** in the same manner that the CBZ tripeptide **16** was coupled(section-IIC). Again, the extent of derivatization was determined using a vancomycin binding study monitored by HPLC. First, increasing concentrations of vancomycin were injected on an HPLC to establish a gradient(Figure 17). Vancomycin was then added to 1 mL of Affigel until a saturation point was reached. Using the HPLC concentration gradient, it was calculated that 12 umol of vancomycin had reversibly bound to the gel. The vancomycin was eluted off the gel using

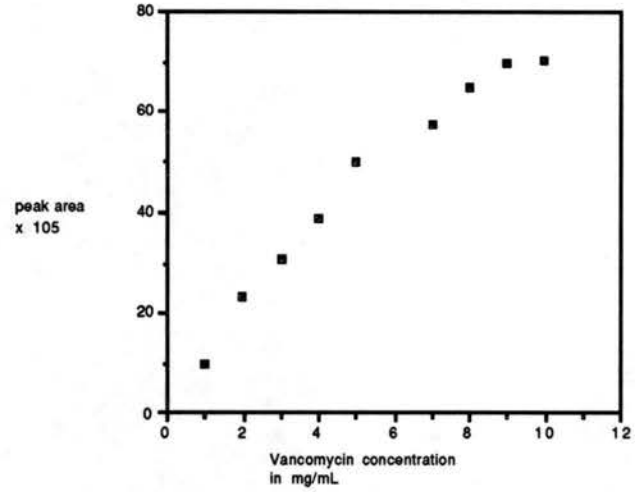
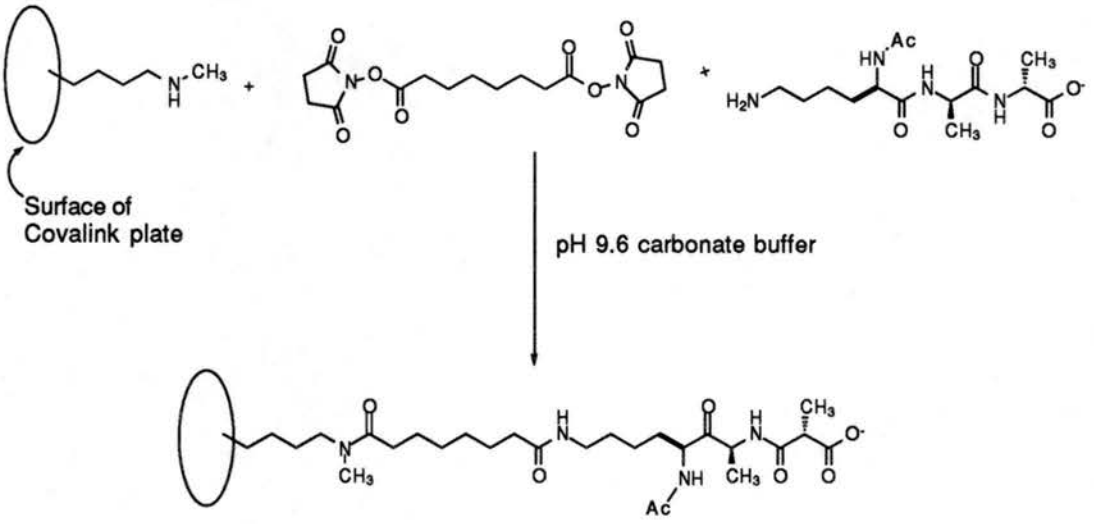


Figure 17: Vancomycin concentration gradient.

D. Determination of Extent of Derivatization of Covalink Plates

Covalink plates contain a N-methylamino group on the polystyrene surface of the plate. The tripeptide is coupled to the plate using the homobifunctional linker disuccinimidyl suberate (DSS).

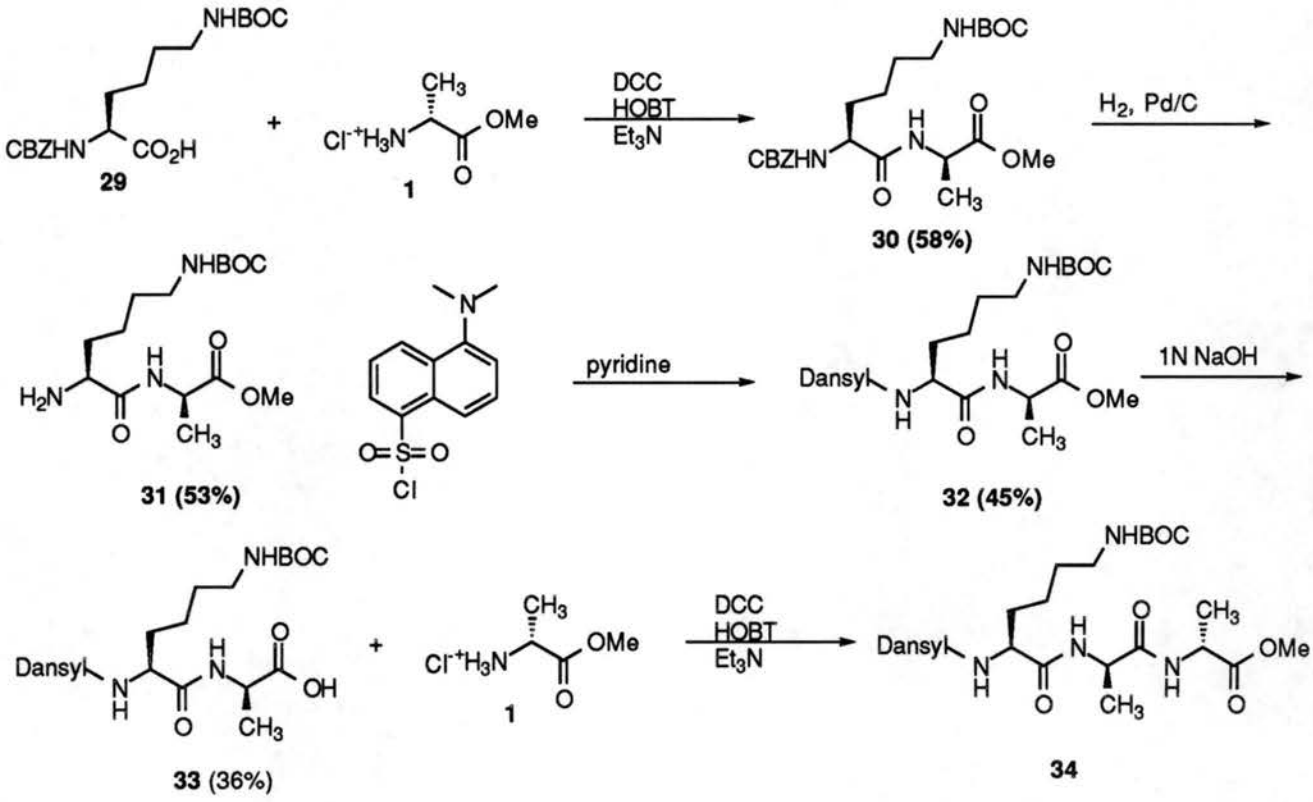


Scheme X: Derivatization of Covalink late with a-Ac-L-lys-D-ala-D-ala

In order to determine the extent of derivatization, several attempts of establishing an HPLC gradient for vancomycin were made. The inherent problem was that the effective concentrations required to perform a vancomycin binding study are in the 1-10 ug/mL range. Our HPLC detector is not sensitive enough to give a linear integration at this concentration range, so several alternative methods were explored.

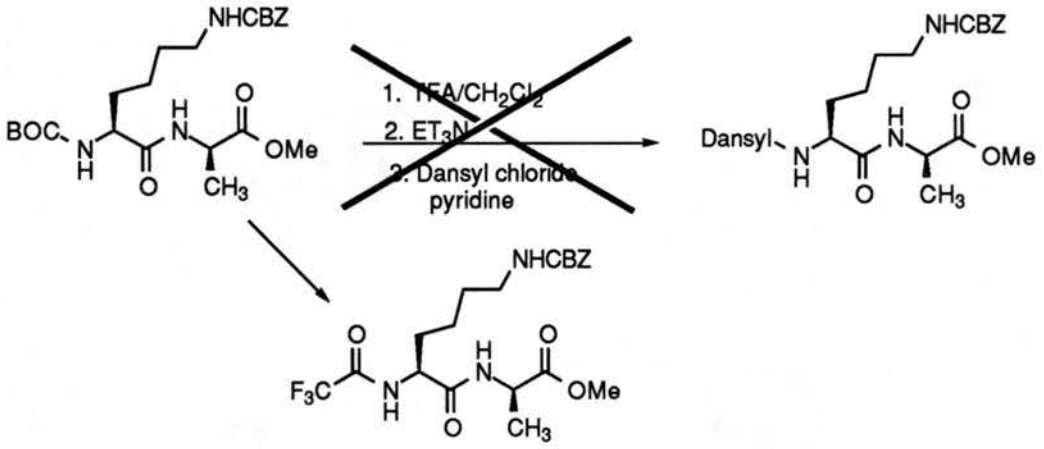
With the advent of fluorescence technology, addition of a fluorescing ligand was considered as a possible way of measuring the extent of plate derivatization. The dansyl group has a known λ_{max} at 245nm, and fluorescence detectors should be sensitive enough to detect ng quantities of this functional group. Thus a dansylated tripeptide was designed (Scheme XI).

The diprotected lysine **29** was coupled to **1** to give the dipeptide **30**. The CBZ group was removed from **30** and the dansyl group was added to give **32**. After adding the dansyl group to **32**, the yields were consistently low due to the sensitive nature of the dansyl group. A new scheme was designed adding the dansyl group at the penultimate step. This next strategy, Scheme XIII, was more fruitful.



Scheme XI

An interesting side reaction occurred while trying to prepare the dansylated tripeptide. α-tBOC-ε-CBZ-L-lys-D-ala-D-ala methyl ester was treated with TFA followed by triethylamine to remove the BOC group, then dansyl chloride and pyridine was added in situ. Instead of dansylating the alpha amine, the trifluoracetamide **35** formed, apparently via a mixed anhydride formed from dansyl chloride and excess TFA.

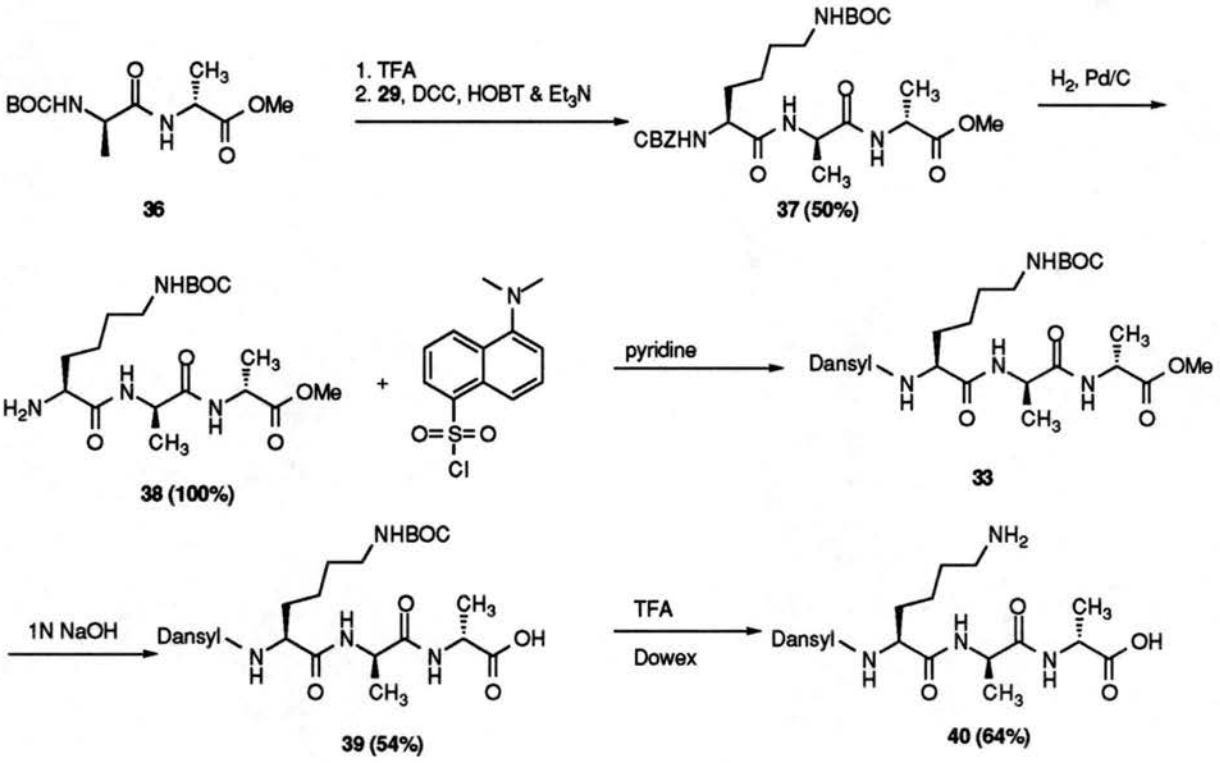


35

Scheme XII

Success was achieved by taking dipeptide **36**, removing the BOC group with TFA, and coupling to diprotected lysine **29** to give tripeptide **37**. The CBZ group was removed and the dansyl group was put into place on the alpha amine. The ester was saponified, and the BOC group removed. The product was purified by ion exchange chromatography to give the dansylated tripeptide **40** in a 17% overall yield.

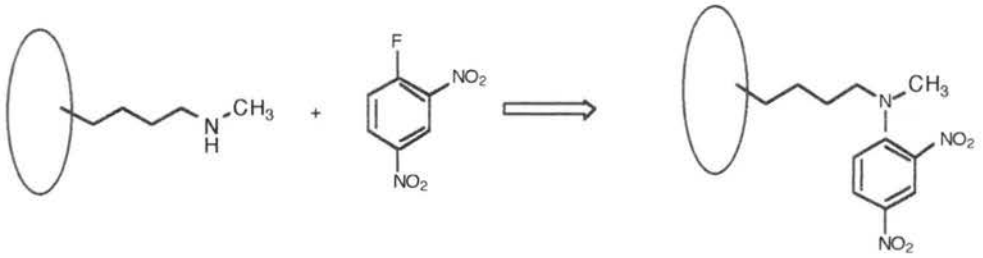
Unfortunately, after linking this fluorescing ligand to the Covalink plates, the fluorimeter was unable to distinguish between derivatized, partially derivatized and blank wells.



Scheme XIII

Since the Covalink are 96 well plates that can be used with a microplate reader, an ELISA assay could be used to amplify the presence of the ligand. This assay would simply require that a group be placed on the tripeptide which is recognizable by an antibody. The 2,4-dinitrophenyl (2,4-DNP) group is a commonly used antibody tag.

A preliminary study was performed in which the 2,4-DNP group was linked directly to the plate(Scheme XIV). According to the manufacturer, there are .14nmol of sites per well of the Covalink plate. A 1:1 molar ratio of flourodinitrobenzene (FDNB) corresponds to 26ng in 100uL or 260ng/mL. Dilutions were made of FDNB ranging from 100 to 500ng/mL and one eight well strip of each of these concentrations was derivatized overnight (see experimental). The plate was then subjected to the ELISA assay conditions, and after stopping the enzymatic reaction, the absorbance of each well was measured at 405nm.



Scheme XIV: Direct Linking of Fluoro-2,4-dinitrobenzene to Covalink Plates.

The average absorbance at each concentration was calculated, and a trend was observed. A linear increase in absorbance occurred with increasing concentration, and as expected, it leveled off above 300ng/mL where complete derivatization should be achieved. Because of the relatively high absorbance observed in the underivatized wells, a reduction in background was desired. Several agents are routinely used to reduce the specific binding of antibodies. The experiment was performed using plates which were treated with Tween-20 and bovine serum albumin (BSA) as blocking agents, compared to no blocking agent.

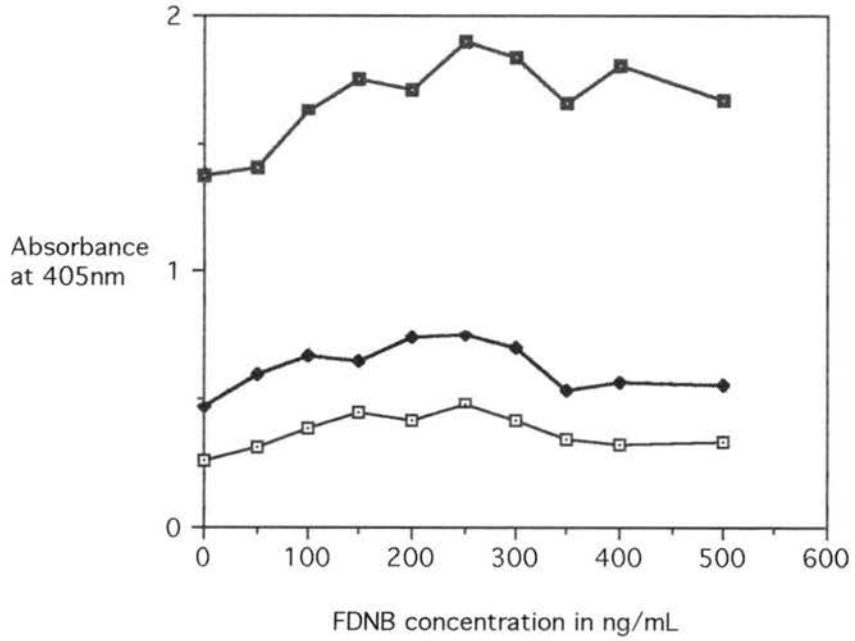


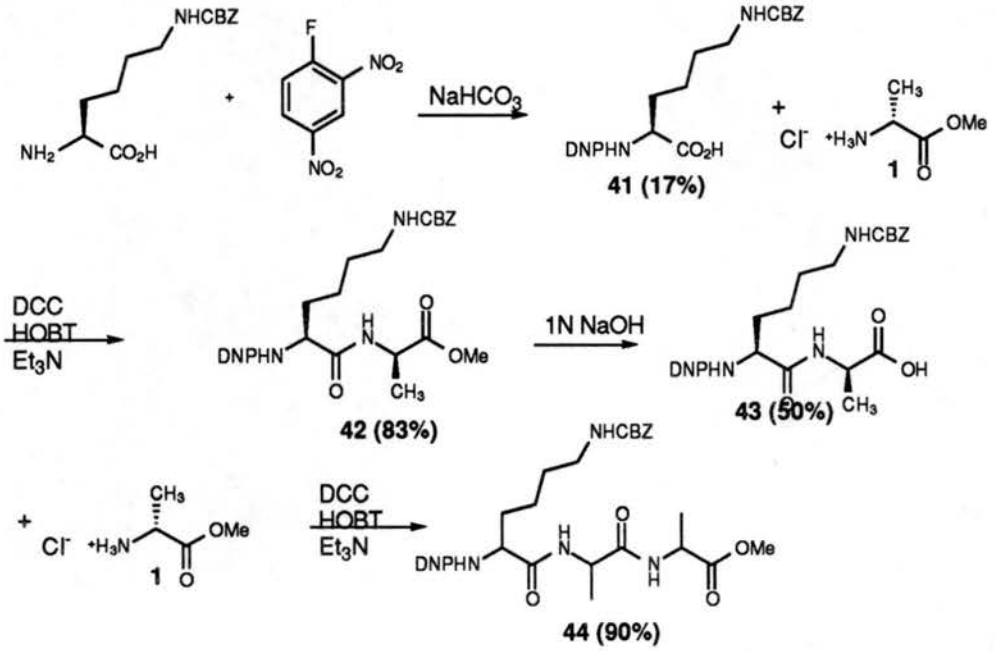
Figure 17: ELISA of Covalink plates derivatized with fluorodinitrobenzene.
Black= BSA blocked, Blue= Tween blocked, Red= No blocking agent

As can be seen, BSA provides the greatest reduction in background absorbance due to nonspecific binding of antibody.

Since it had been determined in the above experiment that the ELISA plate reader can distinguish between partially derivatized and fully derivatized wells, the next experiment was to derivatize the wells with the 2,4-DNP labeled tripeptide. The goal was to mimic the exact conditions used to derivatize plates used in panning, and use the ELISA assay to determine that the plates are truly derivatized. Thus the tripeptide had to be synthesized with a 2,4DNP group replacing the acetyl group.

E. Synthesis of 2,4-DNP-L-lys-D-ala-D-ala

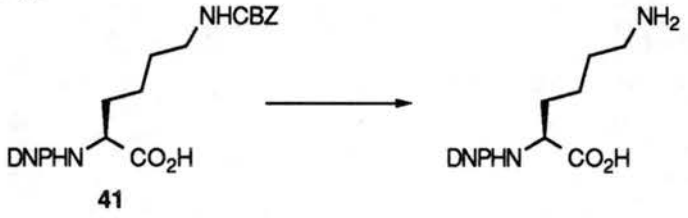
Fluorodinitrobenzene was coupled to ε-CBZ-L-lysine in carbonate buffer to give the DNP labeled lysine **41**. This was coupled to D-alanine methyl ester hydrochloride to give the dipeptide **42.** The dipeptide was then saponified to give acid **43**. The acid was coupled to another D-alanine methyl ester hydrochloride to give the fully protected tripeptide **44** in a 6% overall yield.



Scheme XV

The synthesis of the fully protected tripeptide **44** was completed when some difficulties were encountered. When removing the carbobenzoxy protecting group from the ε-amine of compound **44** using the traditional method of hydrogenation over catalyst, the nitro groups on the dinitrophenyl group are reduced as well.

In order to examine how this problem could be circumvented, a large amount of **41** was prepared to use as a model compound for examining different reaction conditions in which the CBZ group could be removed without destroying the integrity of the DNP group (see scheme XV).



41

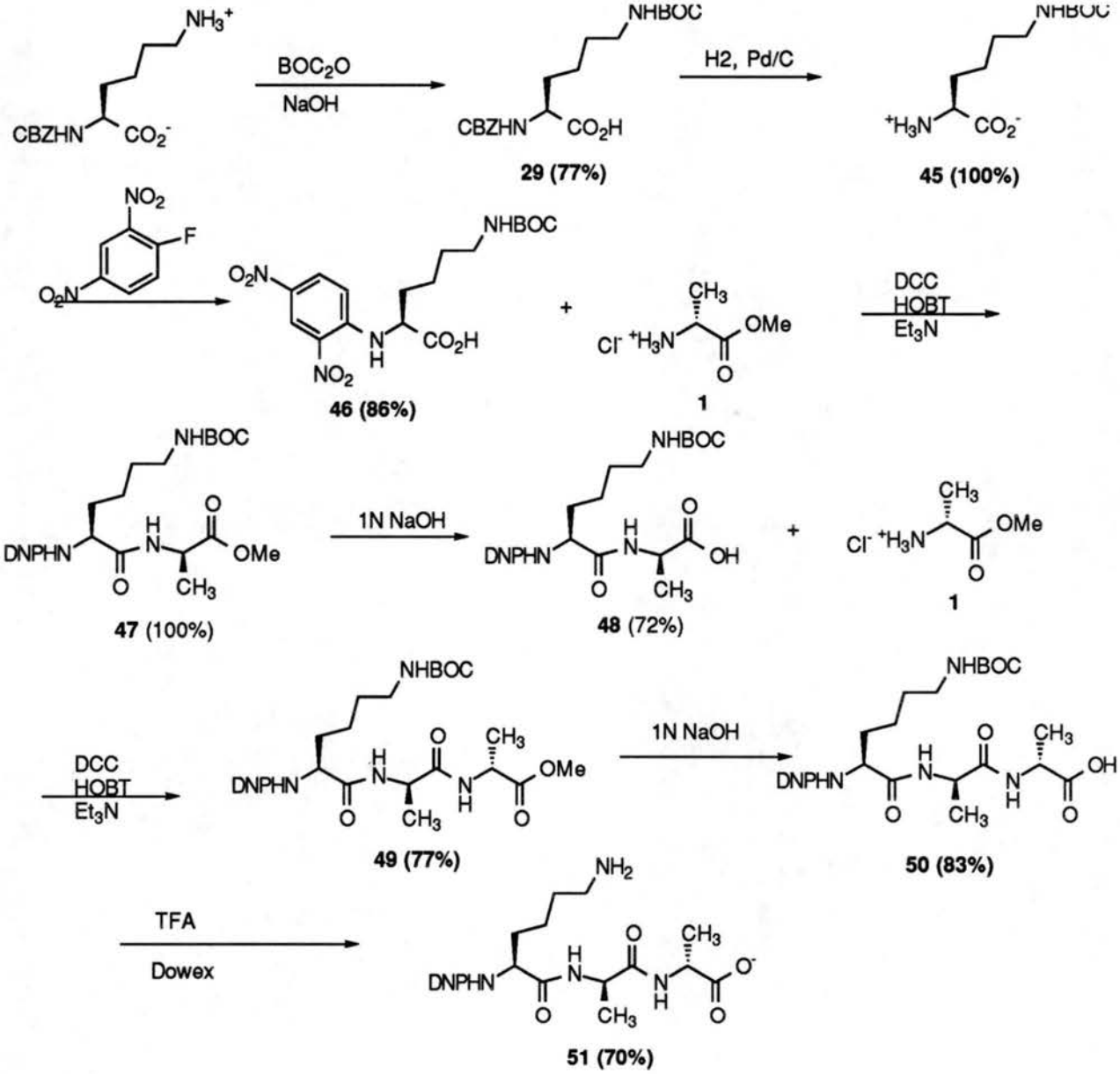
Scheme XVI

Reaction Conditions for Scheme XVI:

Conditions	Results
1) H_2 , 5%Pd/C	Decomposition
2) Et_3SiH, $PdCl_2$	No reaction
3) BBr_3 in CH_2Cl_2	8% yield
4) 40% KOH in MeOH	Decomposition

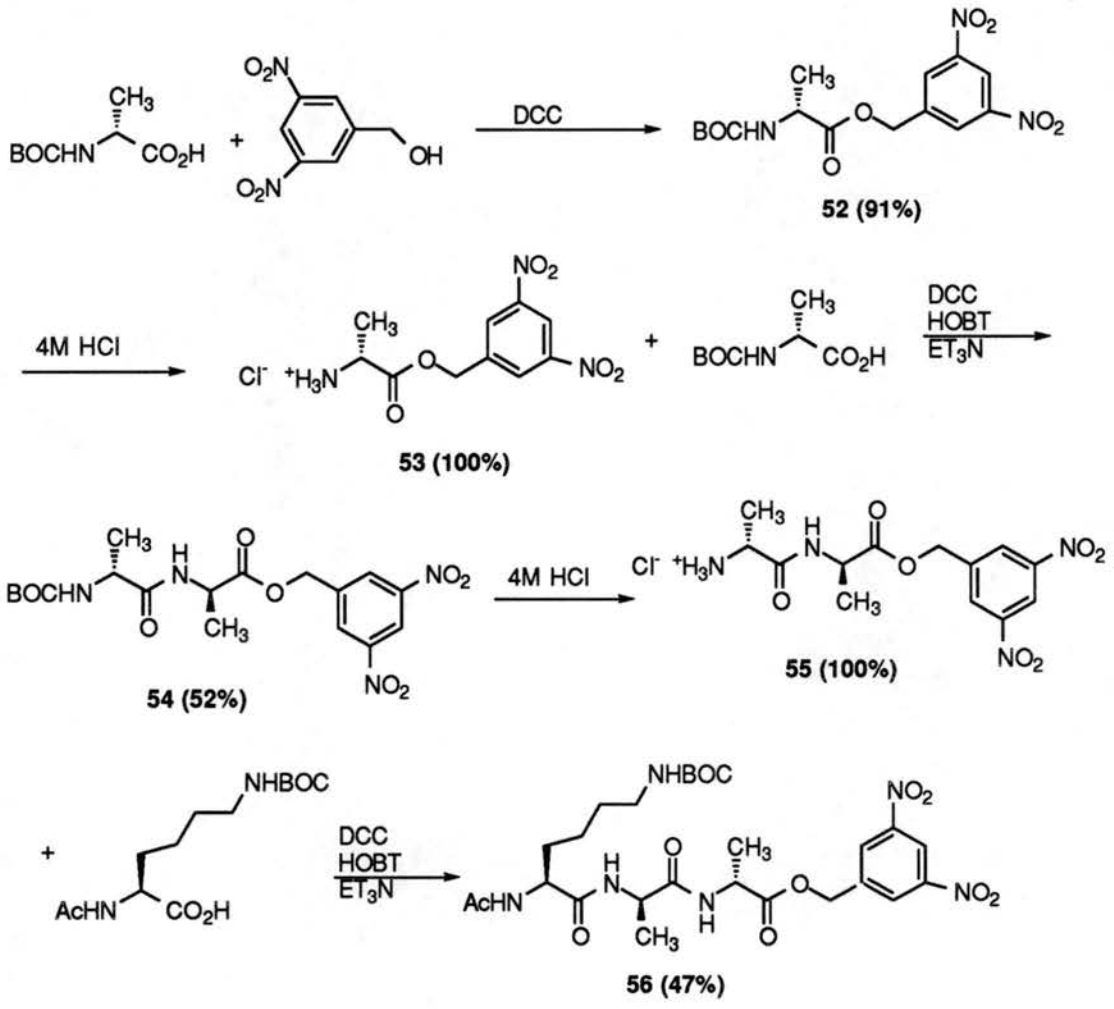
Because of the above limitation, scheme XV was abandoned and scheme XVII was designed employing the t-butyloxycarbonyl group to protect the ε-amine. Thus the tBOC group was placed on commercially available CBZ-L-lysine using tBOC anhydride, the CBZ group was removed and the 2,4-DNP group was added to the α-amine to give **46**. This was coupled with D-alanine methyl ester, saponified, and coupled to another D-

alanine methyl ester. **48** was then deprotected by saponification of the ester, and subsequent removal of the BOC group with TFA gave the final product **51**. This compound was used in the ELISA assays of the Covalink Nunc plates. This eight step procedure gave the final product in a 21% overall yield.



Scheme XVII

The question arose as to whether the 2,4-DNP group would be recognized by the antibody at its internal location on the lysine residue. A different tripeptide was designed with the 2,4-DNP group on the terminus of the tripeptide as the dinitrobenzyl ester (Scheme XVIII). Thus tBOC-D-alanine was coupled to 3,5-dinitrobenzyl alcohol to give the benzyl ester **52**. The BOC group was removed and the amine chloride **53** was coupled to another tBOC-D-alanine to give dipeptide **54**. This was deprotected and coupled to the diprotected lysine to give tripeptide **56**.



Scheme XVIII

F. ELISA Assay of Covalink Plates Derivatized with 2,4-DNP Labeled Tripeptide

In the case of the 2,4-DNP labeled tripeptide(**40**), a 1:1 molar ratio of ligand to sites corresponds to 67ng in 100μL, or 670ng/mL. Thus dilutions were made in the range of 100-1000ng/mL. Each strip of the plates were derivatized with each increasing concentration, then blocked with either Tween-20, BSA, or no block. The ELISA Assay was performed, and figure 18 shows that a maximum absorbance is reached at 700ng/mL and levels off at higher concentrations. Again, BSA provides the best reduction in background absorbance.

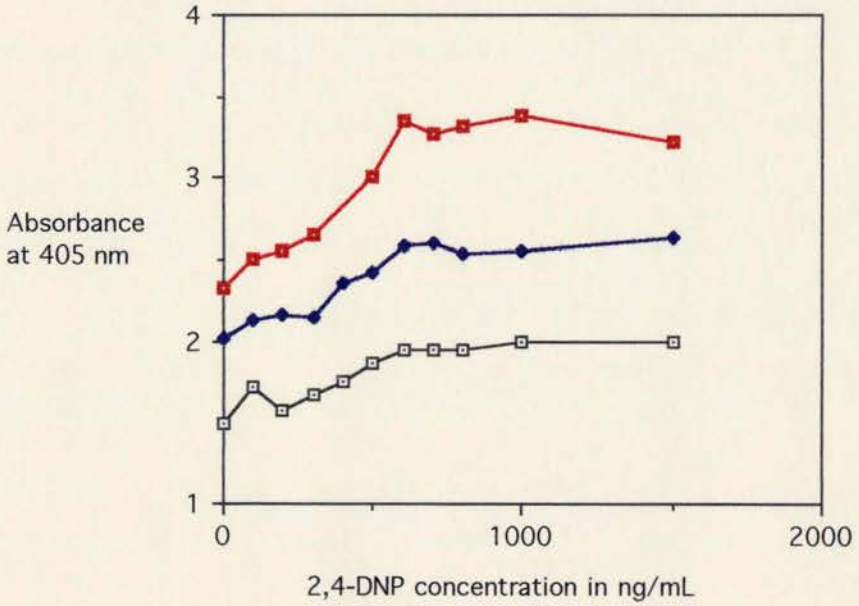


Figure 18: ELISA Assay of 2,4-DNP tripeptide derviatized plates.
Black= BSA blocked, Blue= Tween blocked, Red= No blocking agent

V. PANNING OF THE SECOND LIBRARY

A. Titering

Titering is the method used to determine phage concentration. Knowing how many phage are being panned, how many phage are being washed off, and how many phage are adhering to the solid support can indicate whether enrichment is occurring with each pan. There are two methods used for titering. The first method is by counting plaques. The phage solution is serially diluted(usually 100-fold) and combined with K91 cells in a warm soft agar solution which is spread evenly on an agarose plate and allowed to solidify.

As the bacteria grow within the agar, a grainy texture develops on the surface of the plate. When phage infect the bacteria, a tiny clearing called a plaque appears in the agar. The plaque is about the size of a pin head. Each plaque represents one plaque forming unit, or pfu. Because the plaques are small and very difficult to see, the preferred method of titering is by counting colonies. In the colony counting method, the phage dilutions are simply incubated with K91 cells for half an hour at 37°C to initiate infection. The solution is then spread on LB/tetracycline agarose plates and incubated overnight at 37°C. The colonies are counted the following day and each colony represents one separate phage infection.

To determine enrichment from a pan, the initial phage library is titered. The concentration is usually on the order of 10^{10} phage per mL. The phage are incubated with the derivatized solid support, and the supernatant is removed, the support is then washed, and bound phage are eluted by several different methods. The eluted phage are then titered and compared with original amount of phage (phage in):

$$\text{Phage in} / \text{eluted phage} \times 100 = \% \text{ enrichment}$$

With each successive pan, the highest affinity phage are amplified, therefore, with a successful series of pans, the % enrichment should increase with each pan.

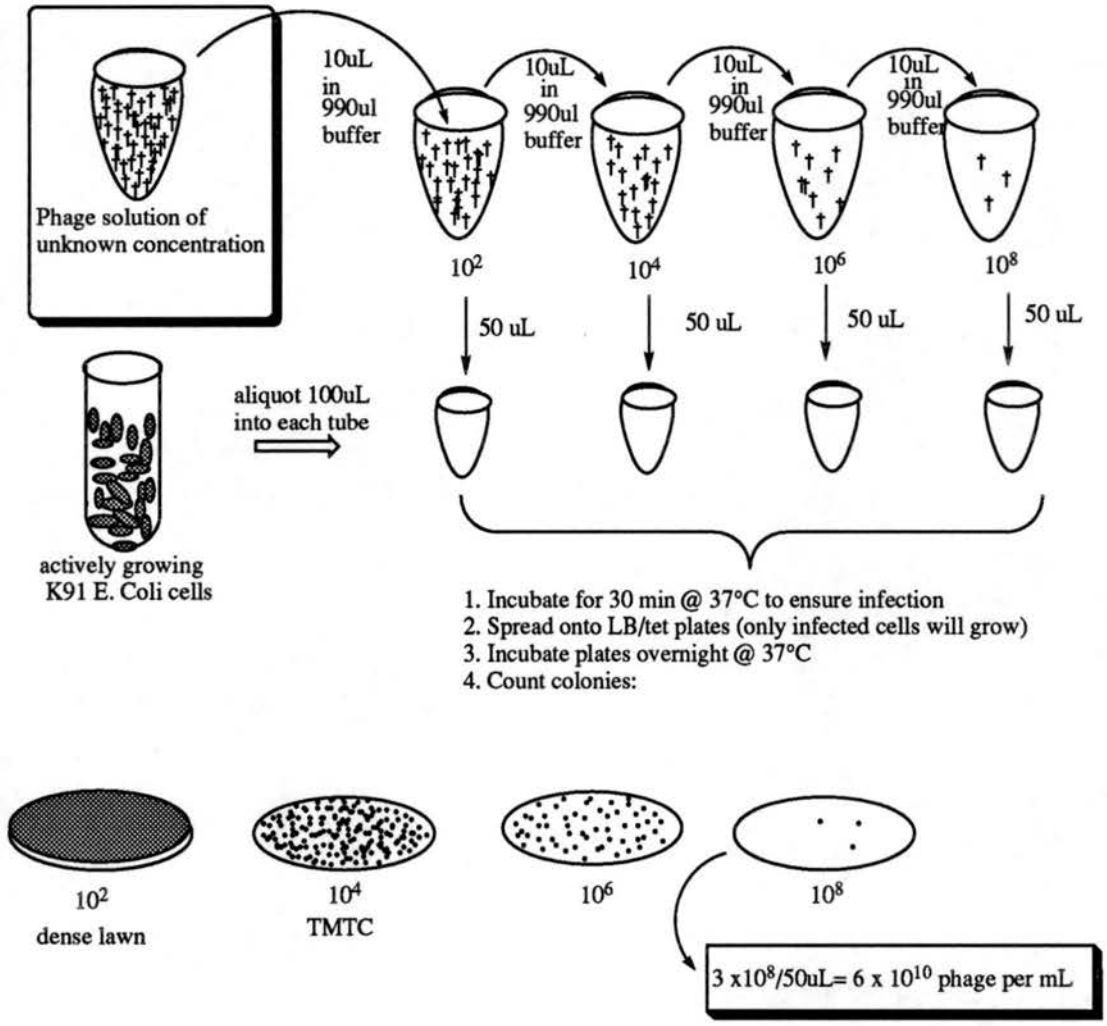


Figure 19: Phage titering by the colony counting method.

B. Panning Experiments

1. 1st Panning Series

The first panning series with the fd/tet-apamin II library was performed on Covalink plates derivatized with α–Ac-L-lys-D-ala-D-ala, and blocked with BSA. The phage were incubated for three hours on the plate and washed ten times with a PBS/BSA solution. The bound phage were removed by treating with 0.1N HCl buffered to pH 2.2 with glycine for 10 minutes. The eluted phage solution was neutralized with 2M Tris and the eluate was titered and amplified for the next round of panning (Table 4).

	Phage In	Phage eluted	% enrichment
Pan 1:			
A	2.4×10^{10}	2.2×10^{6}	.0091
B	1.6×10^{10}	7.4×10^{6}	.046
Pan 2:			
A	1.4×10^{10}	1.6×10^{6}	.011
B	5.6×10^{10}	4.9×10^{6}	.0086
Pan 3:			
A	2.6×10^{10}	1.2×10^{5}	.00046
B	2.2×10^{10}	1.6×10^{6}	.0072

Table 4: Results from panning apamin II library and eluting with .1N HCl buffer. A= library panned on plate derivatized with Ac-L-lys-D-ala-D-ala. B= Control: library panned on ethanolamine capped plate. All the values are phage /mL.

2. Manipulation of Panning Conditions

A. Number of Washes

0.1N HCl is commonly used as an eluant when panning antibody libraries. The premise is that binding is occurring via hydrogen bonding, and a strongly acidic (or basic) solution will break these bonds. Since no enrichment was seen in this series of pans, there was concern over the amount of washing. Could ten washes be too rigorous? Could the high affinity phage be washed out during these steps? Each of the supernatants from the ten washes in the third pan were saved and titered.

Phage In: 2.6×10^{10}	Wash 3: 2.04×10^{6} (.0078%)
Phage Out: 1.85×10^{10} (71%)	Wash 4: 9.8×10^{4} (.00037%)
Wash 1: 2.1×10^{9} (8%)	Wash 5: 1.5×10^{6} (.00057%)
Wash 2: 1.7×10^{8} (.6%)	Wash 8 : 1.64×10^{4} (.00006%)

Table 5: Titer of washes from pan. Each value is in phage/mL

Table 5 clearly shows that after the third wash, only a very small percentage of phage are removed, and the last five washes are probably unnecessary, and could possibly be removing the high binding phage.

B. Nature of Eluant

An experiment was designed to compare a variety of eluants, to see which eluted the highest number of phage bound to the plate. Unpanned library phage were incubated on a derivatized Covalink plate for 3 hours. The supernatants were removed, and the plate was washed five times with PBS/BSA buffer. Each strip of the plate was eluted with a different counterligand and the phage were titered as shown below in table 6.

Eluant	Titer	% recovery
20ug/mL tripeptide(24hrs)	6.06×10^7	1%
20ug/mL tripeptide(2 hrs)	2.12×10^7	.35%
.1N HCl/glycine pH 2.2	5.22×10^5	.0085%
.1N NaOH/glycine pH 10	6.6×10^6	.1%
3.5M MgCl2	1.38×10^6	.022%
6M Urea	1.1×10^7	.18%
1% SDS	1.57×10^6	.025%
PBS	8.8×10^6	.14%

Table 6: % recovery of phage from different eluants.

Table 6 establishes the use of the tripeptide counterligand as the best eluant. Of note are the two different elution times and % enrichment.

C. Length of Elution Times

An experiment was designed to determine the optimum elution time, and whether a series of elutions or a single lengthy elution would provide a higher phage output. Unpanned fd/tet-apamin II library was added to 5 strips of a Covalink plate and each strip was subjected to a different elution regimen with 20ug/mL tripeptide as shown below in table 7. The eluants were collected and titered.

Elution regimen	total phage collected	% enrichment
1 x 10 hour	1.6×10^8	0.41%
2hr + 8hr	9.3×10^7	0.24%
2 x 2hr + 6hr	1.4×10^8	0.35%
3 x 2hr + 4hr	6.9×10^8	1.77%
5 x 2hr	6.7×10^8	1.71%

Table 7: Comparison of different elution times with 20ug/mL tripeptide solution.

D. Blocking agents

In all of the previous experiments, BSA was used as a blocking agent. BSA is a large globular protein, and its role as blocking agent is to cover up the nonderivatized polystyrene surface to prevent any non-specific binding of phage. There was concern over the relative size of the BSA. It could possibly dwarf the ligand and prevent access of the phage to the derivatized surface as well as the polystyrene. Five blocking agents of much smaller molecular size, or more linear structure were chosen and examined in the following experiment. Ficoll is a nonionic synthetic polymer of sucrose. Ficoll 70 is a 70,000 mw polymer, and Ficoll 400 is a 400,000 mw polymer. Polyvinylpyrrolidone has an average molecular weight of 10,000. Heparin is a small polar protein. Antifoam A is a silicone polymer used as a foam suppressor. SDS is sodium dodecyl sulfate, a small anionic detergent. A plate was derivatized with tripeptide, and one strip was treated with each of the five new blocking agents, and the control was treated with PBS buffer only. The plate was treated with library phage for two hours, washed and eluted with 20μg/mL tripeptide (see table 8 below).

Blocking Agent	Titer of eluted phage	% enrichment
1% Ficoll 400	3.8×10^4	.007%
1% Ficoll 70	5.4×10^4	.0096%
1% polyvinylpyrrolidone	5.8×10^4	.01%
50ug/mL heparin	6.8×10^4	.012%
.01% Antifoam A	3.6×10^4	.006%
1% SDS	2.16×10^5	.038%
PBS control	1.54×10^5	.027%

Table 8: Comparison of blocking agents. Original phage concentration for all wells is 5.6×10^8 phage/mL

3. Second Panning Series

The second series of pans were conducted with tripeptide derivatized plates blocked with 1% sodium dodecyl sulfate (SDS). The phage were incubated for two hours, the supernatant removed, and the plates were washed five times with PBS. The bound phage were eluted with 20ug/mL of free tripeptide for two hours. After three rounds of panning, no increase in enrichment is seen (Table 9).

	Phage In	Phage eluted	% enrichment
Pan 1:	7.9×10^9	1.28×10^6	.016%
Pan 2:	1.2×10^{10}	3×10^6	.025%
Pan 3:	4.6×10^9	1.6×10^5	.0035%

Table 9: Panning on tripeptide derivatized Covalink plates blocked with SDS. Eluant is 20ug/mL free tripeptide. Phage in and phage out values are in phage/mL

4.Using Vancomycin to Elute Bound Phage

Because vancomycin has a high affinity for the tripeptide L-lys-D-ala-D-ala, it is possible that adding an excess of vancomycin to a well that has bound phage will release or "bump off" the phage from the ligand. The vancomycin will effectively usurp the binding sites, leaving the phage free in the supernatant to be collected and amplified. Based on this premise, three rounds of panning were conducted (Table 10).

	Phage In	Phage eluted	% enrichment
Pan 1:			
strip 1	3×10^9	6.14×10^5	.02%
strip 2	3×10^9	1.2×10^6	.04%
strip 3	3×10^9	1.3×10^6	.04%
strip 4	3×10^9	1.08×10^6	.036%
Pan 2:			
strip 2	6.9×10^9	1.28×10^7	.18%
strip 3	9.3 x 109	2.8 x 106	.03%
strip 4	7×10^9	1.06×10^7	.15%
Pan 3:			
strip 2	4.6×10^{10}	1.2×10^9	2.6%
strip 3	6×10^{10}	1.16×10^9	1.9%
strip 4	4×10^{10}	8×10^8	2.0%

Table 10: Comparison of vancomycin vs. free tripeptide as eluants. Strip 1: blank wells eluted with tripeptide. Strip 2: blank wells eluted with vancomycin. Strip 3: derivatized wells eluted with tripeptide. Strip 4: derivatized wells eluted with vancomycin. All phage titers are in phage/mL.

The phage were incubated on the plate, the supernatant was removed and the plates were washed three times with PBS. Phage were eluted with either free tripeptide or vancomycin and the eluants titered, then amplified for the next round of panning. In a separate tube, phage was incubated with vancomycin and titered before and after incubation with Vancomycin to determine if the vancomycin would kill any phage. The titer changed by less than an order of magnitude, so vancomycin has no deleterious effect on phage survival. After three rounds of panning, there seemed to be a slight increase in enrichment, so the single stranded DNA was isolated, and sequenced.

The gel showed that no selection had been achieved and the library was still a very random mixture of all possible sequences.

5. Cleavable Disulfide Linker-Panning and ELISA Assays

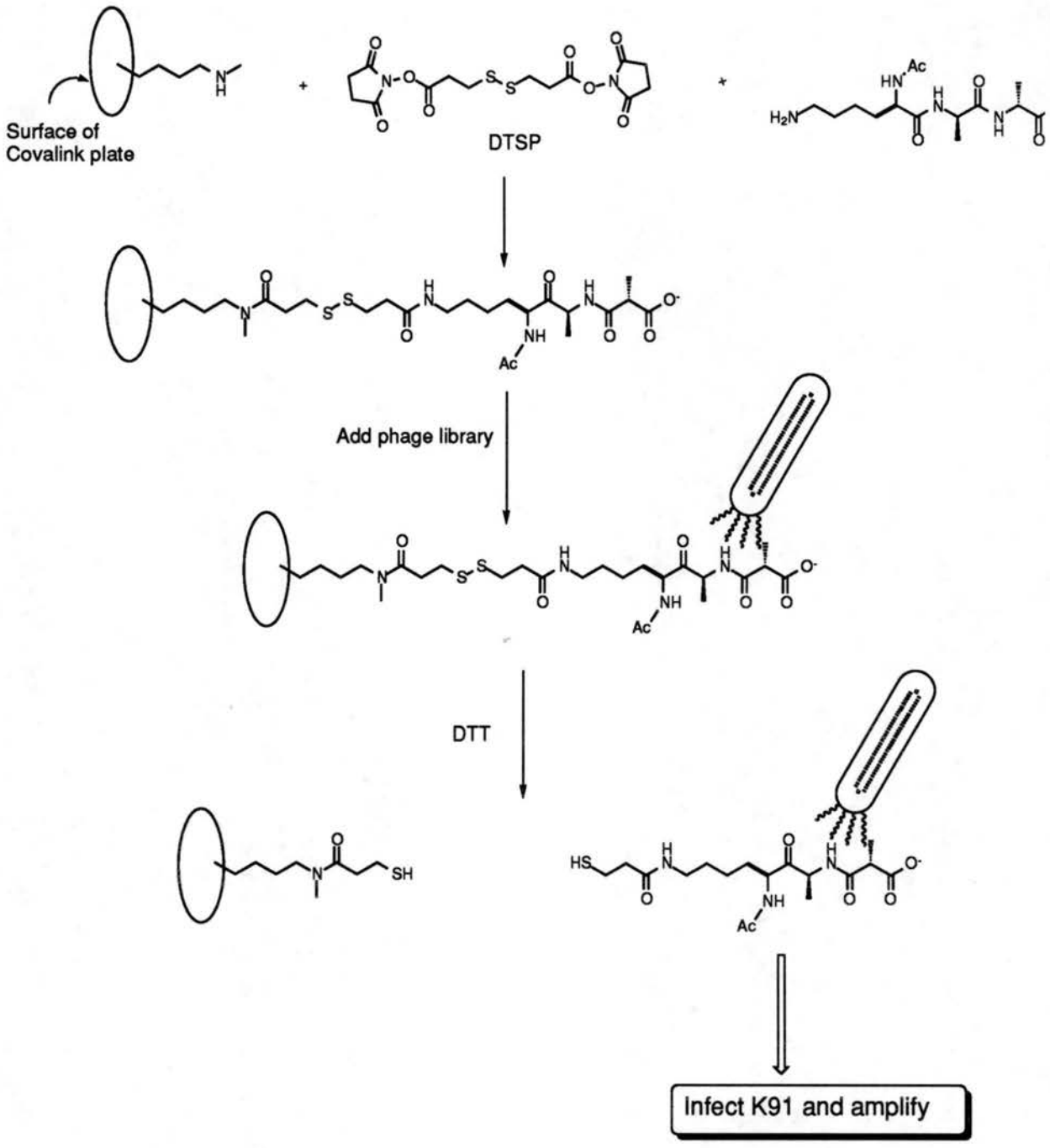
Another method to remove bound phage from solid support would be to cleave the linker from the plate surface while the phage is still bound. Disulfide linkers are commercially available and can be cleaved under reductive conditions. The disulfide linker Dithiobis(succinimidylproprionate),available from Pierce, was used to couple the acetyl tripeptide **22** to Covalink plates (Scheme XVII).

6.Derivatization of Cleavable Disulfide Linker

A considerable amount of time was spent trying to determine the extent of derivatization of the plates using an ELISA assay. Six strips of a Covalink plate were derivatized as follows:

strip 1: Control-SDS only
strip 2: Disulfide linker + 2,4-DNP tripeptide + SDS
strip 3: Regular linker + 2,4-DNP tripeptide + SDS
strip 4: Control-SDS only
strip 5: Disulfide linker + 2,4-DNP tripeptide + SDS
strip 6: Regular linker + 2,4-DNP tripeptide + SDS

After derivatization, the wells from strips 4,5 and 6 were treated with a reducing agent capable of cleaving a disulfide bond. The wells were then washed with PBS and all 6 strips were then subjected to an ELISA and the absorbance readings were measured for each well. The expected results are that strips 1,4 and 5 should all have similar background OD405 values, while the wells from strips 2,3 and 6 should have the absorbance of a fully derivatized well. This experiment was conducted four different times under a variety of reducing conditions(see experimental) with no conclusive results.



Scheme XVII

Although it was never definitively proven that the disulfide linker does quantitatively link tripeptide to the plate and can be cleaved by treatment with a reducing agent, a panning experiment was conducted. Plates were derivatized by linking the acetyl

tripeptide(**22**) with the disulfide linker DTSSP. Library phage were added and subsequently the supernatant was removed, and the plates washed with PBS. The plates were then treated with dithiothreitol (DTT) for two hours, the most commonly used disulfide reducing agent. The reducing solution was removed, titered, and amplified for the next round of panning. In a separate vial phage was incubated with the reducing agent for two hours and titered before and after to ensure that the DTT has no effect on the phage. The results of the three pans are shown below in Table 11. Again no enrichment is seen from this series of pans.

	Phage In	Phage Eluted	% enrichment
Pan 1			
1: Disulfide linked tripeptide	1.46×10^{10}	3.4×10^{6}	.023%
2: Control-SDS only	1.46×10^{10}	6.8×10^{6}	.046%
Pan 2:			
1: Disulfide linked tripeptide	1.8×10^{10}	7.4×10^{6}	.041%
2: Control-SDS only	1.2×10^{10}	6×10^{5}	.005%
Pan 3:			
1: Disulfide linked tripeptide	5.42×10^{9}	9.14×10^{5}	.017%
2: Control-SDS only	1.68×10^{10}	1.08×10^{6}	.006%

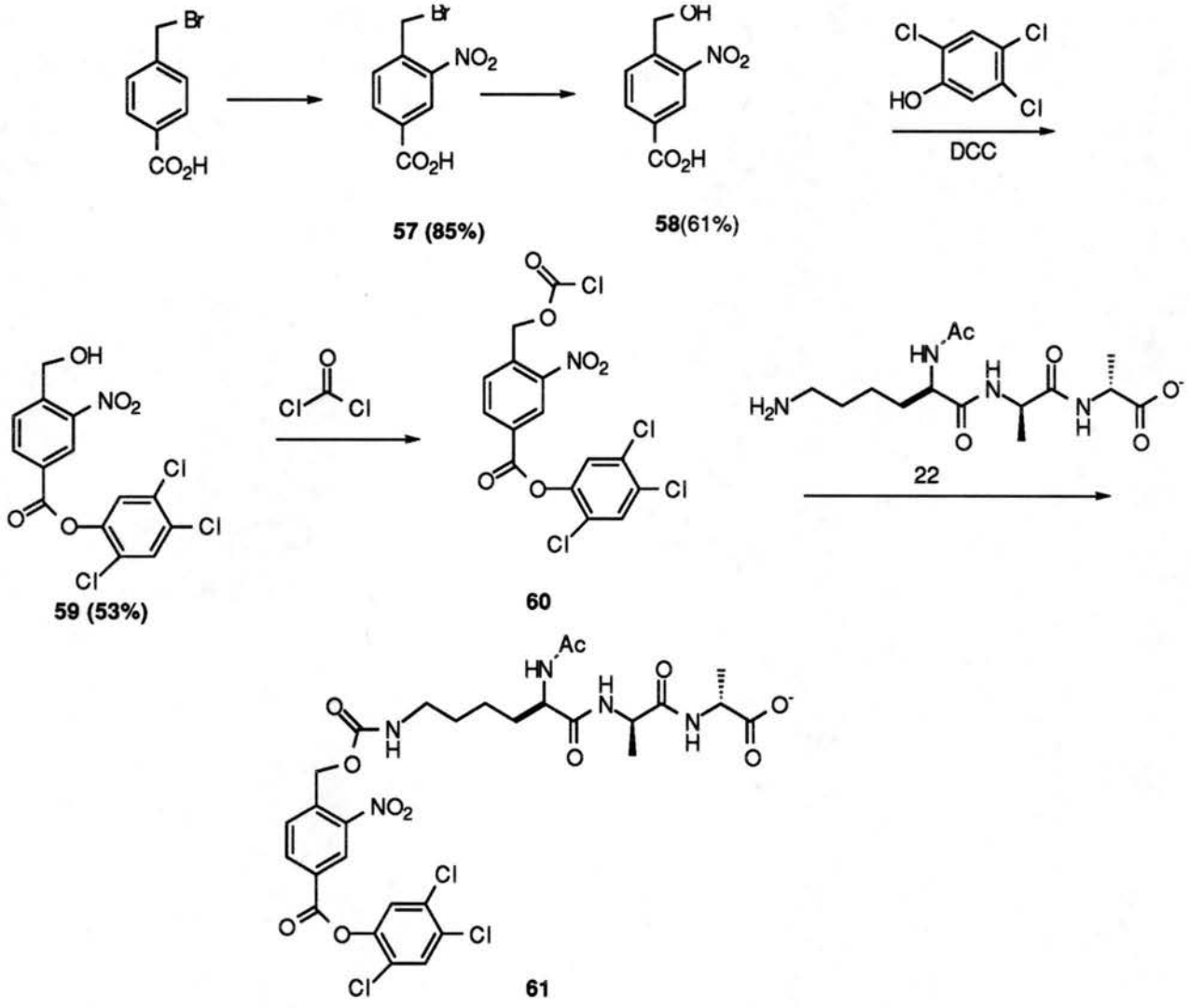
Table 11: Panning on plates derivatized with disulfide cleavable linker.

VI. PHOTOCLEAVABLE LINKER

Another type of linker that could be used to attach the tripeptide to the plate is a photocleavable linker. The phage that bind to the ligand can be released from the surface of the plate by irradiation of the linker. ortho-Nitrobenzyloxy groups are known to be particularly labile to UV light. Based on this premise a tripeptide with a photocleavable linker at the terminus was designed and synthesized (Scheme XIX).

4-Bromomethylbenzoic acid, available from Aldrich, was nitrated with 90% HNO_3 to give **57** in an 85% yield.[23] **57** was converted to the alcohol **58** under Finkelstein conditions using KI and water.[24] **58** was coupled to 2,4,5 trichlorophenol with DCC to give ester **59** in a 53% yield. The alcohol **59** was converted to the chloroformate **60** by

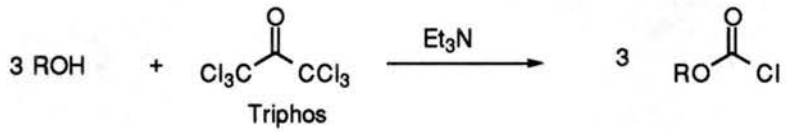
treatment with triethylamine and phosgene, and immediately converted to the carbamate **61** by the addition of tripeptide **22. 61** was purified by prep TLC to give the final product.



Scheme XVIII

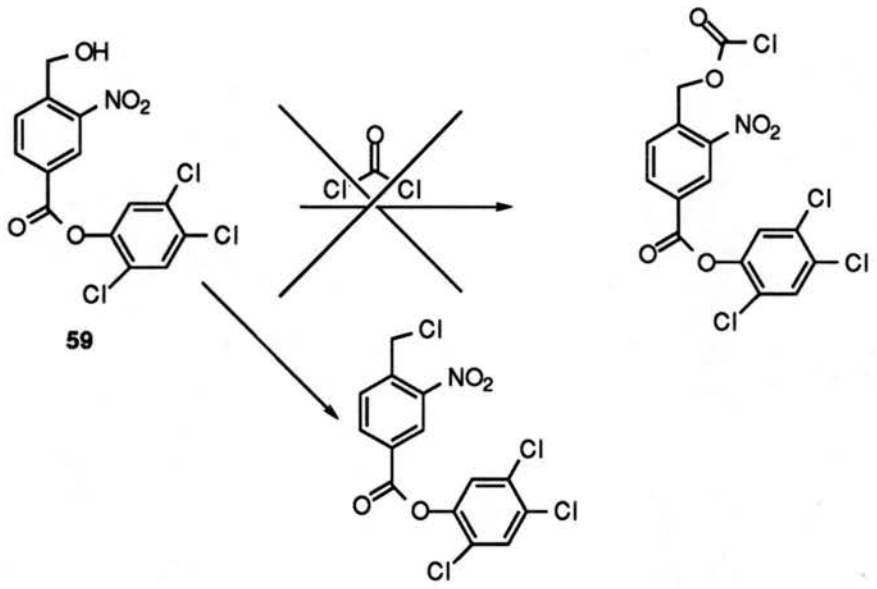
Originally the conversion of **59** to **60** was attempted using the reagent triphosgene(shown below). This is a crystalline solid which is much easier to handle than the highly toxic phosgene.[25] One Triphos molecule contains 3 equivalents of phosgene, so only 1/3 equivalent is necessary for complete conversion (Scheme XIX). However, under

several different attempts, the reaction did not go to completion, and an excess of Triphos was needed. The next reaction is performed directly without workup because of the reactive nature of the chloroformate. When the tripeptide was added , the free amine reacted with the excess Triphos present instead of the chloroformate, forming an isocyanate. Because it is not desirable to have to add excess of the precious tripeptide, this route was abandoned for the traditional phosgene.

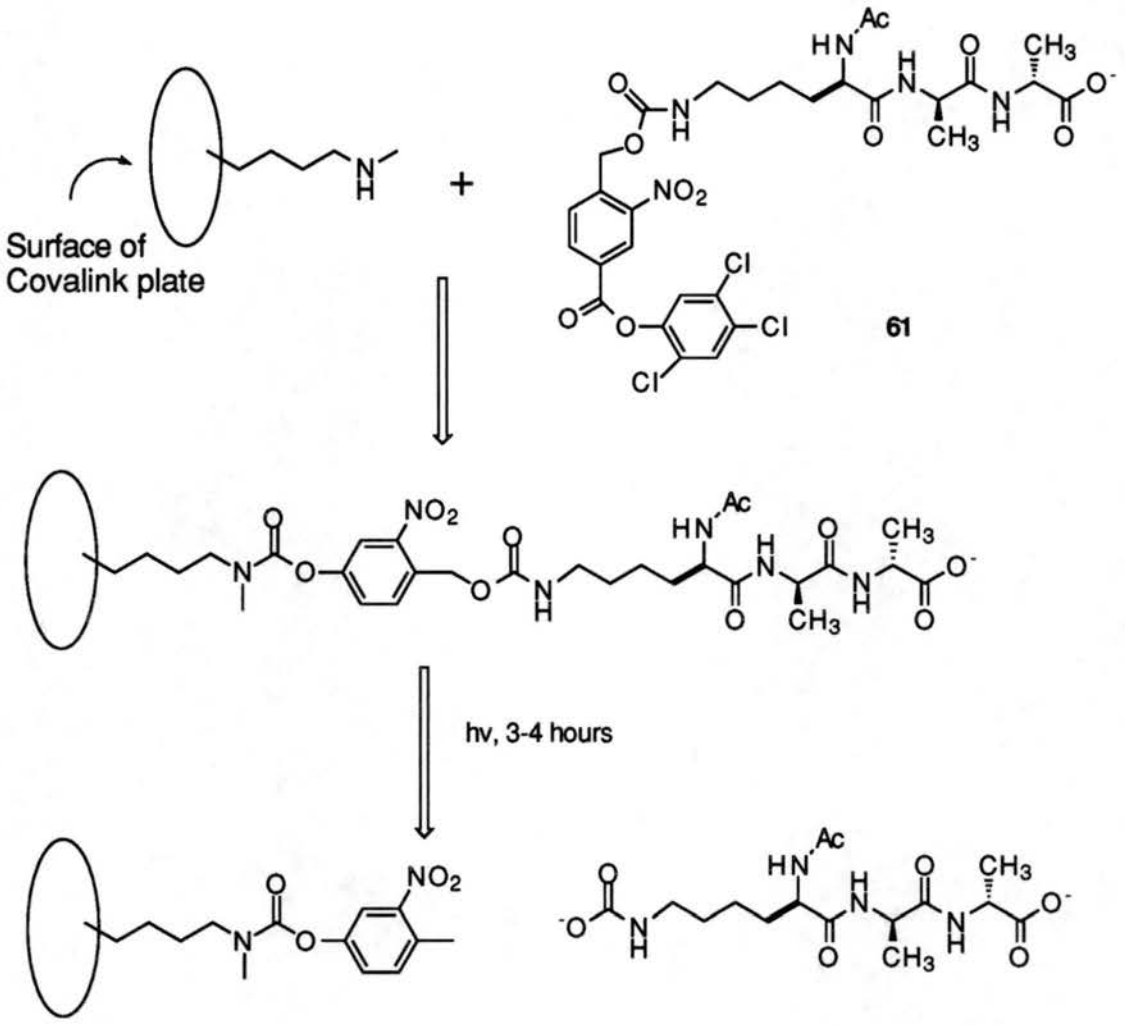


Scheme XIX

In most of the references for synthesizing chloroformates from alcohols using phosgene, a base is not required, and the resultant HCl gas is removed in vacuo. However on substrate **59**, if a base is not added first, chloride **62** is formed, presumably from nucleophilic attack on the benzyl methylene, expelling carbon dioxide and chloride ion. The structure of this compound was confirmed to be the tetra-chlorinated compound **62** by NMR, and mass spectroscopy (see experimental).



Scheme XX



Scheme XXI

With **61** in hand, the plan was to link it to the Covalink plate, add the phage library, and remove the bound phage from the well surface by cleaving the photolabile linker group with light. The question arose as to whether the phage could survive the irradiation conditions. It is well known the UV irradiation causes DNA damage. Since the phage would have to withstand several hours under strong UV light, several experiments were designed to determine if any phage would be damaged under these conditions. The transilluminator is a large broad range UV light source used to visualize ethidium bromide stained gels. When the phage were exposed to the light from the transilluminator, none survived even after 5 minutes of exposure. An Oriel lamp which contains a more

concentrated source of UV light kills phage within 20 minutes. However, when the Oriel lamp is fitted with filter, some phage were able to survive even after three hours of exposure. The filter allows a range of 350 to 500 nm light through with a maximum transmittance at 400nm. (Table 13)

	Phage in	phage out	% survival
control	2.4×10^{10}	1×10^{10}	50%
1 hour	2.4×10^{10}	1.26×10^{9}	12.6%
2 hours	2.4×10^{10}	7×10^{8}	7 %
3 hours	2.4×10^{10}	2.4×10^{8}	2.4 %

Table 13: Phage survival after irradiation periods with filtered Oriel lamp.

Although panning has not yet been conducted with this linker, the next step would be to calculate the amount of time it takes to completely cleave the photolabile linker from the solid support using the filtered UV light.

VII. DYNABEADS

A new solid support became available that avoids some of the handling problems associated with Affigel. Dynabeads are activated magnetic beads that can be covalently derivatized. A magnetic particle concentrator (MPC) is used to separate the beads from the supernatant. The MPC effectively adheres the beads to the side wall of the reaction tube, ensuring none will be drawn into the pipettor when the supernatant is removed.

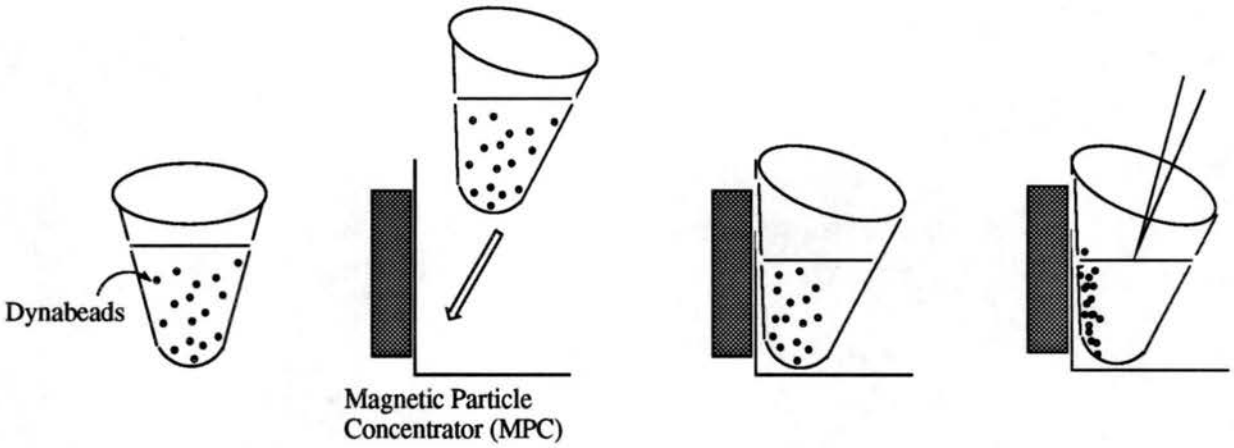


Figure 21: The use of Dynabeads with the Magnetic Particle Concentrator (MPC).

The beads were derivatized with the acetyl tripeptide and panning was conducted with Dr. Monica Baloga. The phage were eluted from the Dynabeads using a 0.5mg/mL tripeptide solution. Three rounds of panning were conducted and the eluted phage were titered to see if enrichment was occurring.

	Phage In	Phage Eluted	% enrichment
Pan 1:			
1-tripeptide	3.4×10^9	2×10^4	.0006%
2-control	3.4×10^9	4×10^4	.0012%
Pan 2:			
1-tripeptide	9.9×10^9	9.6×10^6	.097%
2-control	4.4×10^{10}	2.4×10^7	.054%
Pan 3:			
1-tripeptide	2.4×10^{11}	1×10^7	.0042%
2-control	3.6×10^{11}	2.1×10^7	.0058%

Table 14: Titer results from panning on Dynabeads. Phage in and phage out values are in phage/mL.

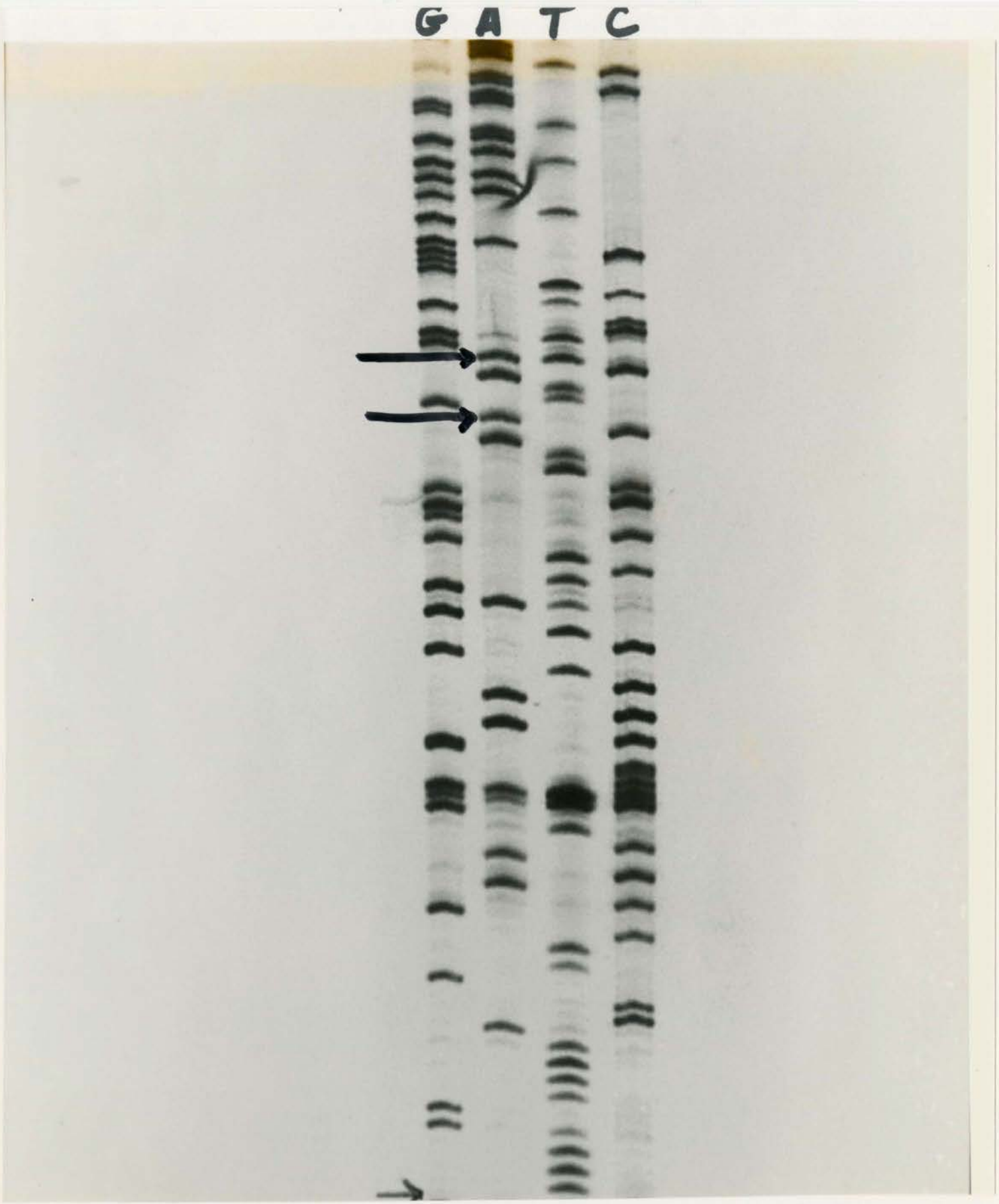
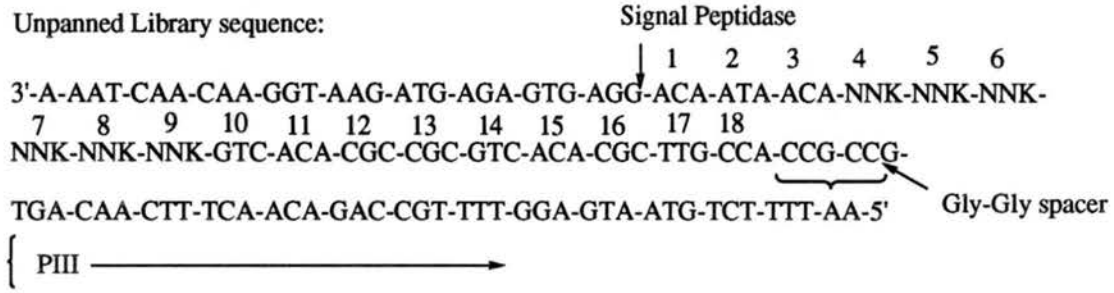


Figure 22: Sanger sequencing gel of mutant.

Although no enrichment occurred, the phage from the third round of panning was sequenced. The sequence was the same for both the control and tripeptide, no randomization was evident, it was a single mutant that appears to have two inserts in the pIII gene. Dr. Baloga repeated the panning several times, and continually obtained the same mutant.



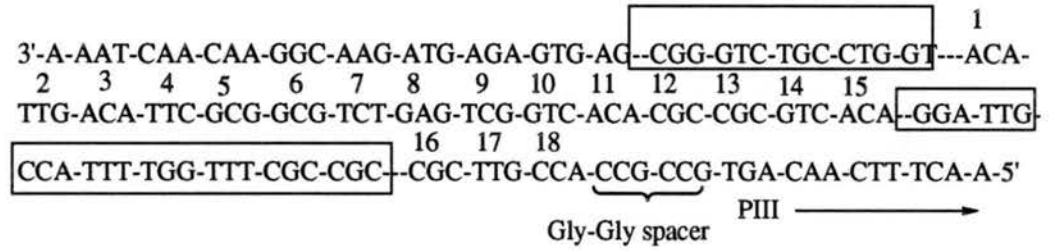


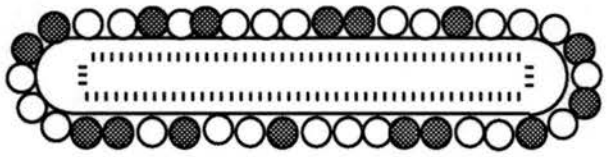
Figure 23: Comparison of library sequence and reccurent mutant . The inserts are boxed.

Direct Infection of Bacteria with Bound Phage

A new strategy was designed which would specifically amplify the high affinity phage without an elution step. The procedure is to add log phase K91 cells into the Covalink well which contains the bound phage. The premise is that with agitation for half an hour at 37°C the bound phage will begin to infect the bacterial cells. several rounds of panning were conducted using this direct infection methodology and the phage were sequenced. Again the mutant showed in figure 22 appeared.

VIII. THE THIRD LIBRARY-LX4

Six new libraries, all derived from the parent vector f88.4, were obtained from the laboratories of Dr. Jamie Scott at Simon Fraser University. These libraries display the randomized region multivalently on the major coat protein pVIII. The f88.4 vector was derived by adding a synthetic recombinant pVIII gene to fd/tet. Both the wild type pVIII and the synthetic pVIII are displayed as a mosaic covering the phage surface. The randomized region is cloned into the synthetic gene just downstream from the signal peptidase site, and it is presumed that the mature recombinant pVIII presents the library at the N-terminus on the phage surface.[26]



● = recombinant pVIII

○ = wild type pVIII

f88.4

The first library chosen for panning is called LX4 and has the sequence shown below.

5'-GTT-CTT-ATG-CTA-TCG-TTT-GCC-NNK-TGT-(NNK)4-TGT-NNK-GCT-GCA
GAA-GGT-GAT-GAC-CCG-GCT-AAA-GCT-GCT-TTT-GAC-TCT-CTT-CAG-3'

From the aliquot of phage donated by Dr. Scott, K91 were infected, and a large scale phage preparation was carried out to give 30 mL of a 10^{10} phage/mL solution (stored at -20°C). The sequencing primer was prepared on the DNA synthesizer and purified by PAGE electrophoresis. The primer is the complement of the underlined region in the

sequence shown above. The single stranded DNA was isolated and sequenced to show that the LX4 library insert was present (figure 23).

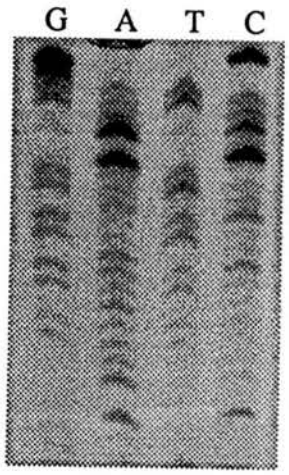


Figure 23: Sanger sequencing gel showing randomized region in the pVIII gene of the LX4 library.

A. Panning of the LX4 Library

The LX4 library was panned on Covalink plates derivatized with N-α-acetyl-L-lys-D-ala-D-ala. The bound phage were eluted from the plates with .1M HCl/glycine. After three rounds of panning the ssDNA was isolated and sequenced. The phage from the second round appears to show some selectivity, but the phage from the third round was entirely a deletion mutant, with no randomized region apparent at all.

The LX4 library was panned again on tripeptide derivatized Covalink plates using 0.1N HCl as the eluant for the first two rounds, then the direct infection method for the third round. The eluted phage from the three rounds were sequenced and it was found that certain residues were predominant (figure 24).

The sequence obtained from the gel in figure 24 is the following:

Library sequence:	NNK	ACA	NNK	NNK	NNK	NNK	ACA	NNK
Panned sequences:	GTC AC	ACA	GGA TC	GAA A T T	GTA C C	TTA CG A	ACA	ATC G
Corresponding amino acids:	Leu Pro	Cys	Arg Ser Thr	Lys Met Ile Asn	Met Ile Leu	Ile Ser Asn	Cys	Leu

There are 216 different possible sequence combinations from the above codons. Thus this library has been culled from 6.4 x 107 possiblities down to a mere 216 sequences. These "high affinity phage" were amplified and subjected to the following competition experiments.

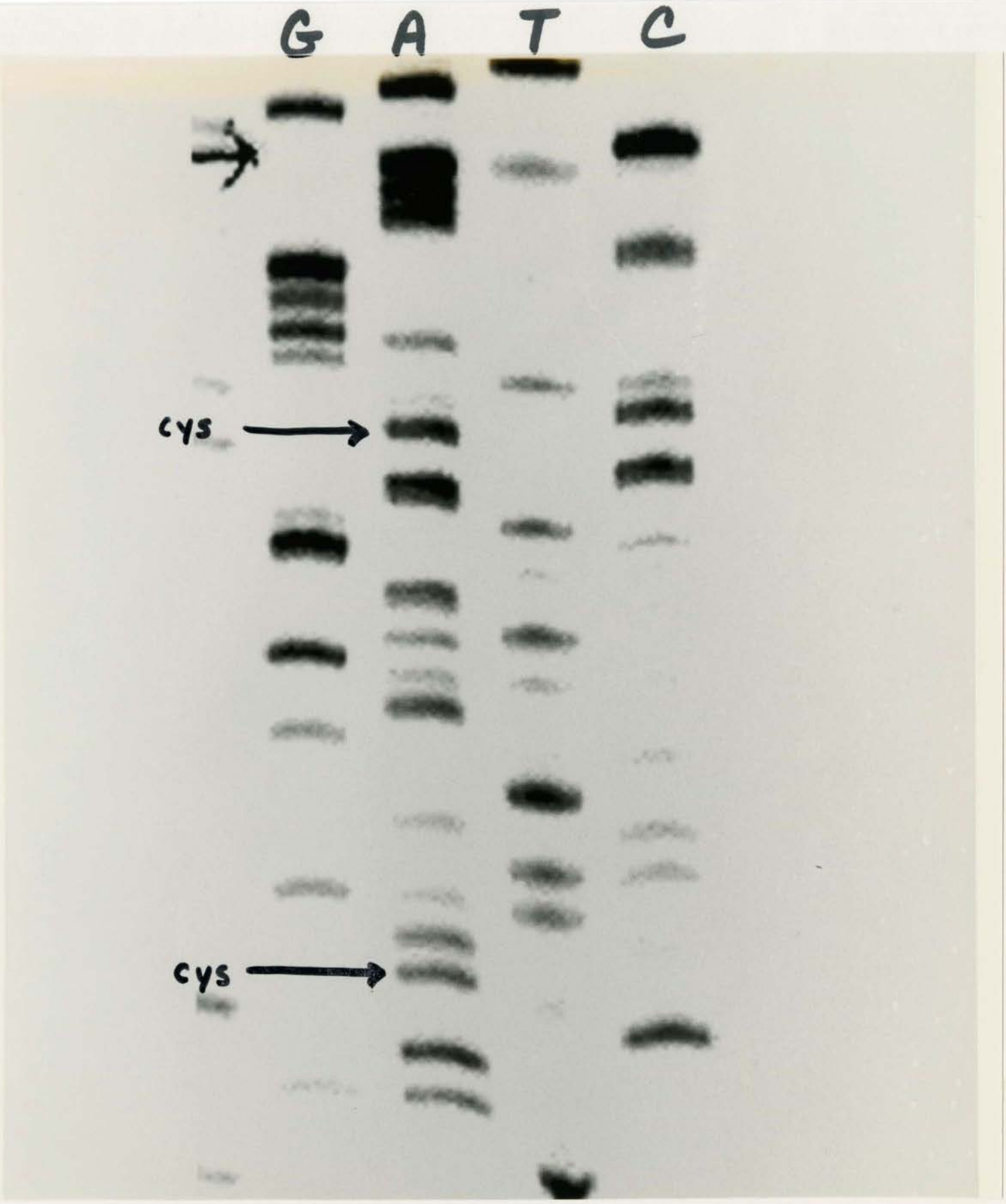


Figure 24: Sequencing gel of Panned LX_4 library.

B. Competition experiments

The high affinity phage from the previous pans on the LX4 library were diluted, combined with K91 cells, spread on plates and incubated. Colonies formed overnight, each colony representing a single phage infection. 96 colonies were picked and grown in LB broth to produce a solution of each clone. An aliquot of these clone solutions were placed into the same well location in each of the three 96 well Covalink plates derivatized with **16**. One of the plates functioned as a positive control: the plates were washed and rabbit anti-phage antibody was added, follwed by anti-rabbit antibody coupled to alkaline phosphatase. The ELISA was performed and the absorbance values recorded represent high binding phage with no competition. The second plate was used for a competition experiment in which the phage were preincubated with anti-phage antibody before being added to the plate. The truly high affinity phage in this experiment should show a reduction in binding, thus a reduction in absorbance in the ELISA. The third plate was pretreated with vancomycin before the phage were added. There should also be a reduction in binding due to the vancomycin masking the binding sites.

The above competition experiments were performed many times with various manipulations of reagents and conditions. No consistent results were obtained from these experiments. It is possible that these phage are not truly high affinity phage, however further experiments need to be performed to determine if that is true.

CONCLUSION

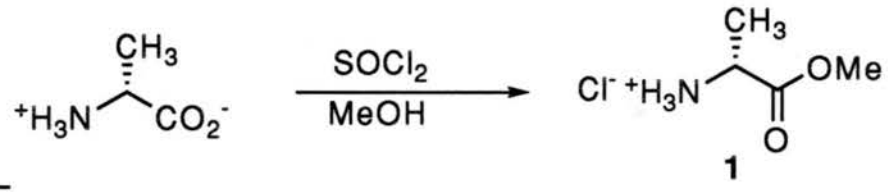
The purpose of this project was to use phage display technology to generate drug candidate leads. While this ultimate goal has not yet been realized, a number of new methodologies have been developed on the way. Conditions for panning have been optimized. Conditions for amplifying and quantitating filamentous phage were optimized. Several methods for quantitating derivatized solid supports were developed.

Because of the ambiguous results of the many panning experiments performed on the first two libraries (both of which expressed the library on the pIII gene), it is possible that the tripeptide target ligand is too small to be recognized by the pIII protein. The LX_4 library which expresses a library throughout the surface of the phage has shown some enhancement, and the possibility of hits from the high affinity phage obtained from this library looks more promising.

EXPERIMENTAL SECTION

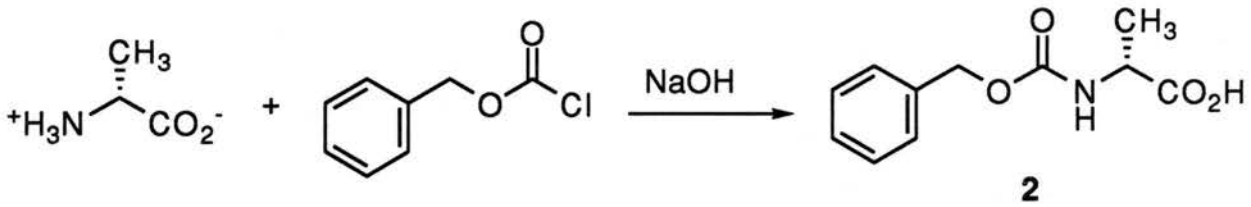
I. CHEMISTRY

General Synthetic Procedures. All melting points were taken on a Mel-Temp apparatus in open-ended capillary tubes. ^1H NMR were taken on a Varian 270 MHz spectrometer, or a Bruker 300MHz spectrometer. Mass spectra were obtained from the CSU Mass Spectrometry facility, Department of Chemistry at Colorado State University. Thin layer chromatography was performed on 25mm coated silica gel glass plates from Merck. Visualization was acheived with UV light and staining with a 2mg/mL ninhydrin solution in 95% ethanol, or a 7% solution of phosphomolybdic acid in 95% ethanol. Column chromatography was performed with silica gel-60 from Merck. The solvents used in chromatography and workup were commercial grade, and used as supplied. The solvents used in reactions were further dried or purified as follows. Methylene chloride, terahydrofuran, and toluene were freshly distilled. Dimethylformamide and triethylamine were dried over 3-A sieves. Pyridine was distilled and dried over 3A sieves.



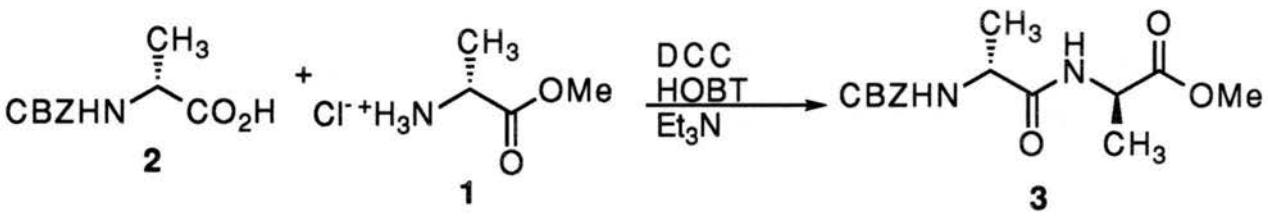
1

1.0g (11.24 mmol) of D-alanine was dissolved in 10mL MeOH and cooled to 0°C. 1.63 mL (22.5mmol) of thionyl chloride was added dropwise over a period of ten minutes. The reaction was stirred for four and 1/2 hours and concentrated in vacuo to a syrup. 20 mL of ether was added and a white solid crystallized out. The solid was filtered, washed with cold ether and dried to give 1.34g of **1** as a white solid (87%). mp= 104-110°C, (lit.= 107°C). $[\alpha]_D$ = - 6.8.° (c=1.6, MeOH) lit. value for L-alanine methyl ester hydrochloride =+7.0°C. ^1H NMR (270 MHz, D_2O) δ1.59 (d, j=7.03Hz, 3H), 3.87 (s, 3H), 4.24 (q, 1H).

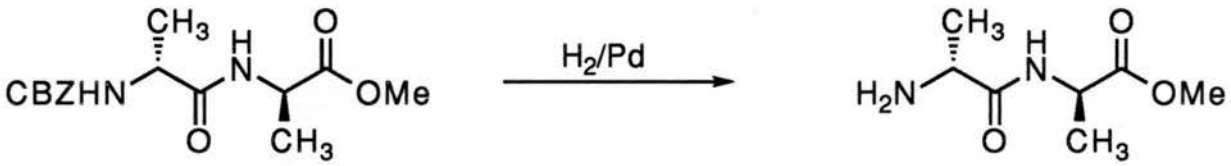


2

1g (11.24mmol) of D-alanine was dissolved in 30mL of water. 2.36g (28.1mmol) of $NaHCO_3$ was added and cooled to 0°C. 10mL of ether was added, and 2.87g (16.9mmol) of carbobenzyloxychloride in 5mL ether was added dropwise over a ten minute period. The solution was allowed to warm to room temperature overnight. After 20 hours, the layers were separated, and the aqueous layer was washed twice with 15 mL of ether. It was then acidified with 10% HCl, then extracted twice with 30 mL of ether. The organic layers were combined, washed with brine, dried with $MgSO_4$, and concentrated in vacuo. The resultant solid was recrystallized from hexane-EtOAc to give 1.26g of **2** as a white solid (54%) mp = 79-83°C, (lit.= 85°C). ^1H NMR (270MHz, $CDCl_3$) δ1.5 (d,3H), 4.4(m,1H), 5.2 (s, 2H) 7.2 (s, 5H).

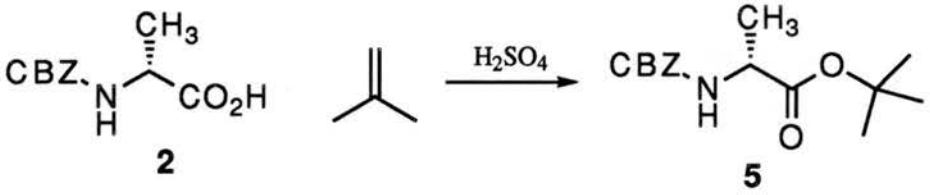


1.0g (4.48mmol) of **2** was dissolved in 3 mL CH_2Cl_2. 623mg (4.48mmol) of **1** and 622uL (4.48mmol) of Et_3N were added and the reaction was cooled to 0°C. 969mg (4.70mmol) of dicylcohexylcarbodiimide (DCC) was added and the reaction was allowed to come to room temperature while stirring overnight. The solution was filtered to remove DCU and chromatographed on silica gel with 95/4/1 CH_2Cl_2/MeOH/AcOH to give 1.14g of **3** (83%). ^1H NMR (270MHz, $CDCl_3$) δ1.35(d, 6H), 3.70 (s, 3H), 4.25 (m, 1H), 4.55 (m, 1H), 5.10 (s, 1H), 5.25 (m, 1H), 6.45 (m, 1H), 7.35 (s, 5H).



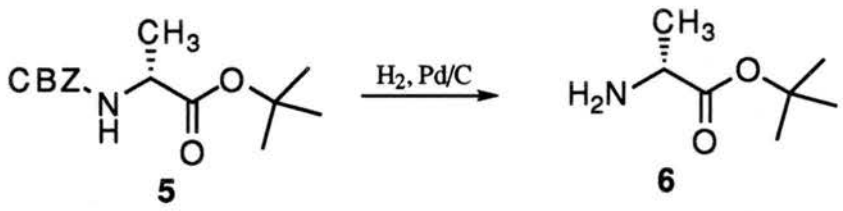
3 **4**

1.12g (3.6mmol) of **3** was dissolved in 30mL of EtOH. 760mg of 5% palladium on carbon was added and the solution was placed in a Parr bottle. The bottle was filled with H_2 gas to a pressure of 60psi, and the reactionwas stirred overnight. The solution was filtered through Celite to give a solid which was recrystallized from methanol to give 200 mg of **4** as white crystals (34%). ^1H NMR ($CDCl_3$, 200MHz) δ1.4 (d, 3H) 1.6 (d, 3H), 3.4 (m, 1H), 3.65 (s, 3H), 4.7 (m, 2H), 7.8 (m, 2H).

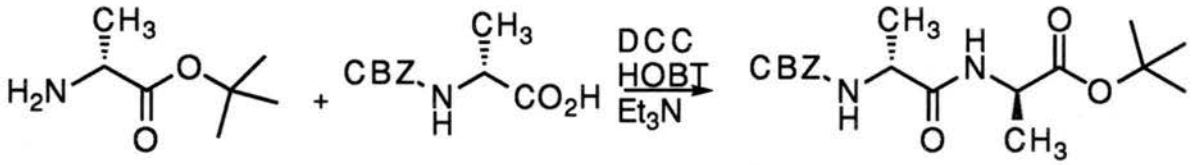


2 5

To 1.29g(5.78mmol) of **2** dissolved in 20 mL CH_2Cl_2 in a high pressure bottle, 10mL of liquid isobutylene (cooled to -78°C) was added. 300 μL of concentrated H_2SO_4 was added and the bottle was closed off and shaken overnight on a Parr apparatus. When the pressure was released after 17 hours, TLC showed no starting material remained. The solution was washed with H_2O, concentrated in vacuo to give 2.0g of a yellow oil. This was purified by flash chromatography on silica gel (95/4/1 CH_2Cl_2/MeOH/AcOH) to give 1.25g of a clear oil **5**. (78% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.35 (3H,d), 1.45 (9H,s) 4.25 (1H,m), 5.10 (2H,s) 5.30 (1H,m), 7.35(5H,s).

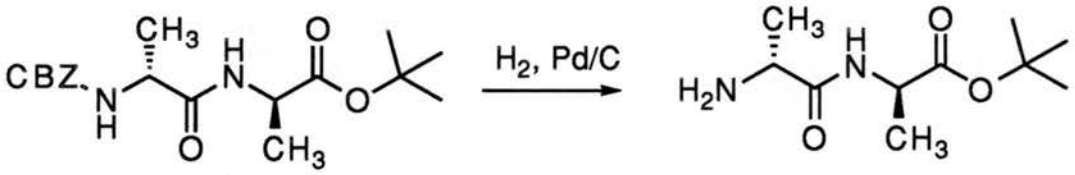


In a 100mL RB flask 1.10g(3.9mmol) of **5** was dissolved in 25 mL of a 3:2 mixture of MeOH/EtOH. The flask was flushed with argon, then 0.42g of 5%Pd/C (.197mmol) was added, and the flask was flushed with hydrogen. A balloon filled with H_2 was then placed over the flask, and sitrred at RT for 5 hours. The solution was then filtered off through filter paper to give 0.58g of an oil, **6**. (100% yield). ^{1}H NMR (270MHz, D_2O) δ1.40 (3H, d) 1.47 (9H,s), 3.90 (1H,m).



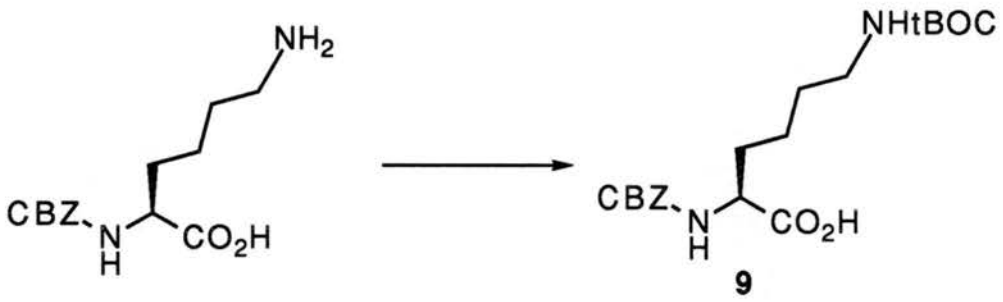
6 2 7

In a 25 mL RB flask, 1.12g (4.0mmol) of **2**, .58g (4.0mmol) of **6**, 0.612mL (4.4mmol) of triethylamine, and 0.54g (4.0mmol) of hydroxybenzotriazole (HOBT) were combined and dissolved in 10mL of DMF and 5 mL of CH_2Cl_2. 0.91g (4.4mmol) of DCC were then added and the reaction was stirred for 18 hrs at RT. The white solid dicyclohexylurea (DCU) was filtered off and 10 mL CH_2Cl_2 was added to the filtrate. This solution was washed with 20 mL of 0.5M NaOH twice, then 25mL of 5% HCl, then saturated brine, dried over $MgSO_4$, and concentrated in vacuo to give 1.21g of an oil **7** in (86% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.25-1.35 (6H,2d), 1.40 (9H,s), 4.20(1H,m), 4.37 (1H,m), 5.05 (2H,s), 5.40 (1H,m), 6.55 (1H,m), 7.30 (5H,s).



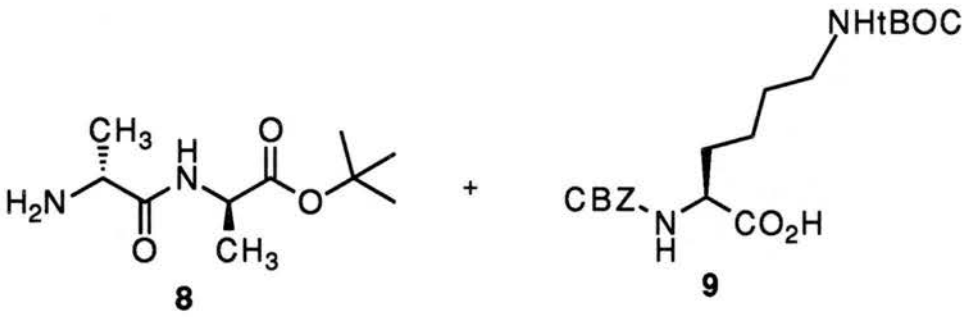
7 **8**

In a 50 mL RB flask, 1.21g (3.4mmol) of **7** in 10mL MeOH was added. The flask was flushed with argon, then 0.36g of 5%Pd/C (5mol%) was added and the flask was flushed with H_2. It was then fitted with an H_2 filled balloon. After 10 hours of stirring at RT, no **7** remained by TLC. The catalyst was filtered through filter paper, and the solution was concentrated in vacuo to afford 0.56g of a white solid **8**. (76% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.6 (3H,d), 1.65 (9H,s), 1.85 (3H,d), 4.5 (1H,m), 4.7 (1H,m), 8.6 (3H,m).



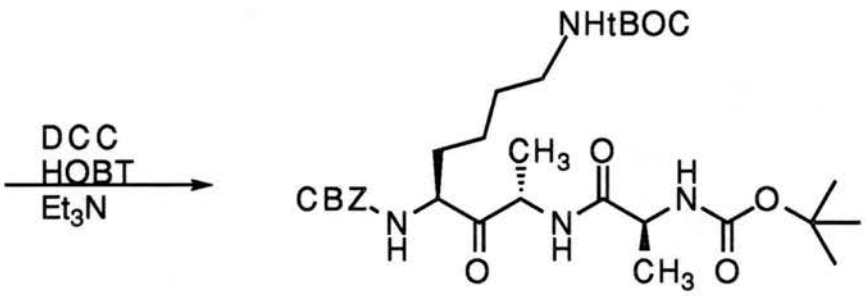
9

1g (3.57 mmol) of ε-tBOC-lysine was dissolved in 15 mL 0.5M NaOH. 3.1g (14.28 mmol) of tBOC anhydride was added and the reaction was stirred at room temperature for 3 hours. Excess tBOC anhydride was removed by extracting twice with 40 mL ether. The aqueous solution was cooled to 0°C, and carefully acidifying to pH 3. The product was immediately extracted with 3X 50 mL Et_2O, dried with $MgSO_4$, and concentrated in vacuo to give 1.044 g **9** as a white solid (77% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.4 (m, 13H), 1.8 (m, 2H), 3.05 (m, 2H), 4.35 (m, 1H), 4.72 (m, 1H), 5.09 (s, 2H), 5.72 (m,1H)



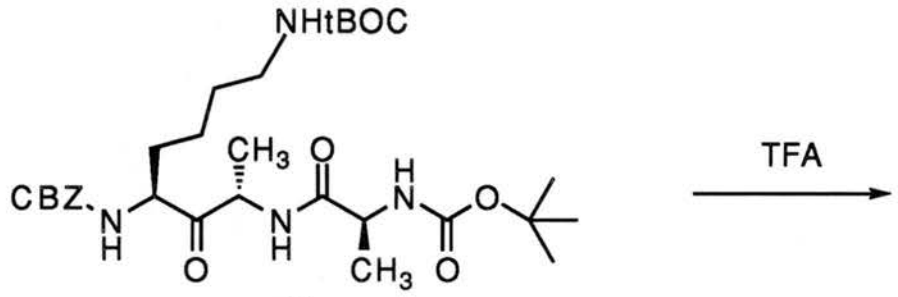
8

9

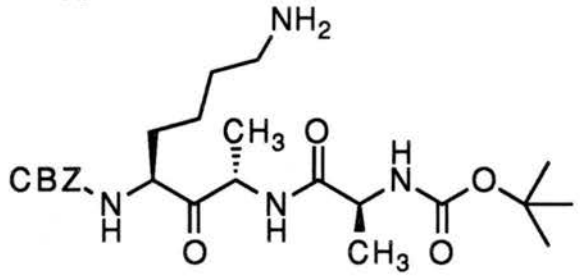


10

In a 25mL RB flask, 0.98g (2.59mmol) of **9**, 0.56g(2.59mmol) of **8**, 396μL (2.85mmol) of triethylamine, and 0.35g (2.59mmol) of HOBT were combined in 10mL DMF/5mL CH_2Cl_2. 0.59g (2.85mmol) of DCC was then added, and the solution stirred for 12 hours at RT. The solid was filtered off, and 15mL CH_2Cl_2 was added. This solution was then washed with 25mL 1N NaOH, 25mL 10% HCl, and 25mL saturated brine, dried over $MgSO_4$ and concentrated to give 1.07g of a sticky solid. It was then purified by chroatography on Silica gel in 98/1/1 CH_2Cl_2/MeOH/AcOH to give 480mg of **10**. (32% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.2-1.8 (30H,m) 3.05 (2H,m), 4.15 (1H,m), 4.37 (1H,m), 4.50 (1H,m) 5.09 (1H,m), 7.05 (1H,m), 7.30 (5H,s).

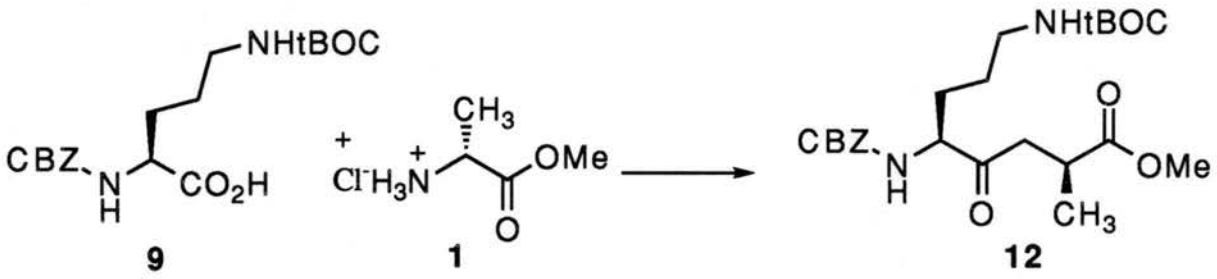


10



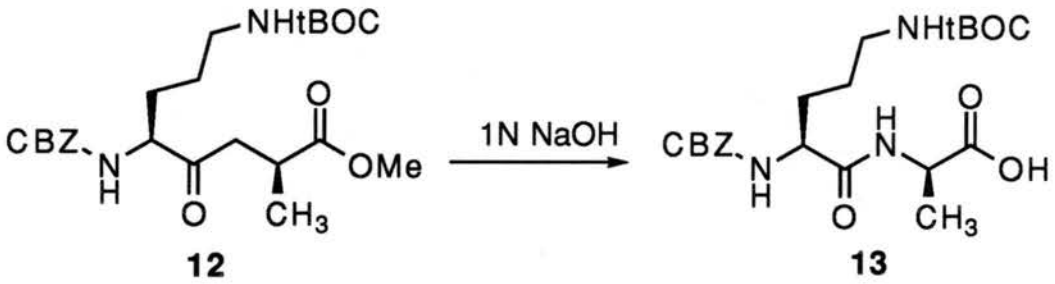
11

480mg(.83mmol) of **10** was dissolved in 10mL of CH_2Cl_2 and cooled to 0°C. 640μL(8.3mmol) of TFA was added and the reaction was stirred for several hours, then allowed to come to room temperature, and stirred for 10 more hours. The solution was concentrated in vacuo, then taken back up in CH_2Cl_2, 200 μL of Et_3N was added and the solution was washed with 10mL H_2O, dried over $MgSO_4$, and concentrated to give 380 mg of a solid. NMR revealed that only the carbamate had been cleaved and the t-butyl ester was still intact. This was subjected to further acidic deprotection conditions including HCl and H_2SO_4 to no avail except decomposition of the tripeptide. After chromatography on silica gel in 4/1 MeOH/AcOH, 160mg of solid **11** was obtained. (30% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.20-1.8 (21H,m), 3.12 (2H,m), 4.18 (1H,m), 4.40 (2H, m), 5.0 (1H,m), 5.07 (2H,s), 7.30 (5H,s).



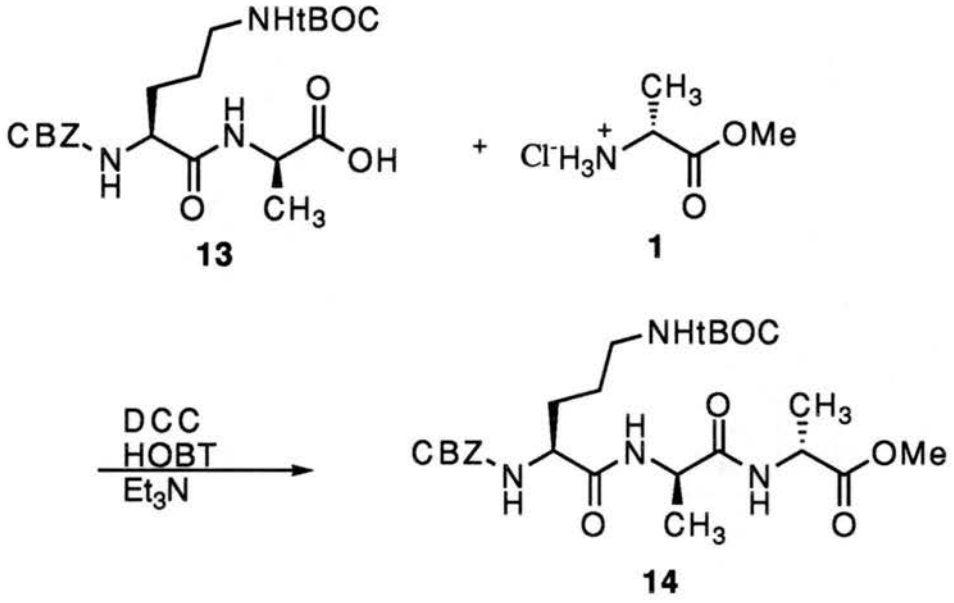
9 1 12

50 mg(1.3mmol) of **9**, 183 mg(1.3mmol) of **1**, 182μL(1.3mmol) of Et_3N, and 177 mg(1.3mmol) of HOBT were combined in 10mL DMF and 5mL CH_2Cl_2. 270mg (1.31mmol) of DCC was then added and the reaction stirred for 12 hours at room temperature. The solution was filtered, washed with 20mL 10% HCl, then 20mL 1M NaOH, concentrated in vacuo and purified by chromatography on silica gel in 95/4/1 CH_2Cl_2/MeOH/AcOH to give 360mg of **12**. (59% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.05-1.9 (18H, m), 3.10 (2H,m), 3.40 (1H,m), 3.70 (3H,s), 4.15 (1H,m), 4.55 (2H,m), 5.1 (2H,s), 5.50 (1H,m), 6.70 (1H,m), 7.35 (5H,s).

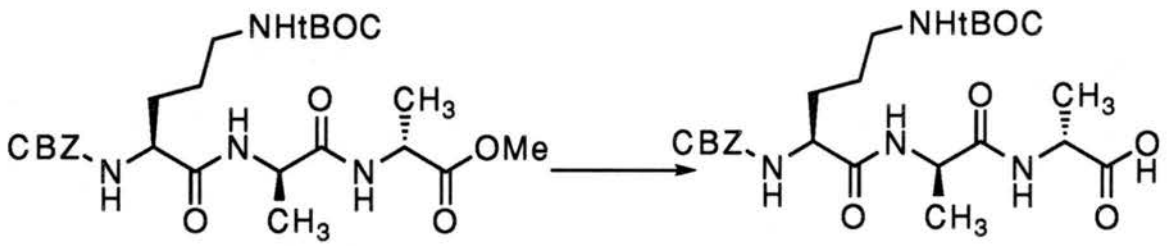


12 **13**

360mg(0.77mmol) of **12** was dissolved in 10mL of EtOH. 2mL of 1N NaOH was added and the reaction stirred at room temerature. After 2 hours, no **3** remained by TLC. The mixture was then acidified with cold 10% HCl until pH<3, and extracted twice with 20 mL Et_2O. The organic layer was concentrated in vacuo to give 290 mg of **13**. (84% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.10-1.95 (18H, m), 3.05 (2H,m), 3.40 (1H,m), 4.30 (1H,m), 4.5-4.7 (2H,m), 5.05 (2H,s), 6.30 (1H,m), 7.30 (5H,s).

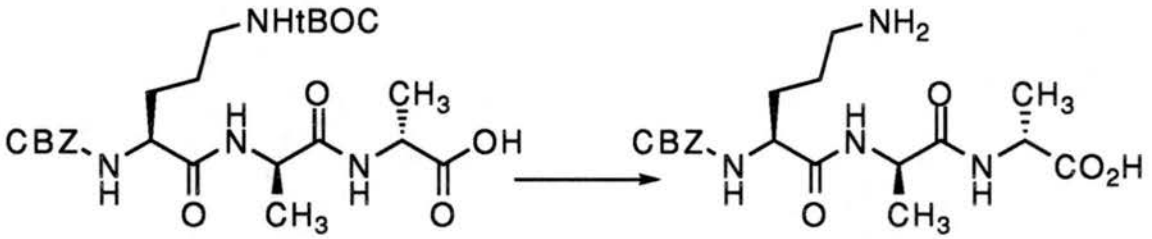


140mg (0.31mmol) of **13**, 43mg (0.31mmol) of **1**, 86μL of Et_3N (.62mmol), and 46mg (.34mmol) of HOBT were combined in 5 mL CH_2Cl_2 and then 70mg(.34mmol) of DCC was added. The reaction stirred at RT for six hours and was then filtered, washed with 20mL 10% HCl, then 20mL 1M NaOH, concentrated in vacuo and chromatographed with 95/4/1 CH_2Cl_2/MeOH/AcOH to give 80mg of **14**. (48% yield). ^1H NMR (270MHz,$CDCl_3$) δ1.10-1.90 (21H,m), 3.0 (2H,m), 3.40 (1H,m), 3.65 (3H,s), 4.20 (1H,m), 4.50 (1H,m), 4.70 (1H,m) 5.05 (2H,s), 5.75 (1H,m), 6.95 (1H,m), 7.30 (5H,s).

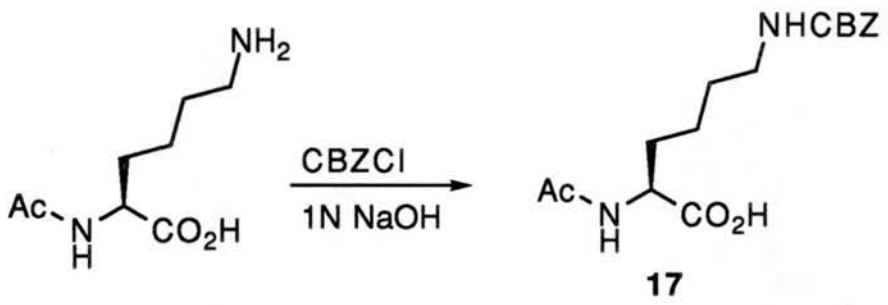


14 **15**

40mg (.075mmol) of **14** was dissolved in 2mL of MeOH. 5mL of 1N NaOH was added and the reaction stirred for several hours. The mixture was slowly acidified with 10% HCl until pH reached 3. The solution was extracted twice with 10mL of EtOAc and dried over $MgSO_4$, then concentrated to give 25mg of **15**. (66% yield). ^1H NMR (270MHz, DMSO-D_6) δ1.15-1.9 (21H,m), 3.10 (2H,t), 4.10 (1H,m), 4.50 (2H,m), 5.15 (2H,s), 7.0 (1H,m), 7.40 (5H,s), 8.0 (1H,m).

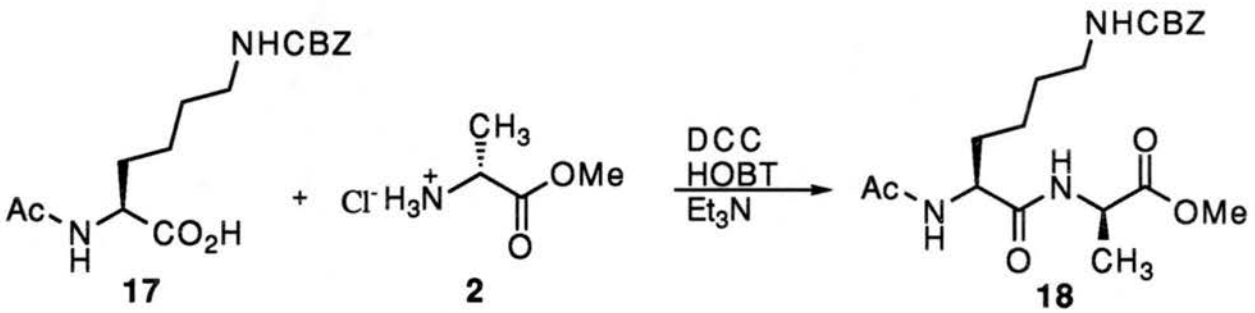


20mg (.039mmol) of **15** was dissolved in 5mL of CH_2Cl_2, and 100μL of TFA was added. The reaction stirred for 16 hours at RT. The solution was concentrated in vacuo, then taken back up in CH_2Cl_2, and treated with Et_3N and concentrated again to give 20mg of **16**. (100% yield). ^1H NMR (270MHz, CD_3OD) δ1.1-1.8 (12H,m), 2.80 (2H,m), 4.0 (1H,m), 4.25 (2H,m), 5.0 (2H,s), 7.25 (5H,s).

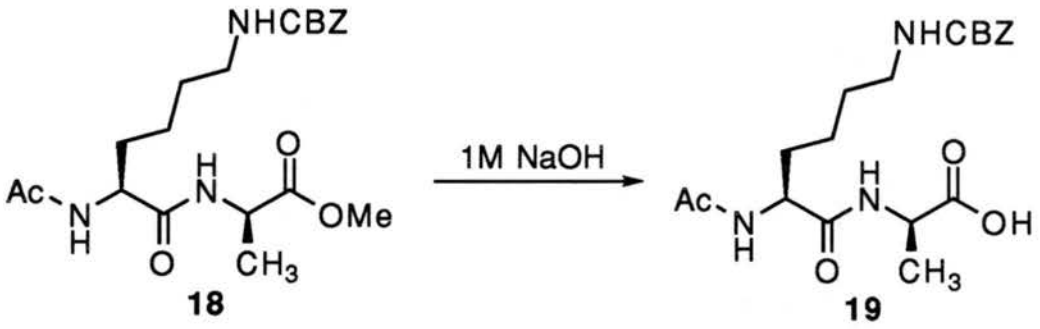


17

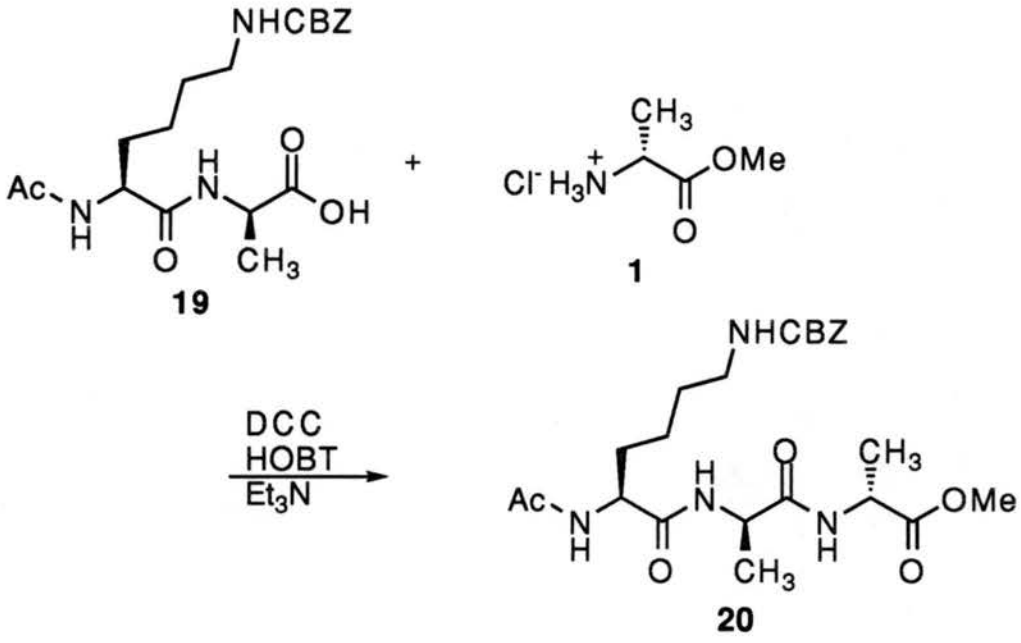
200mg(1.06mmol) of **1** was dissolved in 6mL of 1N NaOH and cooled to 0°C. 227μL(1.59mmol) of carbobenzyloxychloride was dropped in slowly while stirring. After 2 hours the reaction was extracted twice with 20mL Et_2O, and the aqueous layer was carefully acidified to pH3 with HCl. The product was then extracted from the aqueous layer with 3 x 30mL ethyl acetate. Concentration in vacuo gave 228mg of **17** as a clear oil. (67% yield). ^1H NMR (270MHz, $CDCl_3$) 1.34-1.37 (4H,m), 1.67-1.73 (2H,m), 1.97 (3H,s), 3.09-3.14 (2H,q), 4.50 (1H, m), 5.04 (2H,s), 5.25 (1H,t), 6.98 (1H,d), 7.29 (5H,s).



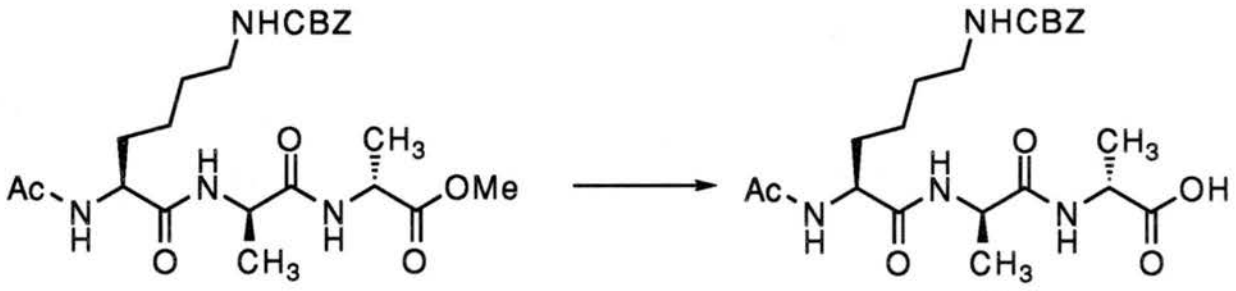
50mg (.155 mmol) of **17**, 25mg (.187mmol) of **2**, 24mg of HOBT (.170mmol) and 35mg (.170mmol) of DCC were dissolved in 3 mL of DMF. 43μL of Et_3N (.31mmol) was added, and the reaction was stirred overnight at room temperature. The mixture was then cooled to 0°C to precipitate the solid DCU which was then filtered off. The filtrate was diluted with 15mL CH_2CL_2 and washed twice with 15mL of dilute aqueous HCl. The organic layer was concentrated in vacuo and chromatographed on silica gel using 90/8/2 CH_2Cl_2/MeOH/AcOH to give 91mg of solid **18** which still contained some DCU. This crude material was carried on to the next step.



91mg of crude **18** from the previous reaction was dissolved in 5mL methanol, and 1mL of 1N NaOH was added. The reaction was stirred at room temperature for one hour. The mixture was cooled to 0°C, and carefully acidified to pH3, then extracted twice with 15mL EtOAc. The organic layer was then concentrated in vacuo to give 52mg of material which was chromatographed on silica gel to give 25mg of **19**, (41% yield) in 2 steps. ^{1}H NMR (270MHz, $CDCl_3$) δ1.08-1.78 (6H,m), 1.37 (3H,d), 1.87 (3H,s), 3.12 (2H,m), 4.36-4.52 (2H,m), 5.06 (2H,s), 7.28 (5H,s).

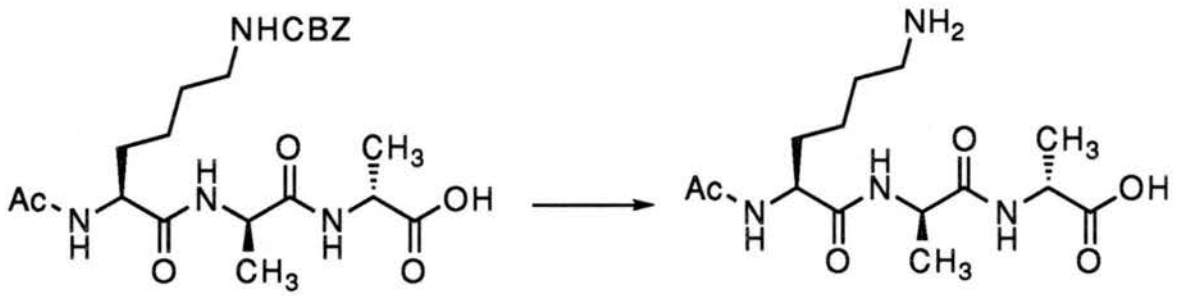


82mg (.209mmol) of **19**, 31mg (.229mmol) of **2**, 32mg (.229mmol of HOBT and 47mg (.229mmol) of DCC were dissolved in 2mL DMF. 58uL(.418mmol) of Et_3N was added and the reaction stirred overnight at room temperature. The reaction was then cooled to O°C, and the insoluble DCU was filtered off. The filtrate was diluted with 15 mL of CH_2Cl_2 and washed twice with dilute aqueous acid and concentrated, the residue was then chromatographed on silica gel with 90/8/2 CH_2Cl_2/MeOH/AcOH to give 88mg of crude **20** which was saponified directly.

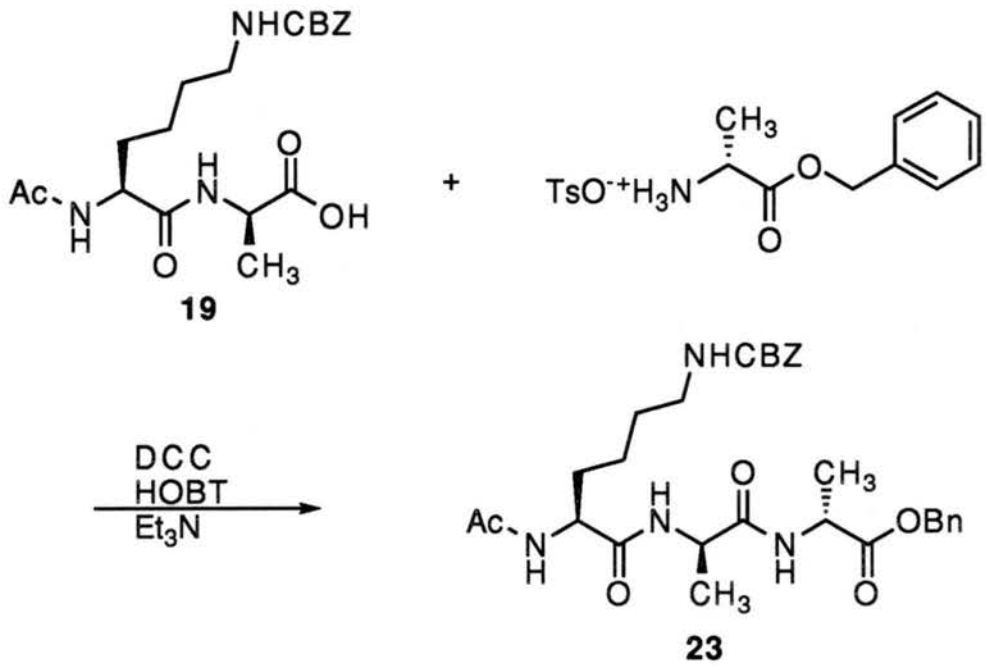


20 **21**

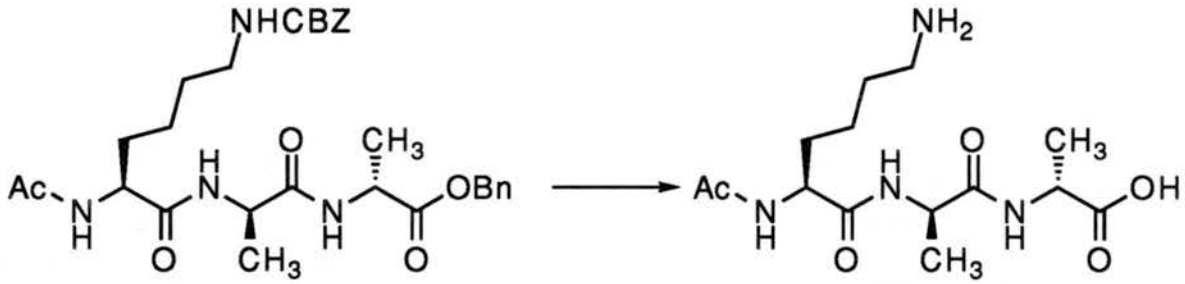
21 was dissolved in 2mL MeOH and 1mL of 1N NaOH was added at room temperature. After 1 hour the reaction was complete. The solution was cooled to 0°C and carefully acidified with the addition of 1N HCl until pH was less than 3. The aqueous solution was then extracted with ethyl acetate (3 x 10mL), dried with MgSO4, and concentrated in vacuo to give 88mg of solid which was then chromatographed on silica gel using 95/4/1 CH_2Cl_2/MeOH/AcOH to give 51mg of a clear oil **22** (53% yield). ^1H NMR (270MHz, CH_3OD) δ1.17-1.66(6H,m), 1.18(3H,d, j=6.83Hz), 1.24(3H,d,j=6.83Hz), 1.83 (3H,s), 2.96(2H,q), 4.09 (1H,m), 4.24 (2H,m), 4.99 (2H,s), 7.21 (1H,t), 7.32 (1H,m), 7.34 (5H,s), 8.0 (2H,m).



14mg(.029mmol) of **21** was dissolved in 1mL of dry absolute ethanol. 16mg(.05mol%) of 10%Pd on carbon was added, and the reaction flask was flushed with hydrogen gas then fitted with a hydrogen balloon. The mixture was allowed to stir overnight at room temperature. The catalyst was removed by filtering through Celite eluting with methanol. The solution was diluted with 20mL and extracted twice with CH_2Cl_2. The water was removed by lyophilization to give 8mg of **22** (87%yield). ^1H NMR (270MHz, $CDCl_3$) δ1.23-1.70 (6H,m), 1.34 (3H,d, j=7.06Hz), 1.37 (3H,d,j=7.06Hz), 1.94 (3H,s), 3.14 (2H,q), 4.33 (1H,m), 4.52 (2H,m), 5.06 (2H,s), 5.12 (2H,d,j=4.98 Hz), 6.52 (1H,d,j=7.89 Hz), 6.89 (1H,d, j=7.47Hz), 6.97 (1H,d,j=7.89), 7.34 (5H,s).

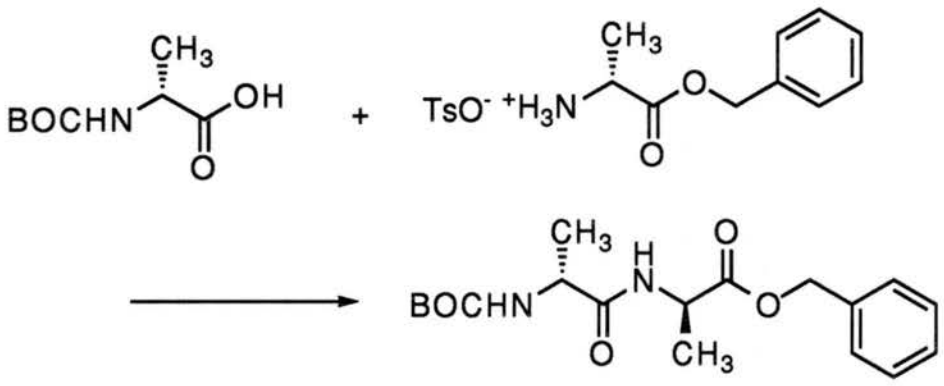


65mg (.165mmol) of **19**, 69mg (.198mmol) of D-alanine benzyl ester tosylate, 24mg (.181mmol) of HOBT , and 37mg (.181mmol) of DCC were dissolved in 7mL of DMF. 46μL(.330mmol) of Et_3N was added and the reaction was stirred at room temperature under N_2. After 18 hours, the solid DCU was filtered off, and the filtrate was diluted with 20mL CH_2Cl_2. This was washed twice with 5% HCl, dried with $MgSO_4$ and concentrated in vacuo to give 80mg of solid. The solid was chormatographed on silica gel with 95/4/1 CH_2Cl_2/MeOH/AcOH to give 66mg of **23** as a white solid (56%yield). ^1H NMR (270MHZ, $CDCl_3$), δ1.33 (d, j=7.47Hz, 3H), 1.37 (d, j=7.47, 3H), 1.49 (m, 2H), 1.65 (m, 2H), 1.78 (m,2H), 1.93 (s, 3H), 3.15 (m,2H), 4.36 (m,1H), 4.4 (m, 2H), 5.06 (s,2H), 5.12 (d,j=5.40 Hz, 2H), 6.55 (d,1H), 6.95 (d, j=7.47,1H), 7.04 (d, 1H), 7.35 (s, 10H).



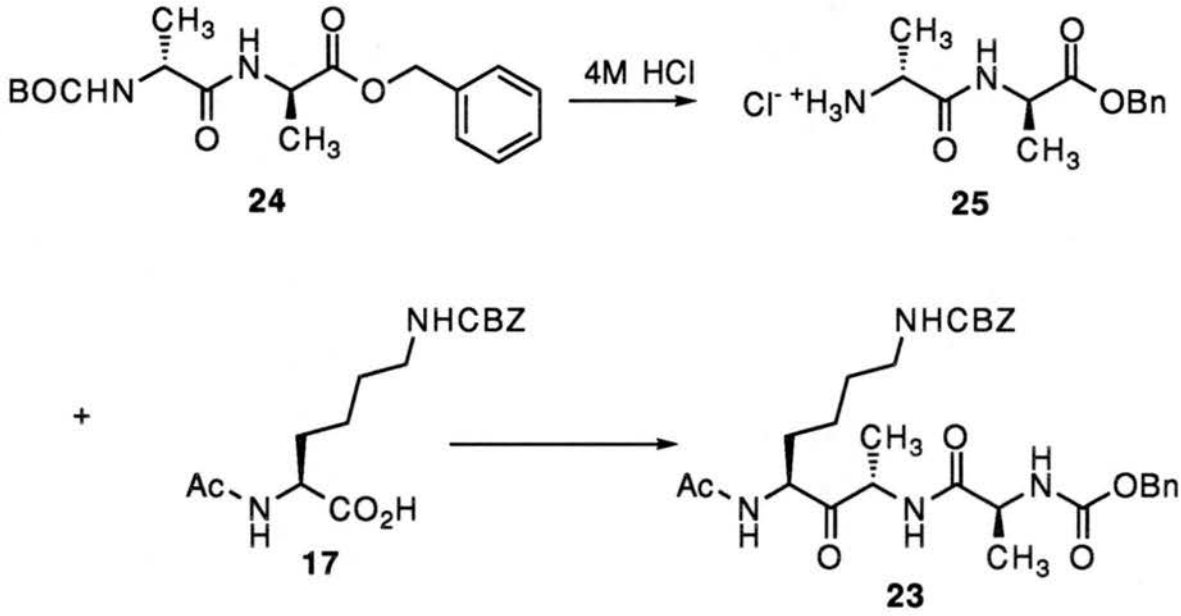
23 22

52 mg (.0939mmol) of 23 was dissolved in 3mL MeOH. 50mg (4.69umol) of 5% Pd on carbon was added, and the mixture was flushed with hydrogen and outfitted with a hydrogen balloon. The reaction stirred overnight at room temperature. The reaction was filtered through Celite to remove catalyst, eluting with methanol. The filtrate was concentrated in vacuo to give 27mg of solid which was purified by ion exchange chromatography on Dowex resin (200-400 mesh) to give 16mg of solid **22** (52% yield). ^{1}H NMR (270 MHz, $CDCl_3$) δ1.35 (d, j=7.62Hz, 3H), 1.39 (d, j=7.03Hz, 3H), 1.42 (m, 4H), 1.67-1.82 (m, 4H), 2.05 (s, 3H), 3.00 (t, j=7.63, 3H), 4.12 (m, 1H), 4.26 (m, 1H), 4.35 (m, 1H).

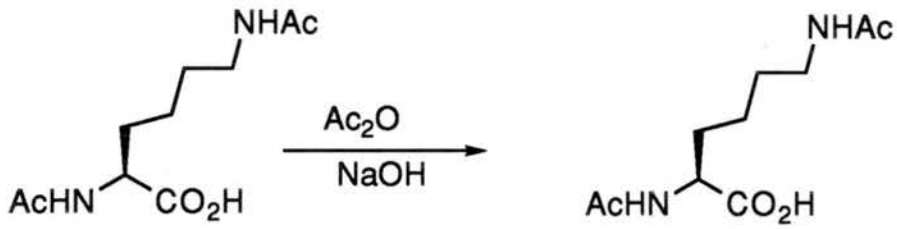


24

1.24g (6.54mmol) of tBOC-D-alanine, 1.0g (7.19mmol) of D-alanine benzyl ester tosylate, .97g (7.19mmol) of HOBT, and 1.48g (7.19mmol) of DCC were dissolved in 25mL of DMF. 1.82mL (13.08mmol) of Et_3N was added, and the reaction was stirred overnight at room temperature. The mixture waas cooled to 0°C and the solid DCU was filtered off. The filtrate was diluted with 40 mL of CH_2Cl_2 and washed with 5% HCl (40ml x 2). The solution was then dried with $MgSO_4$, and concentrated in vacuo to give 2.3g white solid which was chromatographed on silica gel with 95/4/1 CH_2Cl_2/MeOH/ AcOH to obtain 1.598g of **24** (89% yiled).^{1}H NMR (270 MHz, $CDCl_3$) δ1.29 (d, j=7.06Hz, 2H), 1.37 (d, j=7.06Hz, 2H), 1.40 (t, 3H), 4.20 (m, 1H), 4.57 (m, 1H), 5.12 (d, j=4.05, 2H), 5.22 (d,1H), 6.86 (m, 1H), 7.30 (s, 5H).

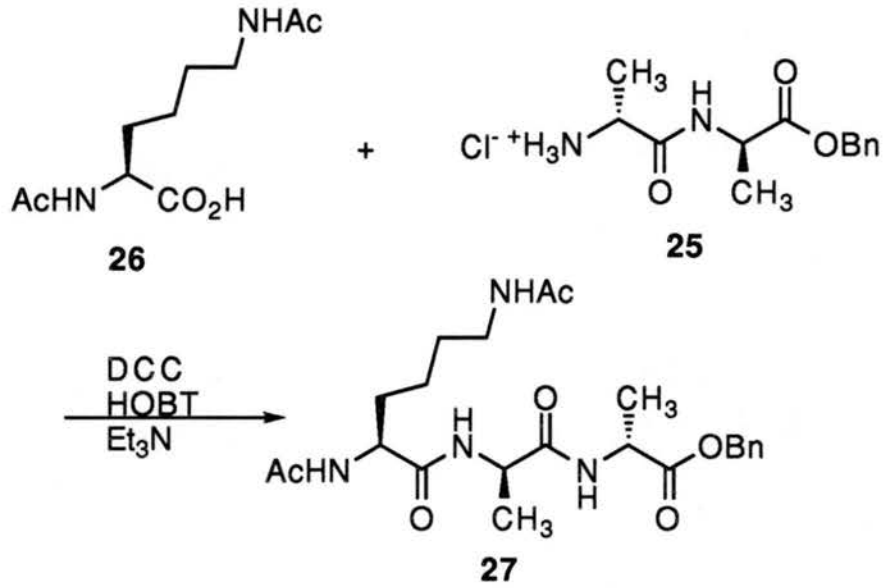


242mg (.692mmol) of **24** was dissolved in 3mL of 4M HCL in dioxane and stirred at room temperature. After one hour, there was no **24** left by TLC. The solution was concentrated in vacuo and redissolved in CH_2Cl_2 twice and reconcentrated. The residue was then taken up in 3mL DMF and 190mg (.629mmol) of **17**, 103mg (.692mmol) of HOBT, 142mg (.692mmol) and 96μL of Et_3N were added. The reaction was stirred overnight at room temperature, then cooled to 0°C with an ice bath. The DCU was filtered off, and the filtrate was taken up in CH_2CL_2 and washed with 1N HCl. The organic layer was concentrated in vacuo to give 424 g of a yellow solid. The solid was chromatographed on silica gel with 95/5 CH_2Cl_2/MeOH to give 357mg of **23** as a white solid (100% yield). ^1H NMR (270MHZ, $CDCl_3$), δ1.33 (d, j=7.47Hz, 3H), 1.37 (d, j=7.47, 3H), 1.49 (m, 2H), 1.65 (m, 2H), 1.78 (m,2H), 1.93 (s, 3H), 3.15 (m,2H), 4.36 (m,1H), 4.4 (m, 2H), 5.06, (s,2H), 5.12 (d,j=5.40 Hz, 2H), 6.55 (d,1H), 6.95 (d, j=7.47,1H), 7.04 (d, 1H), 7.35 (s, 10H).

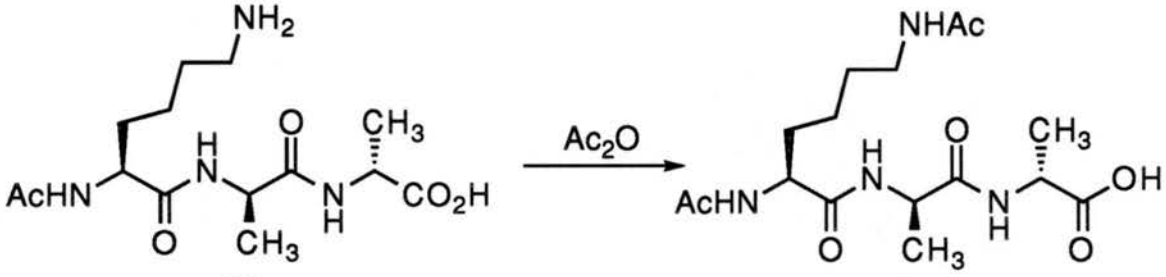


26

200mg (1.064mmol) of α-acetyl-L-lysine was dissolved in 1.064 mL of 1N NaOH (1eq), and 106 μL of 1N NaOH (.1eq) and 10 μL of Ac_2O (.1eq) were added every 5 minutes 11 times. 1 equivalent of HCl was added and the solution was lyophilized to give 260 mg of white solid. This was chromatographed on silica gel, eluting with 1/1 ethyl acetate/ methanol to give 245 mg of **26** (100% yield). $[\alpha]_D$=.179, 1% in methanol at 25°C. ^{1}H NMR (270 MHz, D_2O) δ1.38 (m, 2H), 1.51 (m, 2H), 1.6-1.85 (m, 2H), 1.99 (s, 3H), 2.04 (s, 3H), 3.17 (t, 2H, j=7.03), 4.14 (m, 1H).

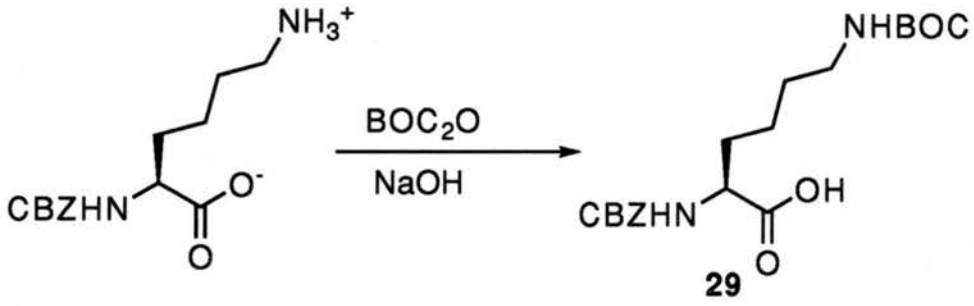


50mg (.218mmol), of **26**, 68mg (.240mmol) of **25**, 36mg (.240mmol) of HOBT, and 49mg (.240mmol) of DCC were dissolved in 1mL DMF. 34μL of Et_3N was added and the reaction was stirred overnight at room temperature under argon. The solution was cooled and DCU filtered off. The filtrate was diluted in CH_2Cl_2, washed with 10mL 0.5N HCl and concnetrated to get 100mg solid. This material was chromatographed with 95/4/1 Me_2Cl_2/MeOH/AcOH to give 40 mg of **27** (40% yield). ^1H NMR (270MHz, $CDCl_3$). δ1.33 (d,j= 7.05), 1.40 (d, j= 7.05), 1.63 (m,4H), 1.84 (m,2H), 1.98 (s,6H), 3.48 (m,2H), 4.48 (m, 3H), 5.15 (d,j=4.57 Hz, 2H), 6.32 (d,1H), 6.74 (d,2H), 7.32 (s,5H).



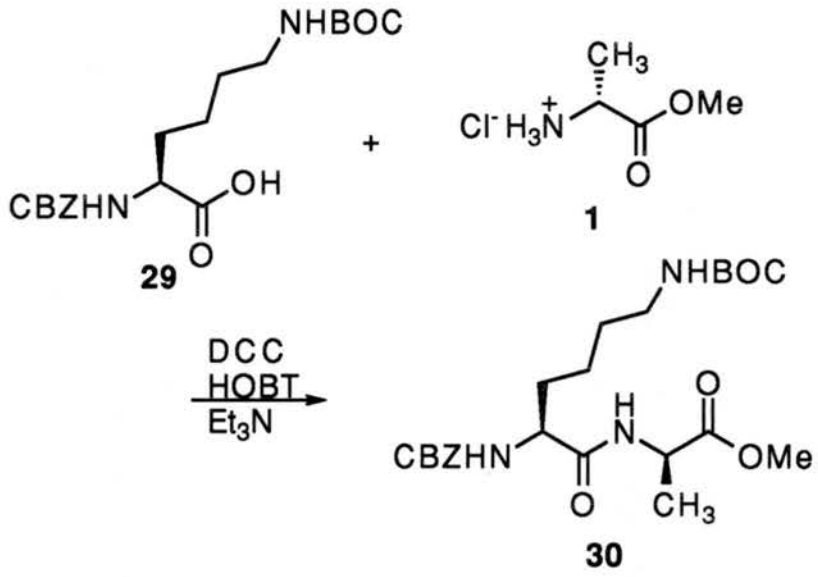
22 **28**

48mg (.145mmol) of **22** was dissolved in 500µL of H_2O, then 145µL (.145mmol) of 1N NaOH was added. 11 aliquots of 14.5µL (0.1 equivalent) of 1N NaOH and 1.4 µL(.1 aliquots) of acetic anhydride were added every 5 minutes. 1 equivalent of 1N HCl was added, then the solution was lyophilized to give 72mg of solid. This solid was chromatographed on silica gel twice with 2/1/1/1 EtOAc/nBuOH/AcOH/H_2O to give 23 mg of **28**. ^1H NMR (270 MHz, D_2O) δ1.38 (d, j=6.44, 3H), 1.39 (d, j=6.44, 3H), 1.4 (m, 2H), 1.53 (m, 2H), 1.75 (m, 2H), 1.98 (s, 3H), 2.04 (s, 3H), 3.17 (t, 2H), 4.19 (m, 1H), 4.24 (m, 1H), 4.36 (m, 1H).

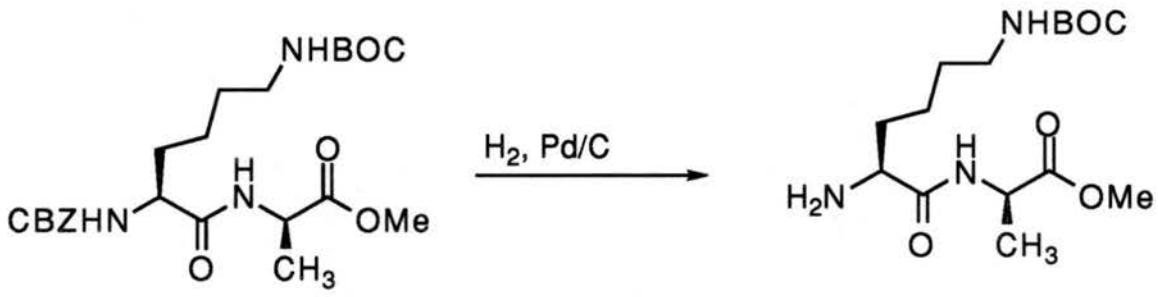


29

1.0 g (3.57 mmol) of CBZ-L-lysine was dissolved in 15 mL 0.5M NaOH. 3.1g (14.28 mmol) of tBOC anhydride was added and the reaction was stirred at room temperature for 3 hours. Excess t-BOC anhydride was removed by extracting twice with 40 mL ether. The aqueous solution was cooled to 0°C, and carefully acidifying to pH 3. The product was immediately extracted with 3X 50 mL Et_2O, dried with $MgSO_4$, and concentrated in vacuo to give 1.044 g **29** as a white solid (77% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.4 (m, 13H), 1.8 (m, 2H), 3.05 (m, 2H), 4.35 (m, 1H), 4.72 (m, 1H), 5.09 (s, 2H), 5.72 (m,1H).

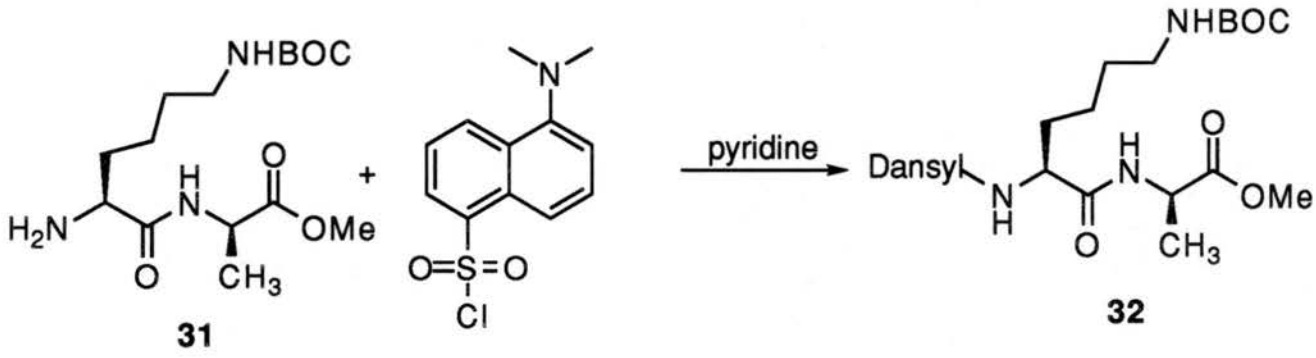


168 mg (.442 mmol) of **29**, 68 mg (.486mmol) of **1**, 66mg (.486 mmol) of HOBT, and 100 mg (.486 mmol) of DCC were dissolved in 10 mL DMF. 68 μL(.486 mmol) of Et_3N was added and the reaction stirred overnight under N_2. The solution was cooled to 0°C, and the DCU was filtered off, the filtrate was dissolved in 20 mL CH_2Cl_2 and washed with 5% HCl, dried with $MgSO_4$, and concentrated in vacuo to give 169 mg of solid. This material was then chromatographed on silica gel with 97/3 Me_2Cl_2/MeOH to give 119 mg of **30** as a white solid, R_f=.67, (58% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.33 (d,3H, j=7.06Hz), 1.37 (s,9H), 1.42 (m, 2H), 1.62 (m, 2H), 1.81 (m, 2H), 3.03 (m, 2H), 3.66 (s, 3H), 4.18 (m, 1H), 4.50 (m, 1H), 4.73 (m, 1H), 5.05 (s, 2H), 5.75 (d, 1H), 6.95 (d, 1H), 7.03 (s,5H).

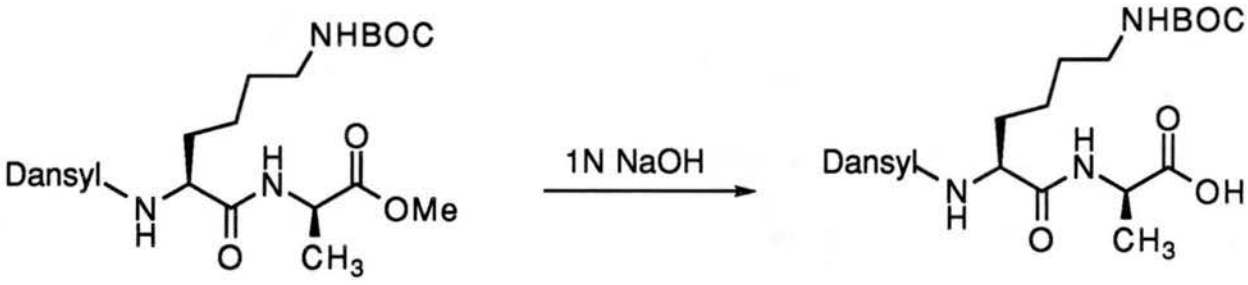


30 **31**

119 mg (.256 mmol) of **30** was dissolved in 3 mL MeOH. 27 mg (.0128 mmol) of 5% palladium on carbon was added and the flask was fitted with a hydrogen balloon and stirred for three hours. The catalyst was removed by filtering through Celite, and the filtrate was concentrated in vacuo to give 75 mg of a white solid. This solid was chromatographed on silica gel with 95/4/1 Me_2Cl_2/MeOH/AcOH to give 45 mg of a white solid **31**, R_f=.13, (53% yield) ^1H NMR (270MHz, $CDCl_3$) 1.47 (m, 16H), 1.58 (m, 2H), 3.04 (m, 2H), 3.68 (s, 3H), 4.53 (m, 1H), 4.67 (m, 1H), 7.70 (d, 1H).

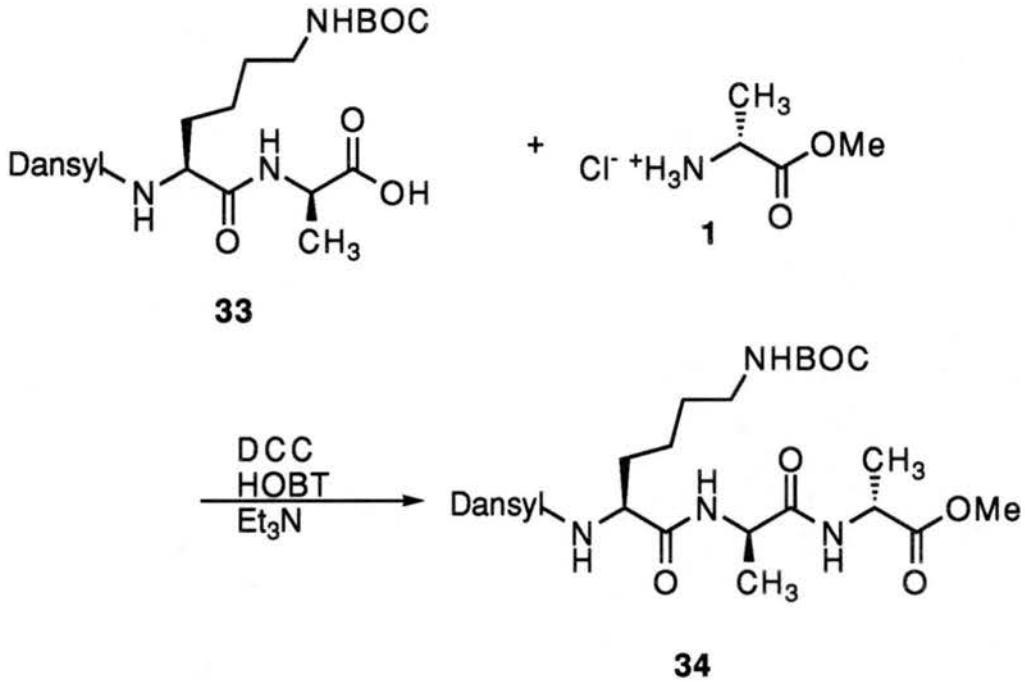


30 mg (.091 mmol)of **31** was dissolved in 2 mL dry pyridine. 29 mg(.109 mmol) of dansyl chloride was added, and the reaction stirred at room temperature for 3 hours. The mixture was then poured into 20 mL water, and then extracted with 20 mL of ether twice. The ether layer was washed with 5% HCl, dried with $MgSO_4$, concentrated in vacuo to give 23 mg of a yellow solid **32** (45% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.32 (d, 3H), 1.48 (s, 9H), 1.6-1.9 (m, 6H), 2.84 (s, 6H), 3.19 (m, 2H), 3.65 (s, 3H), δ4.21 (m, 1H), 4.48 (m, 1H), 5.92 (d, 1H), 6.70 (d, 1H), 7.17 (d, 1H, j=7.89), 7.48 (dd, 1H, j=7.47), δ7.57 (dd, 1H, j=7.47), δ8.21 (dd, 1H, j=1.24), δ8.35 (d, 1H, j=8.72), 8.51(d, 1H, j=8.30).

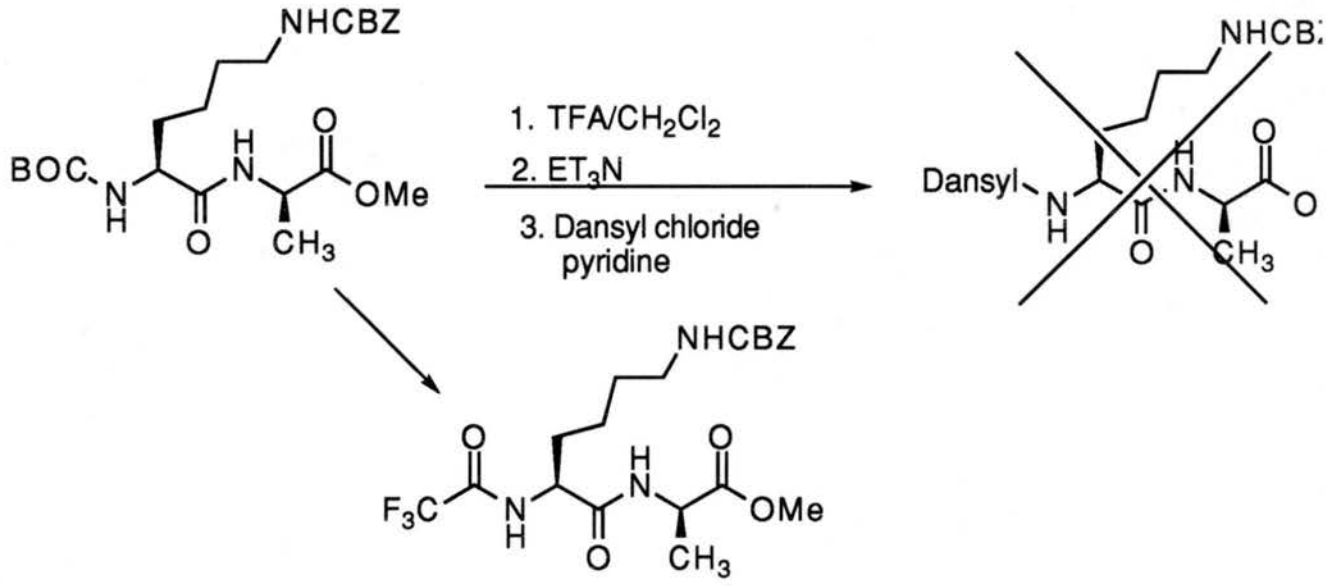


32 **33**

23 mg of **32** was dissolved in 1mL MeOH and cooled to 0°C. 1 mL 1N NaOH was added and the reaction stirred for 2 hours. It was then diluted with 3 mL H_2O, extracted with ether, acidified with HCl until pH 3, then the product was extracted out of the aqueous layer with 10 mL ether twice. The organic layer was dried with $MgSO_4$, concentrated in vacuo to give 8 mg of **33** (36 % yield). ^1H NMR (270MHz, $CDCl_3$) δ1.08 (d, 3H), 1.23 (m, 2H), 1.43 (s, 9H), 1.57 (m, 4H), 2.87 (m, 8H), 3.73 (m, 1H), 4.26 (m, 1H), 7.19 (m, 1H), 7.48 (m, 2H), 8.23 (d, 1H), 8.37 (d, 1H), 8.53 (d, 1H).

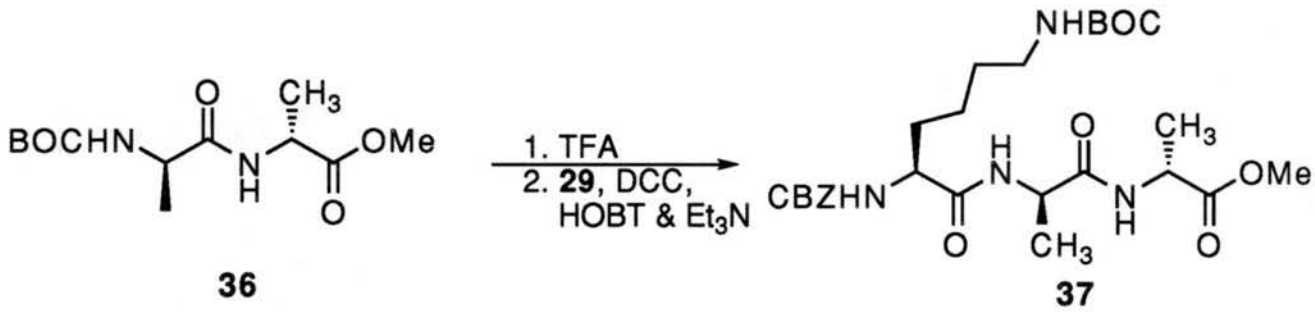


8mg (14.4umol) of **33**, 2mg (15.9umol) of HOBT, 2mg (15.9umol) of 1, 3mg (15.9 umol) of DCC were combined in 100μL of DMF. The reaction was stirred overnight at room temperature, then cooled to 0°C. The DCU was filtered off and the filtrate was taken up in 5mL CH_2Cl_2 and washed with 5mL of 1N HCl . The organic layer was concentrated to give 10 mg of material which was purified by preparative TLC with 95/4/1 CH_2Cl_2/MeOH/AcOH to obtain 7mg of **34** which was still impure.



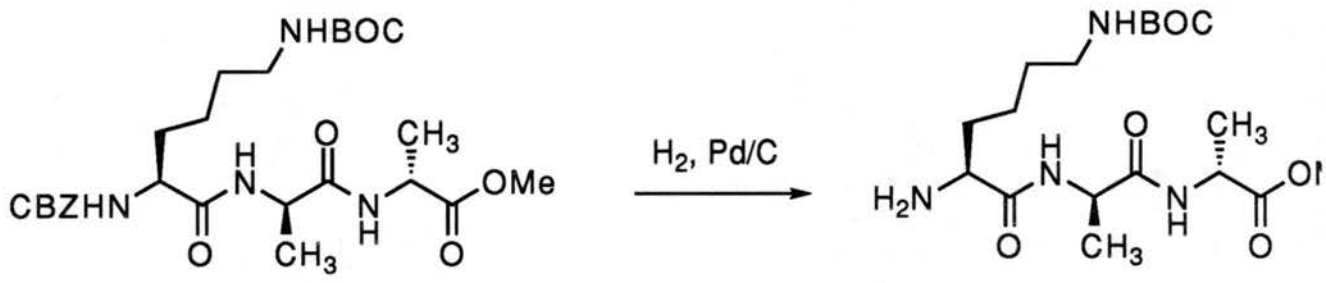
35

136mg (.303mmol) of α-tBOC-ε-CBZ-L-lys-D-ala methyl ester was dissolved in 1mL CH_2Cl_2 and cooled to 0°C. 1mL of TFA was added. The reaction was stirred for two hours, until no evidence of starting material remained by TLC. The mixture was concentrated in vacuo, then flushed with nitrogen and redissolved in 2 mL of dry pyridine. 98 mg (.364 mmol) of dansyl chloride was added and the reaction was stirred under nitrogen for 8 hours. 15mL of H_2O was added and the aqueous solution was extracted three times with 20mL of Et_2O . The ether layer was dried to give 67 mg of **35** as a white solid. ^1H NMR (270MHz, $CDCl_3$) δ1.39 (d, j= 7.05, 3H)), 1.52 (m, 2H), 1.76 (m, 2H), 1.83 (m, 2H),3.16 (m, 2H), 3.72 (s, 3H), 4.53 (m, 2H), 4.91 (m, 1H), 5.06 (s, 2H), 6.81 (m, 1H), 7.32 (s, 5H), 7.43 (m,1H).



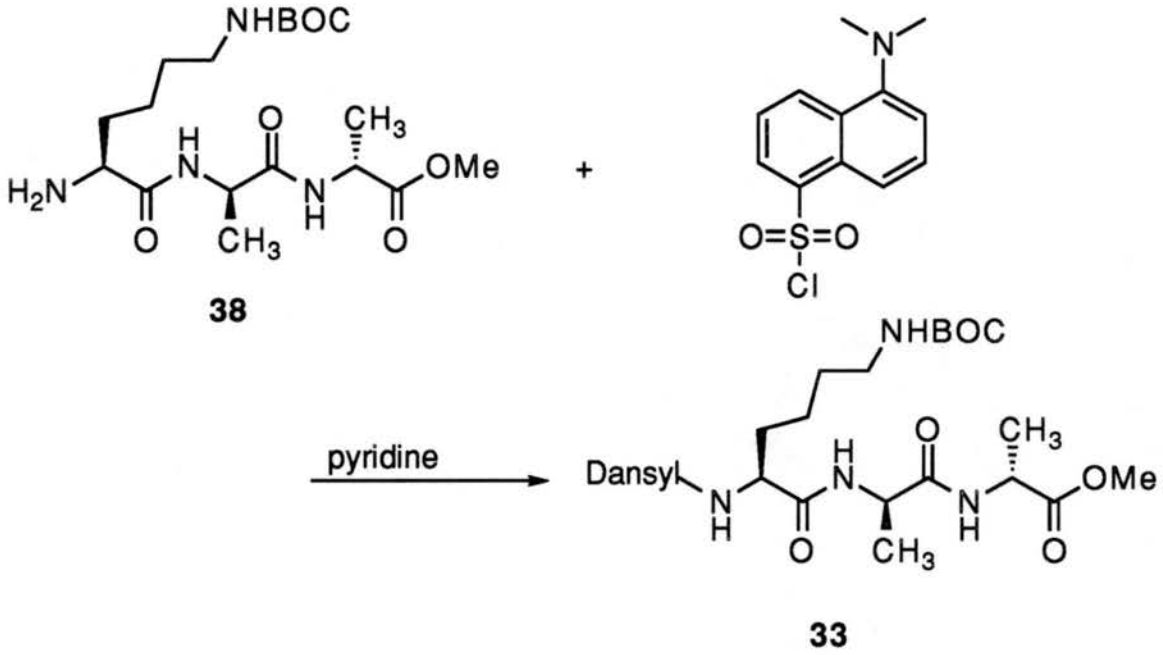
36 **37**

86 mg (.316 mmol) of **36** was dissolved in1 mL CH_2Cl_2 and cooled to 0°C. 1 mL TFA was added and the reaction stirred for 30 minutes. The mixture was concentrated in vacuo, and redissolved in CH_2Cl_2, 200 μL of Et_3N was added to neutralize any remaining TFA, and the mixture was concentrated again. 100 mg (.263 mmol) of **29**-CBZ-L-lys(tBOC) OH, 39 mg(.289 mmol) of HOBT, 59 mg (.289 mmol) of DCC were added and the mixture was dissolved in 2 mL DMF. 40 υL of Et_3N was added and the reaction stirred overnight at room temperature under N_2. The DCU was filtered off, and the filtrate was diluted with 15 mL CH_2Cl_2, which was washed with HCl, dried, and concentrated. The resulting residue was chromatographed on silica gel with 97/3 Me_2Cl_2/MeOH to give 85 mg of **37** as a white solid, R_f=.42 (50% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.35 (d, 3H, j=7.06), 1.39-1.45 (m, 14H), 1.63 (m, 2H), 1.79 (m, 2H), 3.03 (m, 2H), 3.69 (s, 3H), 4.12 (m, 1H), 4.50 (m, 2H), 4.66 (m, 1H), 5.06 (s, 2H), 5.71 (d, 1H), 6.87 (d, 1H), 6.97 (d, 1H), 7.32 (s, 5H).

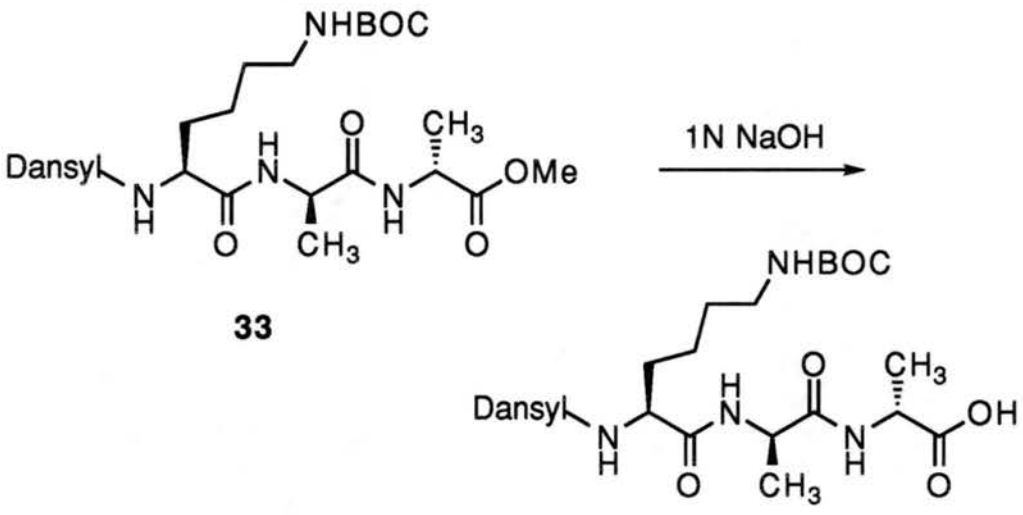


37 **38**

77 mg(1.437 mmol) of **37** was dissolved in 3 mL MeOH. 15 mg (.0718 mmol) of 5% Pd on carbon was added and the reaction was fitted with a hydrogen balloon. After stirring for 5 hours at room temperature, the catalyst was filtered off through Celite, and concentrated to give 58 mg of **38** (100% yield). ^{1}H NMR (270 MHz, $CDCl_3$) δ1.35 (d, j= 7.47, 3H), 1.37(d, j=4.98, 3H), 1.48 (m, 2H), 1.55 (m, 2H), 1.79 (m, 2H), 3.06 (m, 2H), 3.42 (m, 1H), 3.69 (s, 3H), 4.48 (m, 2H), 4.75 (m, 1H), 7.15 (m, 1H), 7.89 (m, 1H).

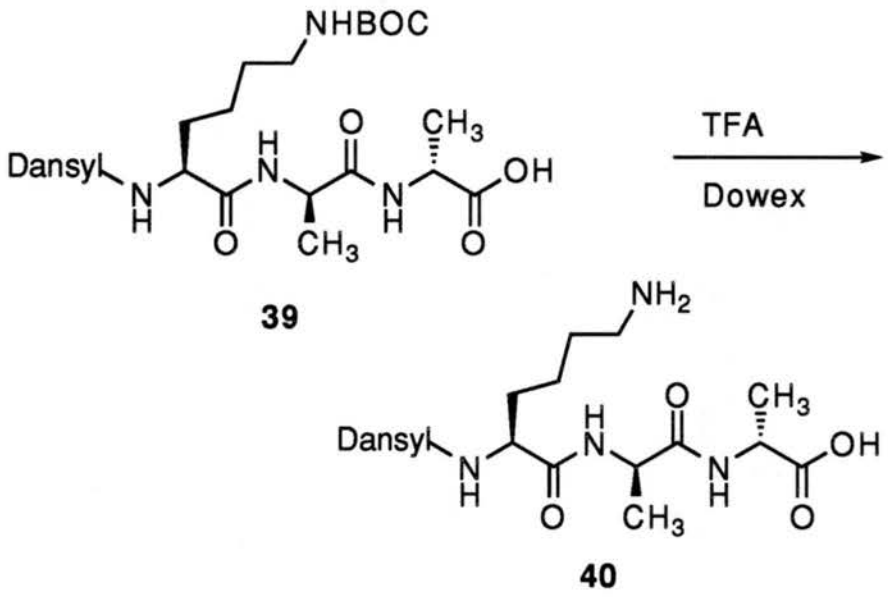


58 mg(.1437mmol) of **38** was dissolved in 2 mL dry pyridine. 116 mg(.4311 mmol) of dansyl chloride was added and the reaction was stirred under N_2 at room temperature for 3 hours. The solution was then diluted with 15 mL Et_2O, and the excess dansyl chloride was removed by washing with 5% HCl. The ether layer was dried and concentrated to give 133 mg of a yellow oil, which was chromatographed on silica gel with 97/3 CH_2Cl_2/MeOH to give 58 mg of **33** (61%yield) ^1H NMR (270 MHz, $CDCl_3$) δ1.07 (d,j=7.05Hz, 3H), 1.1-1.3 (m, 4H), 1.35 (d, j=7.06 Hz, 3H), 1.39 (s, 9H), 1.55 (m, 2H), 2.75 (m, 1H), 2.85 (s, 6H), 3.66 (m, 1H), 3.70 (s, 3H), 4.27 (m, 1H), 4.48 (m, 2H), 7.19 (d, j= 7.47 Hz, 1H), 7.49 (t, j= 7.06 Hz, 1H), 7.59 (t, j= 7.48 Hz, 1H), 8.22 (d, j=7.47 Hz, 1H), 8.35 (d, 1H), 8.53 (d, j= 8.71Hz, 1H).

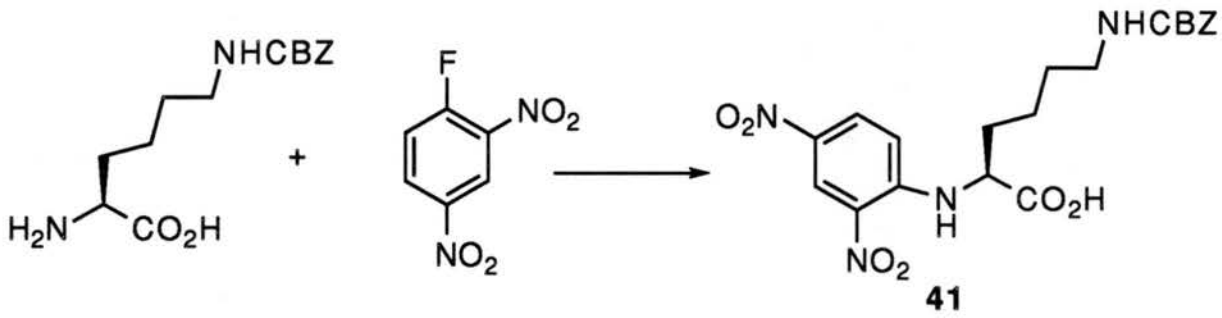


33 **39**

56 mg(.0882 mmols) of **33** was dissolved in 2mL MeOH. 1mL of 1N NaOH was added and the reaction stirred for three hours at room temperature. 3 mLs of H_2O was added and the reaction was extracted with ether, acidified to pH 3 with 5% HCl, and the product was extracted out of the aqueous layer with 2X 15 mL Et_2O, dried with $MgSO_4$, and concentrated to give 30 mg of **39** (54% yield). After examination by NMR to ensure the methyl group was gone,this material was carried on to the next step.

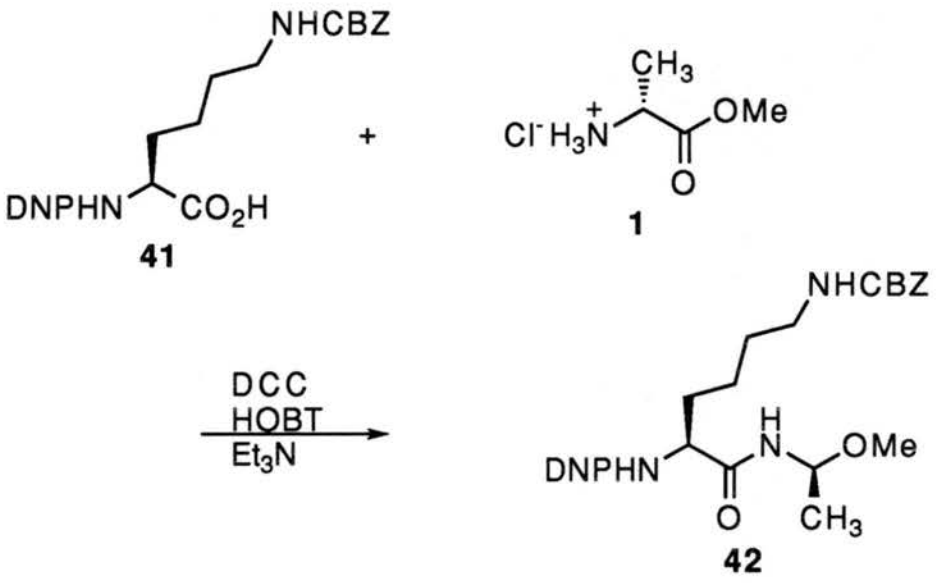


30 mg(.0483 mmol) of **39** was dissolved in 1 mL CH_2Cl_2. 500 uL of TFA was added and the reaction stirred at room temperature for 2 hours. The mixture was concentrated, then dissolved in 2 mL Me_2Cl_2,and 100 μL of Et_3N was added to neutralize any excess TFA. The mixture was concentrated again, then dissolved in 1mL H_2O and purified by ion exchange chromatography on Dowex 50W-X8 resin. The ninhydrin staining fractions were collected and lyophilized to give 16 mg of **40** (64% yield). ^1H NMR (270 MHz, D_2O) δ0.88 (d, 3H, j=7.03 Hz), 1.2 (m, 2H), 1.31 (d, 3H, j=7.03 Hz), 1.43 (m, 2H), 1.63 (m,2H), 2.71 (t, 1H), 2.87(s, 6H), 3.68 (m,1H), 3.75 (m,1H), 4.04 (m,1H),

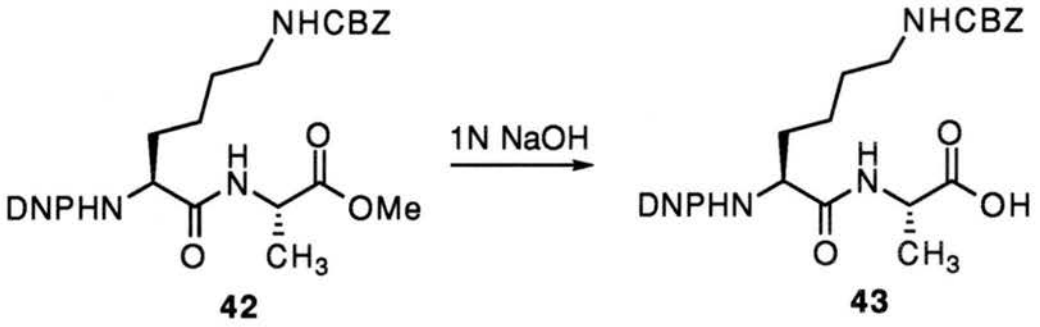


41

200mg(.714mmol) of ε-CBZ-L-lysine was dissolved in 10mL H_2O, and 24 mg(2.86 mmol) of $NaHCO_3$ was added. 266 mg(1.43mmol) of fluorodintirobenzene was dissolved in 2 mL MeOH and added to the aqueous mixture. The solution was then heated to reflux for 1 hour, cooled, and concentrated to remove MeOH. The mixture was then extracted twice with 25 mL Et_2O to remove excess fluorodinitrobenzene, then acidified with HCl to pH 3. Crude product was extracted twice with 50mL Et_2O, dried with $MgSO_4$ and concentrated to give 230 mg of yellow solid. The solid was then chromatographed with 95/4/1 CH_2Cl_2/MeOH/AcOH to give 56 mg of **41** R_f=.30, (17% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.51 (m, 4H), 2.03 (m, 2H), 3.2 (m, 2H), 4.33 (q, 1H), 5.06 (s, 2H), 6.81 (d, 1H), 7.32 (s, 5H), 8.25 (d, 1H), 8.9 (d, 1H), 9.12 (d, 1H).

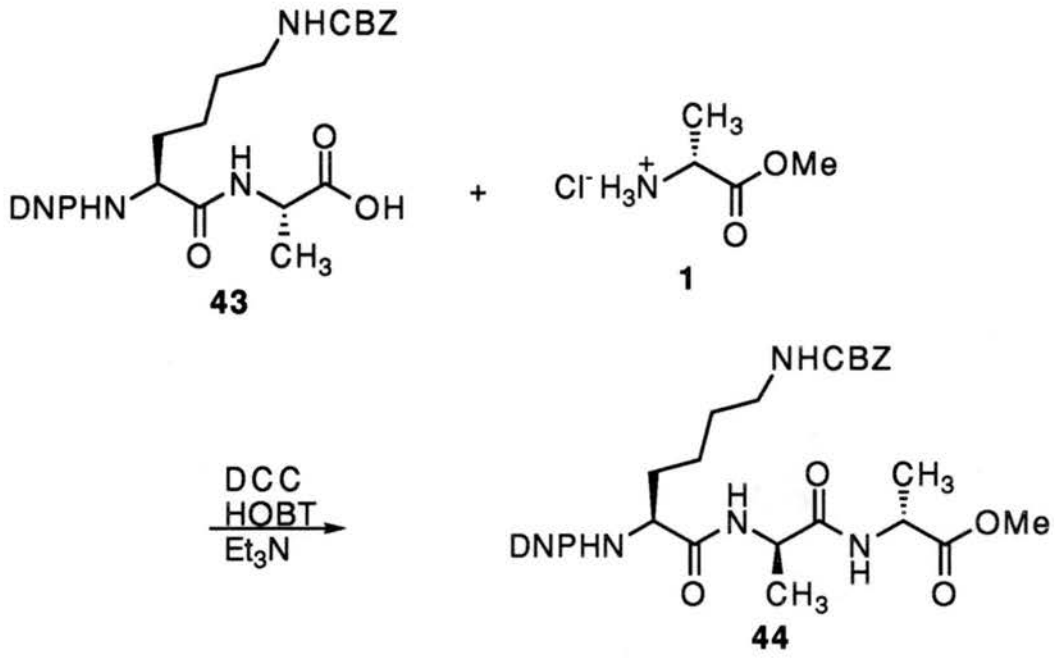


54 mg (.121mmol) of **41**, 18mg (.133mmol) **1**, 27 mg (.133mmol) DCC, and18 mg (.133mmol) HOBT were dissolved in 3mL DMF under N_2. 18uL (.133mmol) of Et_3N was added and the reaction was stirred overnight at room temperature. The cloudy mixture was cooled to 0°C, and the DCU was filtered off. The filtrate was diluted with 20mL CH_2Cl_2 and washed twice with 5% HCl, dried with $MgSO_4$, and concentrated in vacuo to give 67 mg of yellow solid. This solid was chromatographed on silica gel with 95/4/1 CH_2Cl_2/MeOH/AcOH, and 53 mg of **42** was obtained as a yellow solid R_f=.44 (83%yield). ^1H NMR (270MHz, $CDCl_3$) δ1.35 (d, 3H, j=7.06 Hz), 1.55 (m, 4H), 1.91 (m, 2H), 3.20 (m, 2H), 3.70 (s, 3H), 4.10 (m, 1H), 4.56 (m,1H), 4.94 (t, 1H), 5.06 (s, 2H), 6.77 (d, 1H), 6.82 (d,1H), 7.33 (s, 5H), 8.25 (dd,1H), 8.75 (d, 1H), 9.12 (d,1H).

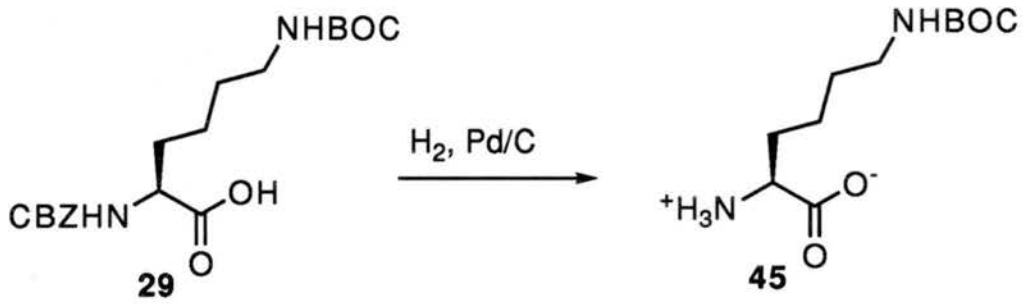


42 **43**

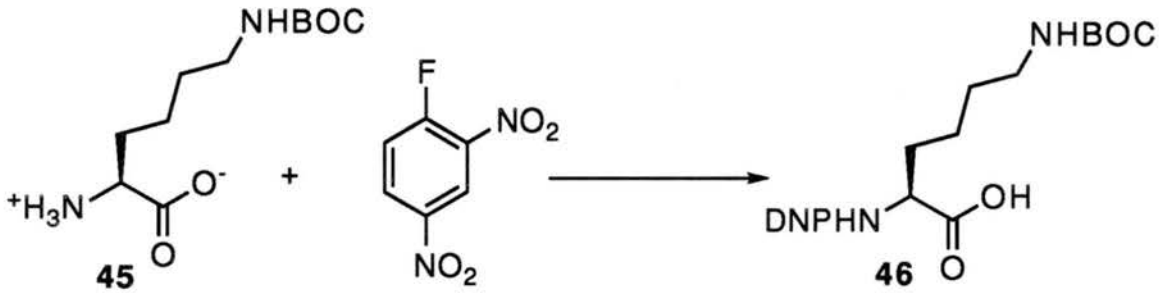
53 mg(.0996 mmol) of **42** was dissolved in 3 mL MeOH, 3 mL 1N NaOH was added, and the reaction stirred at room temperature for 1 hour. It was then acidified to pH 3 and extracted twice with 15 mL Et_2O, dried with $MgSO_4$, and concentrated in vacuo to give 39 mg of a yellow semi-solid. This material was then chromatographed on silica gel with 95/4/1 CH_2Cl_2/MeOH/AcOH, to give 26 mg of **43** as a yellow solid R_f=.14, (50% yield). ^1H NMR (270MHz, $CDCl_3$). δ1.38 (d,3H), 1.2-1.49 (m, 4H), 1.9 (m, 2H), 4.12 (m, 1H), 4.61 (m, 1H), 5.05 (s,2H), 6.80 (m, 1H), 7.31 (s, 5H), 8.34 (m, 1H), 8.72 (m,1H), 9.18 (s, 1H).



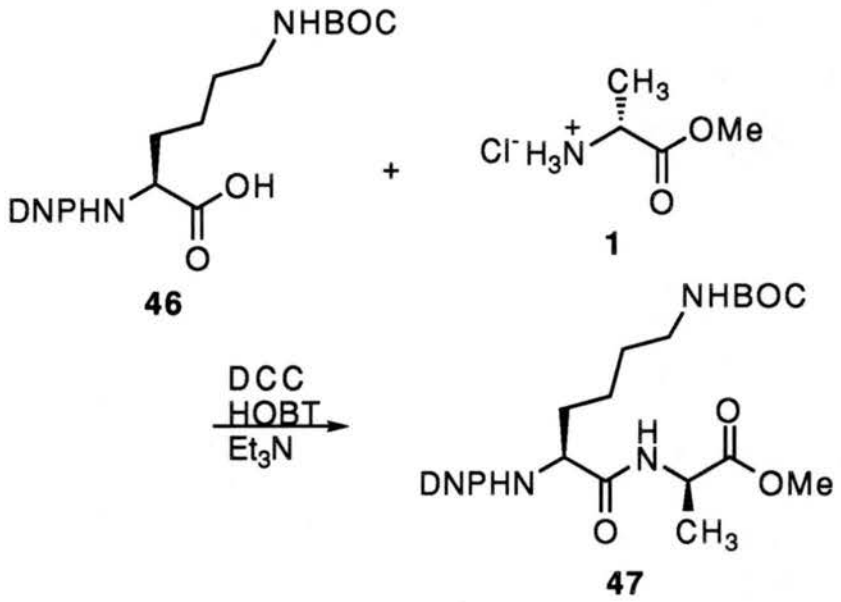
26 mg (.05mmol) of **43**, 8 mg(.055 mmol) of **1**, 11mg (.055mmol) DCC, and 8 mg (.055 mmol) of HOBT were dissolved in 1.5 mL DMF. 8μL of Et_3N was added and the mixture was stirred overnight under N_2 at room temperature. The mixture was then cooled and the DCU was filtered off. The filtrate was dissolved in 15 mL CH_2Cl_2, washed with 5% HCl, dried with $MgSO_4$, and concentrated in vacuo to give 38 mg of yellow solid. The solid was then chromatographed on silica gel with 95/4/1 CH_2Cl_2/MeOH/AcOH to give 27 mg of **44** as a yellow solid, R_f= .48 (90% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.36 (t, 6H), 1.52 (m, 4H), 1.96 (m, 2H), 3.19 (m, 2H), 3.73 (s, 3H), 4.09 (m, 1H), 4.50 (m, 2H), 4.92 (m, 1H), 5.07 (s, 2H), 6.40 (d, 1H), 6.75 (d, 1H), 6.85 (d,1H), 7.32 (s, 5H), 8.23 (dd, 1H), 9.13 (d, 1H).



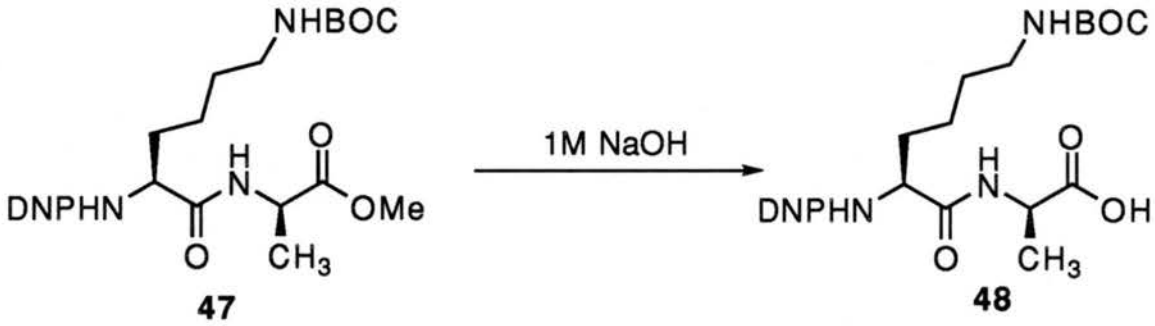
130 mg (.342 mmol) of **29** was dissolved in 12 mL MeOH. 36 mg (.017mmol) of 5% Pd on carbon was added and the flask was fitted with a hydrogen balloon and stirred for three hours at room temperature. The catalyst was filtered off through celite, the solvent removed in vacuo, and 108 mg of **45** was recovered as a white solid (100% yield). ^{1}H NMR (270MHz, D_2O) δ1.45 (s, 9H), 1.45-1.57 (m, 4H), 1.88 (m, 2H), 3.10 (t, 2H), 3.73 (t, 1H).



300 mg (1.22 mmol)of **45** was dissolved in 8 mL 10% $NaHCO_3$ and 3mL 1N NaOH. fluoro-2,4-dinitrobenzene (FDNB) was dissolved in 3 mL EtOH and added to the aqueous solution. After one hour, the excess FDNB was removed by extracting with 30 mL ether. The aqueous layer was carefully acidified to pH 3 and the product was extracted out with 3X 30 mL Et_2O. The ether layer was dried with $MgSO_4$, and concentrated in vacuo to give 433 mg of yellow solid **46** (86% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.45 (m, 13H), 2.03 (m, 2H), 3.13 (m, 2H), 4.35 (m, 1H), 6.85 (d, 1H), 8.25 (d, 1H) 9.11 (s, 1H).

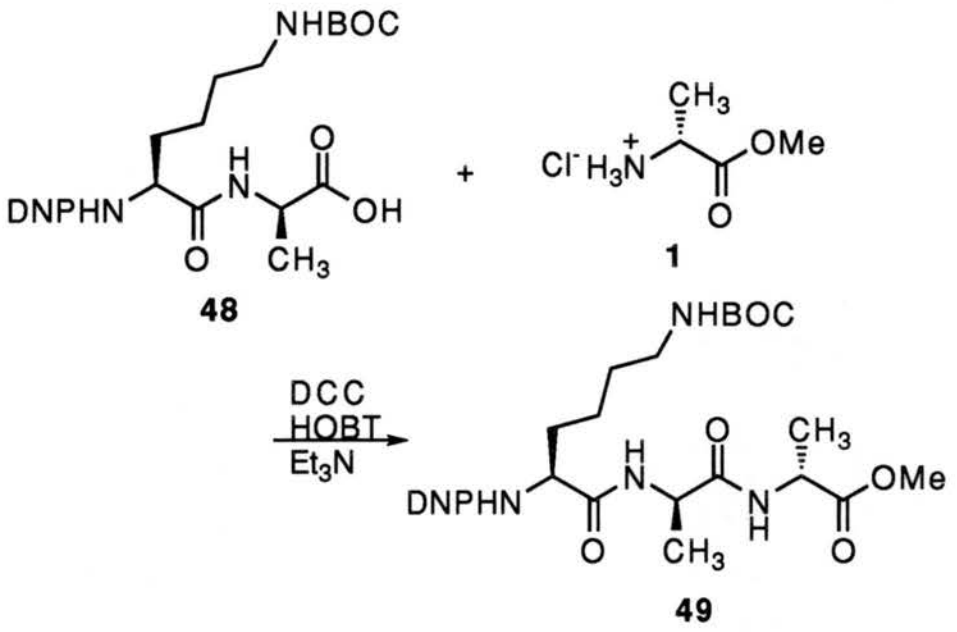


461mg (1.12mmol) of **46**, 171mg (1.23mmol),of **1**, 166mg (1.23mmol) of HOBT, and 253mg (1.23mmol) of DCC were dissolved in 10 mL DMF. 171μL (1.23mmol) of Et_3N was added and the reaction stirred overnight at room temperature under N_2. The DCU was filtered off and the filtrate was diluted with 25 mL CH_2Cl_2, washed twice with 20 mL 5% HCl, concentrated in vacuo to give a solid which was chromatographed on silica gel with 9/1 CH_2Cl_2/MeOH. 580 mg of **47** was isolated as a yellow solid, R_f= .39, (100% yield). ^1H NMR (270 MHz, $CDCl_3$) δ1.33 (d, 3H, j=7.05), 1.36 (s, 9H), 1.49 (m, 4H), 1.90 (m, 2H), 3.06 (m, 2H), 4.20 (m, 1H), 4.51 (m, 1H), 4.73 (m, 1H), 6.85 (d, 1H), 7.35 (d, 1H), 8.20 (dd, 1H), 8.76 (d, 1H) 9.04 (d, 1H).

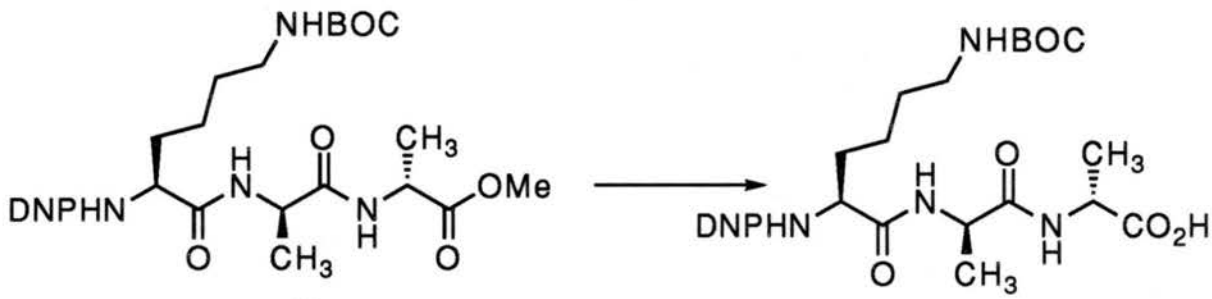


47 **48**

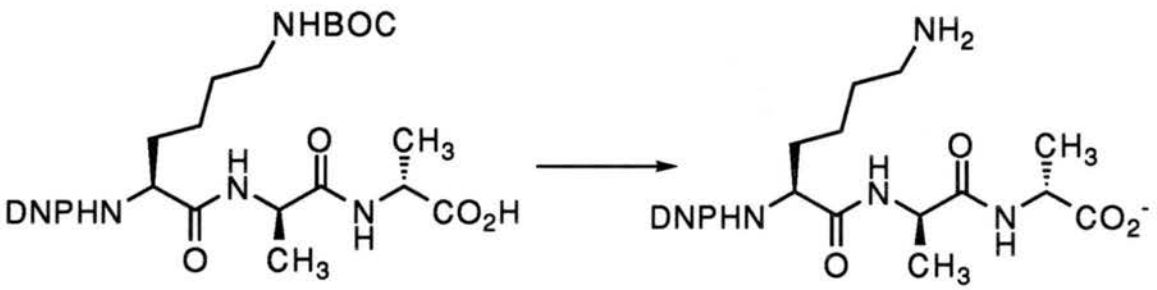
580mg (1.167mmol) of **47** was dissolved in 5 mL of MeOH and cooled to 0°C. 3 mL 1N NaOH was added. After one hour the mixture was diluted with water, acidified to pH 3, and extracted with 3X 30 mL ether. This was dried with $MgSO_4$, and concentrated in vacuo, to give 405 mg of **48** (72% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.4 (m, 16H), 1.98 (m, 2H), 3.07 (m, 2H), 4.30 (m, 1H), 4.57 (m, 1H), 6.88 (d, 1H), 7.47 (d, 1H), 8.80 (d, 1H), 8.95 (s, 1H).



100mg (.207 mmol) of **48**, 32mg (.228mmol) of **1**, 31mg (.228mmol) of HOBT, and 47mg (.228 mmol) of DCC were dissolved in 3 mL DMF. 32μL (.228mmol) of Et_3N was added and the reaction stirred overnight at room temperature under N_2. The DCU was filtered off, and the filtrate was diluted with 10 mL CH_2Cl_2, washed with 15 mL of 5% HCl, dried with $MgSO_4$, and concentrated in vacuo to give 167 mg of a yellow solid. This material was chromatographed on silica gel with 97:3 CH_2Cl_2/MeOH to give 90 mg of **49** as a yellow solid (77% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.34 (d, 3H), 1.41 (m, 12H), 1.5 (m, 2H), 1.58 (m, 2H), 1.98 (m, 2H), 3.12 (m, 2H), 3.75 (s, 3H), 4.14 (m, 1H), 4.50 (m, 2H), 4.64 (m, 1H), 6.47 (d, 1H), 6.78 (d, 1H), 6.93 (d, 1H), 8.27 (dd, 1H), 8.78 (d, 1H), 9.13 (d, 1H).

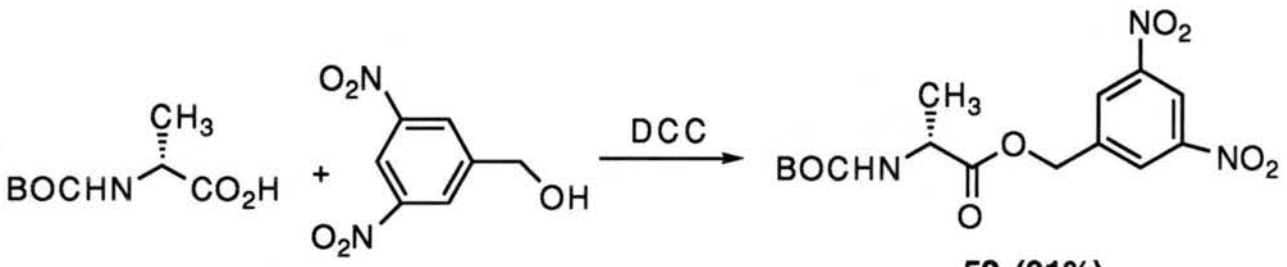


90mg (.158 mmol) of **49** was dissolved in 3 mL MeOH, 1 mL 1N NaOH was added. The mixture was stirred for 1 hour at room temperature. The mixture was washed with Et_2O, acidified to pH 3 with 5% HCl, and the product was extracted twice with 20 mL ether, dried with $MgSO_4$, and concentrated in vacuo to give 73 mg of a yellow solid **50** (83% yield). ^1H NMR (270 MHz, D_2O) δ1.33 (d, 3H, j=7.03 Hz), 1.38 (d, 3H, j=7.04 Hz), 1.56 (m, 2H), 1.78 (m, 2H), 2.09 (m, 2H), 3.05 (m, 2H), 4.13 (m,1H), 4.42 (m, 2H), 6.94 (d, 1H), 8.34 (d, 1H), 9.09 (s, 1H).



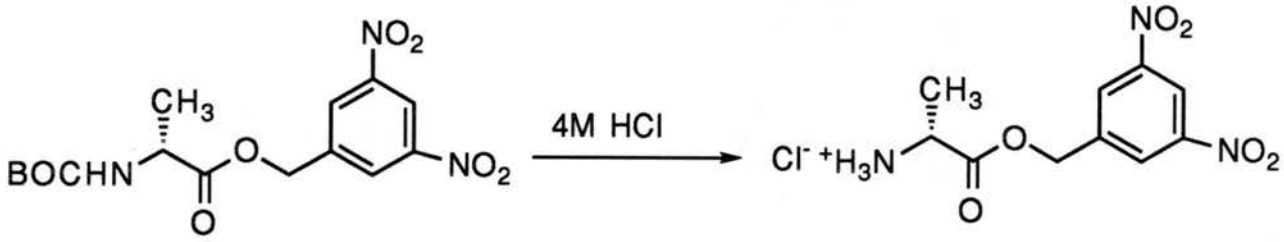
50 **51**

73mg (.132 mmol) of **50** was dissolved in CH_2Cl_2, and cooled to 0°C. 1 mL Trifluoroacetic acid (TFA) was added and the reaction was stirred for 2 hours, allowing it to come slowly to room temperature. The mixture was concentrated in vacuo then redissolved in 3 mL CH_2Cl_2. 500 uL of Et_3N was added to neutralize any remaining TFA and the solution was extracted with H_2O. This was added to a column of Dowex ion-exchange resin (200 mesh), which was washed with water. The product was eluted off with 5 % NH_4OH, and the fractions which stained red with ninhydrin were combined and lyophilized to give 42 mg of **51** as a golden solid (70% yield). ^1H NMR (270MHz, D_2O) δ1.33 (d,3H, j=7.03 Hz), 1.38 (d,3H, j=7.04 Hz), 1.58 (m,2H), 1.78 (m,2H), 2.09 (m,2H), 3.05 (t,2H), 4.13(q,1H), 4.42 (m,2H), 6.95 (d,1H), 8.34 (d,1H), 9.09 (s,1H).



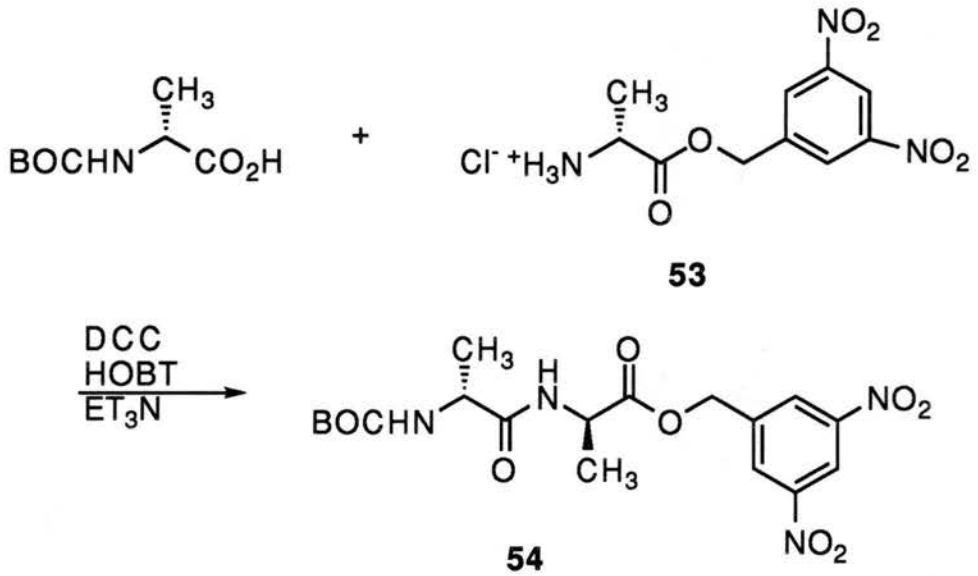
52 (91%)

200mg (1.06mmol) of tBOC-D-ala was combined with 231mg (1.17mmol) of 3,5-dinitrobenzyl alcohol, 241mg (1.17 mmol) of dicyclohexylcarbodiiimide (DCC), and 16 mg (.106mmol) of pyrrolidinopyridine. The mixture was dissolved in 5 mL CH_2Cl_2 and stirred overnight under argon. The DCU was filtered off, and the solution was diluted to 20mL with CH_2Cl_2. It was then washed with 20 mL H_2O, 20 mL 5% AcOH, and 20 mL H_2O. After drying with $MgSO_4$, it was concentrated to give 400mg of grainy yellow solid. This was chromatographed on silica gel with 95/5 CH_2Cl_2/MeOH to give 355 mg of **52,** R_f=.83 (91% yield) (rf=.83). ^1H NMR (270MHz, $CDCl_3$) δ1.40 (s, 9H), 1.43 (d,3H), 4.36 (m, 1H), 4.98 (d, 1H), 5.34 (s, 1H), 8.54 (s, 2H), 8.98 (s, 1H).

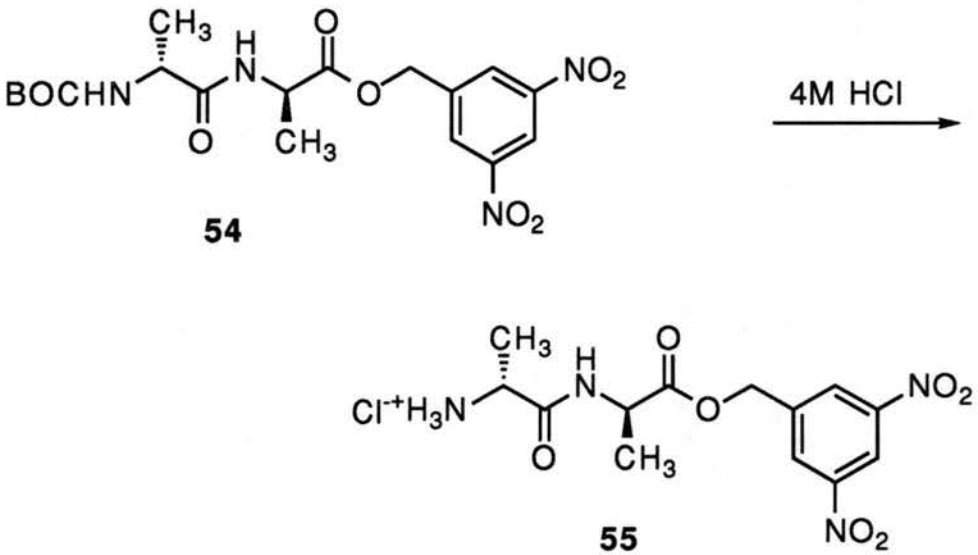


52 **53**

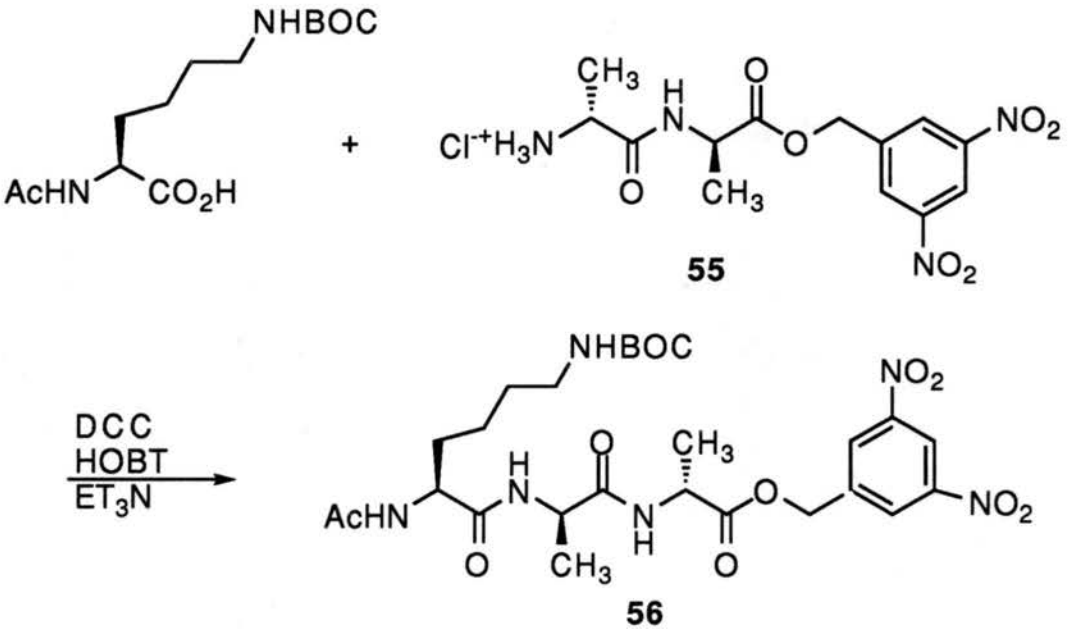
350mg (.972 mmol) of **52** was dissolved in 7 ml of a 4M solution of HCl in dioxane. After stirring for one hour at room temperature the solution was concentrated to give 276 mg of **53** (100%). ^{1}H NMR (270 MHz,D_20) δ1.64 (d, 3H, j=7.03), 4.35 (m, 1H), 5.54 (s, 2H), 8.74 (s, 2H), 9.11 (s, 1H).



156 mg (.823 mmol) of tBOC-D-alanine, 276mg (.905 mmol) of **53**, 126mg (.905 mmol) of HOBT, and 186mg (.905 mmol) of DCC were dissolved in 5 mL of DMF. 126μL (.905 mmol) of Et_3N was added and the solution was stirred overnight under Argon. The DCU was filtered off and the filtrate was diluted with CH_2Cl_2 to 20mL. It was then washed with 20 mL of 0.1N HCl twice, dried with $MgSO_4$, and concentrated to get 292 mg of yellow solid. This solid was then chromatographed on silica gel eluting with a 10% methanol in methylene chloride solvent to give 190 mg of **54,** R_f=.50, (52% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.30 (d, 3H, j=7.05), 1.37 (s, 9H), 1.41 (d, 3H, j=7.06), 4.17 (m, 1H), 4.56 (m, 1H), 5.13 (d, 1H, j=7.47), 5.32 (s, 2H), 8.50 (d, 2H, j=2.08), 8.94 (s, 1H).



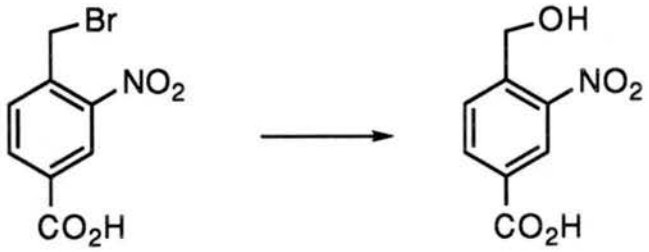
190mg (.432mmol) of **54** were dissolved in 5mL of a 4M HCl solution in dioxane. After stirring at for 2 hours at room temperature the mixture was concentrated in vacuo to give 172 mg of **55** (100% yield) ^{1}NMR (270MHz, D_2O) δ1.51 (d, 6H, j= 7.62), 4.10 (m, 1H), 4.59 (m, 1H) 5.45 (s, 2H) 8.69 (s, 2H), 9.07 (s, 1H).



70mg (.241 mmol) of α-acetyl-ε-tBOC-L-lysine, 100mg (.266 mmol) of **55**, 37mg (.266mmol) of HOBT, and 55mg (.266 mmol) of DCC were dissolved in 2 mL DMF under Ar. 40μL (.266mmol) of Et_3N was added and the solution was stirred overnight. The DCU was filtered off, and the residue was concentrated in vacuo to give 224 mg of a brown sticky solid. This was chromatographed on silica gel with 5% MeOH in methylene chloride to give 70 mg of **56** as a yellow foamy solid, R_f=.21, (47% yield). ^1H NMR (270MHz, $CDCl_3$) δ1.37 (d, 3H, j=7.06), 1.40 (s, 9H), 1.43 (d, 3H, j= 7.47), 1.35-1.45 (m, 4H) 1.55-1.82 (m, 2H), 1.98 (s, 3H), 4.26 (m, 1H), 4.52 (m, 2H), 4.74 (m, 1H), 5.31 (s, 2H), 6.70 (d, 1H), 6.94 (d, 1H), 7.28 (d, 1H), 8.53 (d, 2H, j=1.66), 8.96 (d,1H,j=1.25).

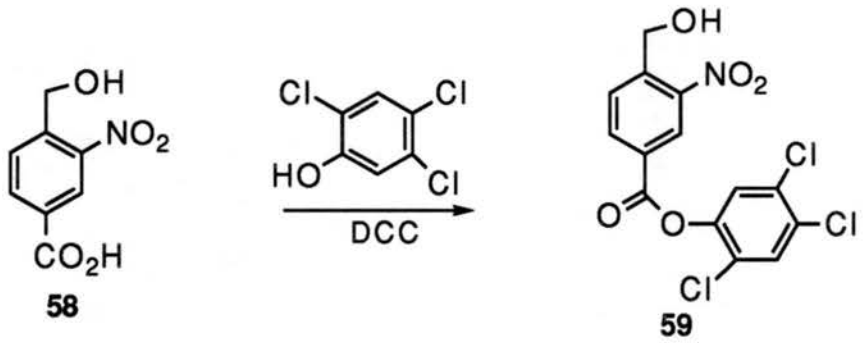
57

5 mL of 90% HNO_3 was cooled to -15°C with a dry ice/glycerol bath. 125mg (.58 mmol) of **1** was added every fifteen minutes, four times until a total of 500mg (2.32 mmol) was added. After 2 hours, the reaction was done by TLC. The mixture was poured over ice and a white solid precipitated out of solution. It was washed repeatedly with ice cold water and dried overnight under vacuum to give 530 mg of **57** (83% yield), mp= 121-124°C, (lit=125-126°C). ^1H NMR (270MHz, $CDCl_3$) δ4.85 (s, 2H), 7.73 (d, 1H, j= 7.88 hz), 8.31 (dd, 1H, j=1.66 hz, 8.30 hz), 8.73 (d,1H, j=1.66 hz).

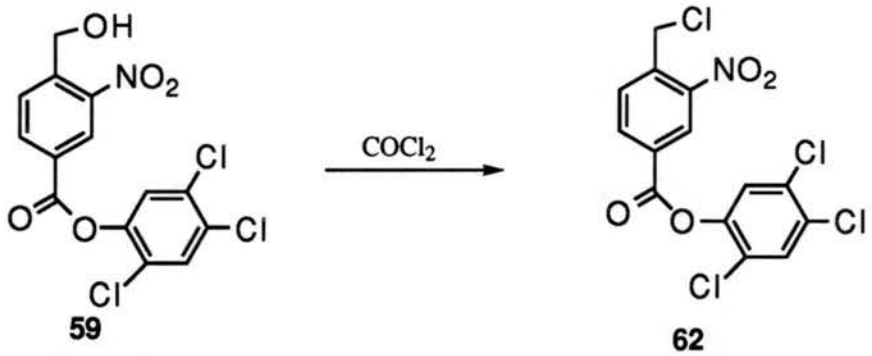


57 **58**

200mg (.769 mmol) of **57** was dissolved in H_2O, then 20 mg of KI was added. The mixture was brought to reflux and allowed to reflux overnight. An insoluble orange solid had collected around the edge of the flask. This was filtered off and the filtrate was cooled to 4°C overnight. Long yellow crystals formed and were collected and dried to give 98 mg of **58** (61% yield), mp =155-159°C, (lit=154-156°C). ^1H NMR (270MHz, D_6-acetone) δ5.11 (s, 2H), 8.10 (d, 1H, j=8.3hz), 8.35 (dd, 1H, j=1.46 hz, 8.3hz), 8.61 (d, 1H, j=1.96).

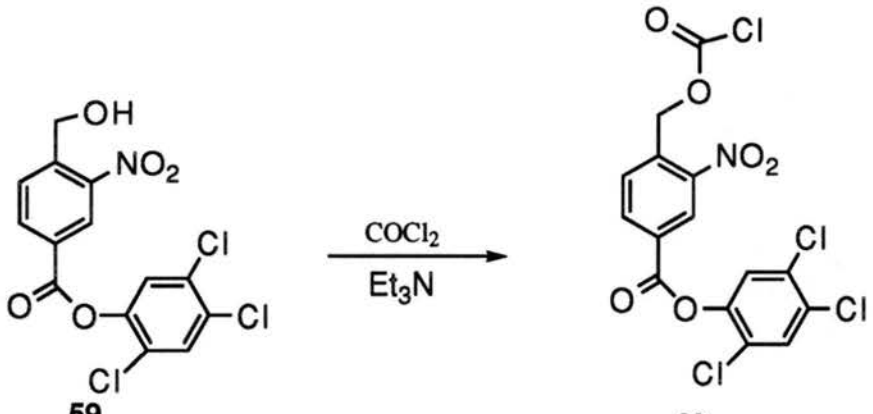


98mg (.497mmol) of 2,4,5-trichlorophenol, and 103mg (50mmol) of DCC were dissolved in 2 mL of CH_2Cl_2. This mixture was added to a suspension of 98mg (.497 mmol) of **58** in 5 mL of CH_2Cl_2. The reaction was stirred at RT overnight under a N_2 atmosphere. The solution was cooled to -15°C for one hour to precipitate the DCU, which was filtered off. The solvent was removed in vacuo to give 94 mg of yellow powder. This was chromatographed on silica gel with 95/5 CH_2Cl_2/MeOH. 57 mg of material was obtained which was further chromatographed by PTLC in 2/1 Hexane/EtOAc to give 12 mg of pure **59**. ^1H NMR (270MHz, $CDCl_3$), δ5.12 (s, 2H), d7.44 (s, 1H), 7616 (s, 1H), 8.04 (d, 1H, j=8.30 hz), 8.46 (dd,1H, j=1.66 hz, 8.30 hz), 8.88 (d, 1H, j=1.66).

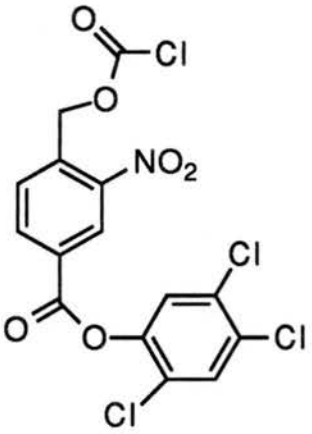


10mg (.0267mmol) of **59** was placed in 5 mL toluene under N_2. 83μL (.16mmol) of phosgene solution (1.93M in toluene) was added. After 2 hours, there was no sign of the desired chloroformate by NMR (The benzyl methylene group should shift >.5ppm downfield) but a new product had formed by TLC. The reaction was flushed with nitrogen for one hour and concentrated in vacuo to give 10 mg of a yellow solid **62**. ^1H NMR ($CDCl_3$, 270MHz) δ5.05(s, 2H), 7.44 (s, 1H), 7.62 (s, 1H), 7.93 (d, j=8.30Hz, 1H), 8.43(dd,j=1.66, 7.88, 1H), 8.83 (d, j=2.06Hz, 1H). Mass spec (EI): M=392.913. For tetrachlorinated compounds, there should be an M+2, M+4, M+6 and M+8 peak.

abundance as % of M peak:	M+2	M+4	M+6	M+8
trichloro compound(calculated):	97.8%	31.9%	3.47%	
terachloro compound(calculated):	131.0%	63.9%	14.0%	1.15%
actual found:	129.8%	64.3%	13.6%	0

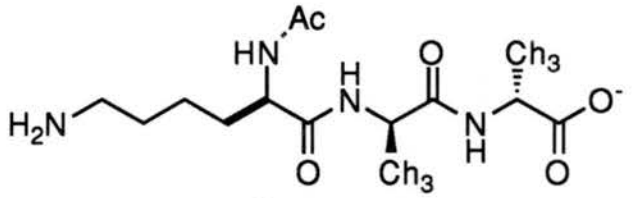


10mg (26.7μmol) of **59** was dissolved in freshly distilled toluene, and 8μL(53.4μmol) of Et3N was added. 83μl of a 2M phosgene solution in toluene(160μmol) was added and the reaction was stirred at room temperature for two hours. Nitrogen gas was bubbled through the mixture for one hour to purge the premaining phosgene, and the mixture was then concentrated in vacuo. A crude NMR showed the benzyl methylene peak had shifted from 5.1ppm in the starting material to 5.8, indicating the chloroformate **60** had formed. The residue was dissolved in dry toluene and carried on to the next step without isolation. ^{1}H NMR (270MHz, $CDCl_3$) δ5.80(s, 2H), 7.42(s, 1H), 7.59(s, 1H), 7.83 (d,j=8.3Hz, 1H), 8.46(dd, j=1.66, 7.88, 1H), 8.93(d, j=1.66Hz, 1H).

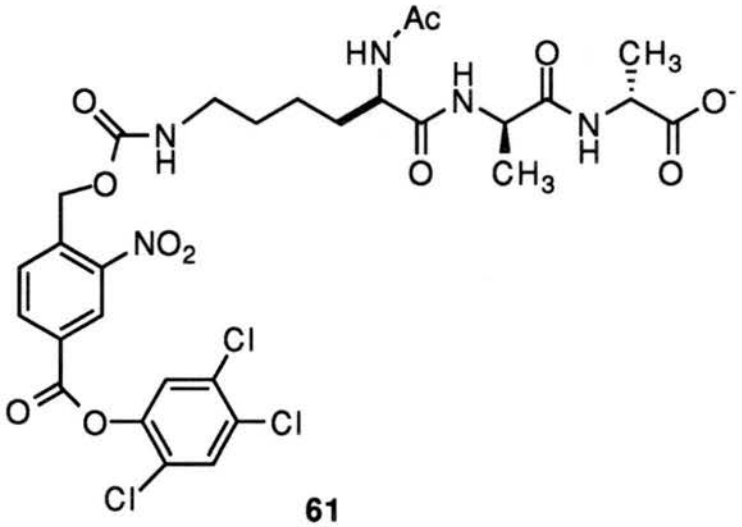


60

+



22



61

26.7umol of **60** was dissolved in 2 mL of CH_2Cl_2 was combined with 10mg of **22** dissolved in 3mL of pH 9.2 carbonate buffer and the reaction was stirred for four hours at RT. The aqueous layer was then acidified(without separating from the organic) and stirred for another 20 minutes. The organic layer was then concentrated in vacuo to give 14 mg of yellow solid which was chromatographed by TLC to give 10 mg of **61**. ^1H NMR (270MHz,$CDCl_3$) δ1.37 (d, 3H, j=7.06), 1.43 (d, 3H, j= 7.47), 1.35-1.45 (m, 4H) 1.55-1.82 (m, 2H), 1.98 (s, 3H), 4.26 (m, 1H), 4.52 (m, 2H), 4.74 (m, 1H), 6.47 (m, 1H), 5.21(s, 2H), 7.41(s, 1H), 7.53(s, 1H), 7.81 (d,j=8.3Hz, 1H), 8.44(dd, j=1.66, 7.88, 1H), 8.92(d, j=1.66Hz, 1H).

II. BIOLOGY

All aqueous biological solutions were prepared with distilled deionized water. Agarose for electrophoresis was obtained from FMC corporation. Urea, acrylamide, phenol, PEG,tryptone, yeast extract and tris were obtained from the Sigma chemical company. Cells were obtained from ATCC or donated as designated herein. Sanger sequencing reactions were prepared using the Sequenase kit. α-^{35}S-dATP was obtained from New England Nuclear Corporation. Agarose for culture plates was obtained from Difco.

Minigels were performed on a Hoefer apparatus. Sanger sequencing gels were performed on a Biorad electrophoresis apparatus. Gels were developed using Kodak X-ray film, developer and fixer.Electroporations were performed with a Biorad electroporator. ELISA plates were read on a Molecular Dynamics microtiter plate reader. HPLC was performed on a waters chromatographic system witha microbond-pak C-18 reverse phase analytical column.

PAGE Purification of Oligonucleotides

Example: Oligo A3- primer used for sequencing.

sequence = 5'-CGATCTAAAGTTTTGTCGTCT-3' corresponds to 1785-1805 on the fd/tet-apamin hybrid genome.

1. Mark Flanagan synthesized the above oligonucleotide on the DNA synthesizer on a 1μmole scale. The crude DNA solution was separated into four Eppendorf tubes and lyophilized to give a white powder.

2. The solid from one tube was first dissolved in 100μL sterile H_2O, then 100 μL of formamide was added. The tube was heated to 55°C for five minutes.

3. 100 μL of the above solution was loaded into each of two wells of a 20% polyacrylamide-urea gel. A solution of the dyes cyanol and bromophenol blue were loaded in an adjacent lane.

4. The gel was run at a current of 800 volts for four hours.

5. The gel was transferred onto Saran wrap and the DNA bands were located halfway between the two blue dyes by visualizing with a hand held UV light. The gel bands were excised with a razor blade.

6. The gel pieces were added to a 15 mL sterile tube and crushed. 2mL of elution buffer was added, and the mixture was gently shaken overnight at 37°C.

7. The tube was centrifuged to pellet the gel pieces in the bottom of the tube. The supernatant was then passed through a .45 micron filter(Millipore).

8. The solution was then passed through a C-18 Sep-pak column, and the column was washed twice with 10mL H_2O.

9. The oligo was eluted off the column with three1 mL aliquots of 60:40 MeOH/H_2O. Optical density readings showed most of the DNA to be in the first elution.

10. The solvent was removed on the Speedvac, and the pellet was taken up in 50μL H_2O.

The OD reading of .463 translates to about 440pm/μL of purified DNA.

Phage Preparation by Infection and Amplification of K91 cells-Liquid Method

K91 E. coli cells are stored on M9 minimal media plates at 4°C. This ensures the presence of the F'pilus because the F'pilus gene is linked to the proline biosynthesis gene. Growth in a minimal media such as M9 neccessitates that the cells make their own proline, and thus the F' pilus will also be produced under these conditions.

1. A single colony of K91 on an M9 plate is transferred to 3mL LB broth and grown overnight at 37°C with shaking.

2. The 3mL overnight culture is transferred to 30mL fresh LB and shaken vigorously at 200 rpm for 1-2 hrs. OD_{280} readings should be between 0.60 -0.80 for mid log phase cells to give maximum infectivity rates.

3. The volume of phage to be amplified is combined with twice the volume of mid log phase K91 cells in an eppendorf tube (typically 400μL phage + 800μL cells). The tube is incubated for 30 minutes at 37°C.

4. This solution is then added to 30mL of fresh LB broth and shaken vigorously for fifteen minutes at 37°C. Tetracycline is then added to give a concentration of 18ug/mL, and the culture is shaken overnight, usually 12-18 hours.

5. The supernatant is cleared by three centrifugations at 15,000 rpm for 20 minutes.

6. A 0.2 volume of 16.7% PEG in 3.3M NaCl is added and the solution is thoroughly mixed and stored at 4°C for at least four hours, usually overnight.

7. The phage are precipitated from this solution by centrifugation for one hour at 30,000 rpm at 4°C.

8. The supernatant is poured off, and the tube is carefully wiped to remove any remaining PEG, and the phage pellet is dissolved in the desired volume of PBS (200μL-4mL).

Amplification of Phage via the Agarose Plate Method

1. 3mL of an overnight culture of K91 cells in LB is poured into 30 mL of fresh LB broth and shaken at 220rpm for 1-2hours at 37°C. The OD_{600} readings should be between 0.2 and 0.4 OD units.

2. The phage to be amplified (usually 100-800μL) is combined with an equal volume of fresh K91 cells as prepared above, and incubated for 30 minutes at 37°C.

3. 250 μL of the infected cell mixture is spread onto LB/tet plates and allowed to dry for 30 minutes, then incubated overnight at 37°C.

4. 5mL of LB broth is added to the surface of each plate (now containing a bacterial lawn). The plates are allowed to sit undisturbed for 15 minutes at room temperature.

5. The plates are then gently scraped with a glass rod spreader, and the supernatant is collected into a sterile centrifuge tube.

6. The tubes are centrifuged twice at 12,000 rpm for 15 minutes to clear the supernatant.

7. A 0.2 volume of 16.7% PEG in 4M NaCL is added to each supernatant and the solution is stored for a minimum of four hours at 4°C.

8. The phage are then precipitated by centrifuging at 30,000 rpm for one hour at 4°C.

9. The supernatant is poured off, and the walls of the tube are wiped free of any residual PEG solution.

10. The phage pellets are dissolved in the desired amount of PBS.

Isolation of Single Stranded DNA from Phage

1. The phage solution(usually 100-500μL in PBS) is placed in an Eppendorf tube and an equal volume of phenol is added. The tube is vortexed for 30 seconds, then centrifuged to separate the two layers. Most of the lower phenol layer is removed with a pasteur pipet, then the tube is centrifuged again to drive the white interfacial material to the bottom of the tube. The upper layer is removed and placed in a clean tube. Sometimes this step is repeated a second time.

2. An equal volume of phenol/chloroform (buffered at pH 8 with Tris) is added and the solution is again vortexed and centrifuged, and the layers are separated as above, transferrring the upper layer to a new tube.

3. An equal volume of 24/1 chloroform/isoamyl acetate is added and the solution is vortexed and centrifuged, and the upper layer is carefully removed. The DNA can now either be precipitated-method A, or concentrated-method B shown below.

Method A-Ethanol Precipitation

1. In an eppendorf tube, 1/10 volume of 4M LiCl is added to the aqueous DNA , then 2 volumes of absolute ethanol. The solution is mixed thouroughly, and cooled to -80°C for an hour.

2. The DNA is then pelleted by microfuging at maximum speed for 30 minutes at 4°C.

3. The supernatant is carefully decanted off, then cold 70% ethanol, equal to the volume of the original DNA solution started with in step 1, is added, swirled gently, and remicrofuged for 5 minutes at maximum speed.

4. The pellet is allowed to either air dry (>1hour) or dried under vacuum, and the DNA can then be dissolved in the desired volume of TE.

Method B- Microconcentration

1. The DNA solution is placed in the filtration cup of a Microcon-30 and centrifuged on the microfuge at a speed of 8,000 rpm for 3 minutes. The DNA adheres to the surface of the filtration membrane.

2. The filtration cup is inverted and placed over a new eppendorf tube, and the desired amount of TE is added. The device is then centrifuged for 10 at 3000 rpm. The DNA is now in the retentate with the desired volume.

Agarose Gel Electrophoresis = Minigel

1. 0.4g of agarose and 50 mL of TAE buffer is placed in a 125 mL Erlenmeyer flask and heated to just boiling in the microwave to dissolve the gel. This may be done several times to ensure complete dissolution. The solution is allowed to cool to 75°C and 2μL of 10mg/ml ethidium bromide is added and the warm solution is poured into a Hoefer minigel apparatus. A comb is inserted to create wells. The gel polymerizes within an hour and 450 mL TAE is poured over it. This is the standard 0.8% gel used as a minigel unless otherwise noted.

2. A bromophenol blue dye in 20% glucose is usually added to the DNA placed in each well. The dye serves as a visual marker, and the glucose weights the solution so it remains in the well and doesn't diffuse out into the buffer.

3. The gel is usually run at room temperature at 55 volts for varied lengths of time unless otherwise noted.

Purification of DNA by Low Melt Agarose Gel

1. The gel is prepared by dissolving 2.4g of low melt agarose gel in 500mL TAE buffer. 15uL of 10mg/mL ethidium bromide is added and the solution is poured into a large horizontal gel apparatus and ftted with a large comb for polymerization.

2. A 0.1 volume of bromophenol blue dye in 20% glucose is added to the crude DNA sample.

3. The gel is run at 75 volts at 4°C for 2-4 hours or until the desired band is clearly separable from the undesired bands.

4. The desired band is visualized by a hand held UV light and excised with a razor blade.

5. The gel pieces are placed in a 50mL Pyrex tube and heated to 70°C for about 15 minutes, or until the gel has completely melted(clears).

6. 2 volumes of 0.3M NaCl is added to the melted gel solution and further heated for 10 min, then shaken for 10 minutes at 37°C.

7. The melted agarose is extracted with an equal volume of phenol, shaking vigorously, then centrifuging to separate the layers.

8. The DNA is precipitated from the aqueous layer by adding two volumes of absolute ethanol and chilling for an hour, then centrifuging and washing with cold 70% EtOH.

9. The DNA pellet is dried and taken up in the desired amount of TE buffer.

Electroelution of DNA from Agarose Gel

1. The crude DNA is placed in the well of a 1.5% agarose gel and run for 2-3 hours at 55 volts.

2. The desired band is excised from the gel with a scalpel.

3. The band is placed in the trough of an electroeluter which contains TAE buffer, and the current is rum at 300V for 1 1/2 hours so the DNA migrates onto to the furthest of two membranes.

4. The current is reversed and run for 30 seconds at 400V to pull the DNA off the membrane. The solution between the two membranes is removed.

5. The aqueous solution is extracted three times with an equal volume of butanol. This reduces the aqueous volume.

6. The DNA soltuion is then ethanol precipitated or concentrated using the amicon microconcentrator.

Single Stranded Sanger Sequencing of DNA

1. The polyacrylamide gel is prepared by combining 63g of urea, 15mL of 40% acrylamide solution, 45 mL TBE, and 220mg of ammonium persulfate. The solution is stirred until the urea completely dissolves. It is then filtered through a .45 micron filter and 30 uL of TE is added and the solution is poured between two glass plates and polymerizes.

2. The sequencing reaction is begun by combining 7μL of ssDNA with 2μL of sequencing buffer and 1μL of primer. The primer is annealed to the ssDNA by heating to 65°C, and cooling slowly to RT, then chilling on ice.

3. While cooling, 2.5μL of each termination mixture -G,A,T and C are filled and labelled.

4. The kit's labelling mixture is diluted 1:5.

5. The Sequenase enzyme (DNA polymerase) is diluted in buffer 1:8.

6. The termination tubes from step 3 are prewarmed to 37°C.

7. To the annealed DNA prepared in step 2, add 1μL of DTT, 2μL of diluted labeling mix (step 4), .5μL of [^{35}S]dATP, and 2μL of the diluted enzyme prepared in step 5. This solution is mixed and incubated at room temperature for 5 minutes.

8. 3.5μL of the above labelling reaction is transferred to each of the four termination tubes (G,A,T andC) and mixed and incubated for another 5 minutes at 37°C.

9. The reaction is stopped by adding 4uL of stop solution from the kit.

10. The samples are heated to 75°C for 2 minutes before loading onto the gel.

11. The gel is run at 1200 volts for 3-5 hours depending on the region of interest to be sequenced.

12. The upper plate is removed form the gel and the lower plate with the gel attached is fixed in a 5%AcOH/ 15%MeOH bath for 30 minutes to remove the urea.

13. The gel is then carefully transferred to filter paper and covered with Saran wrap.

14. The gel is then fixed onto the filter paper by placing in the gel dryer at 80°C for 45 minutes.

15. The Saran wrap is removed and the gel is placed in an XRay cassette with a piece of XRay film for 12-48 hours, depending on the activity of the ^{35}S.

16. The film is developed by dipping in Kodak developer for 2 minutes, rinsed, and fixed by submerging in Kodak fixer solution for 1 minute.

Phage Titering by Plaque Formation

1. Prepare 100 fold serial dilutions of the phage solution to be titered. This is usually done by dissolving 10 µL of the phage solution into 990 µL of PBS. Five dilutions are sufficient: 10^2-10^{10}

2. Combine 100µL of each phage dilution with 400µL of overnight K91 cells, and add to 3mL of warm soft agar (47-50°C).

3. Immediately pour the agar solution over LB plates, and let set at room temperature for 30 minutes.

4. Incubate overnight at 37°C.

5. Count plaques to determine original phage concentration.

6. Example of typical results:

plate:	10^2	10^4	10^6	10^8
# of plaques:	TMTC	TMTC	52	2

use 52 x 10^6 plaques per 100µL = 5.2 x 10^8 pfu per mL

pfu = plaque forming unit

TMTC= Too many to count

Phage Titering by Colony Formation

1. Add a 3mL overnight culture of K91 to 30mL LB, shake at 150-200 rpm at 37°C for 1-2 hrs.

2. Prepare 100-fold serial dilutions of the phage solution to be titered. Usually 10^2-10^{10}.

3. Combine 50 uL of diluted phage with 100 uL of log phase K91 in an Eppendorf tube and incubate for 30 minutes.

4. Spread each infected cell solution on LB/tet plates and allow to air dry for 30 minutes to an hour.

5. Incubate the plates overnight at 37°C

6. Count colonies to determine the number of phage in the original solution.

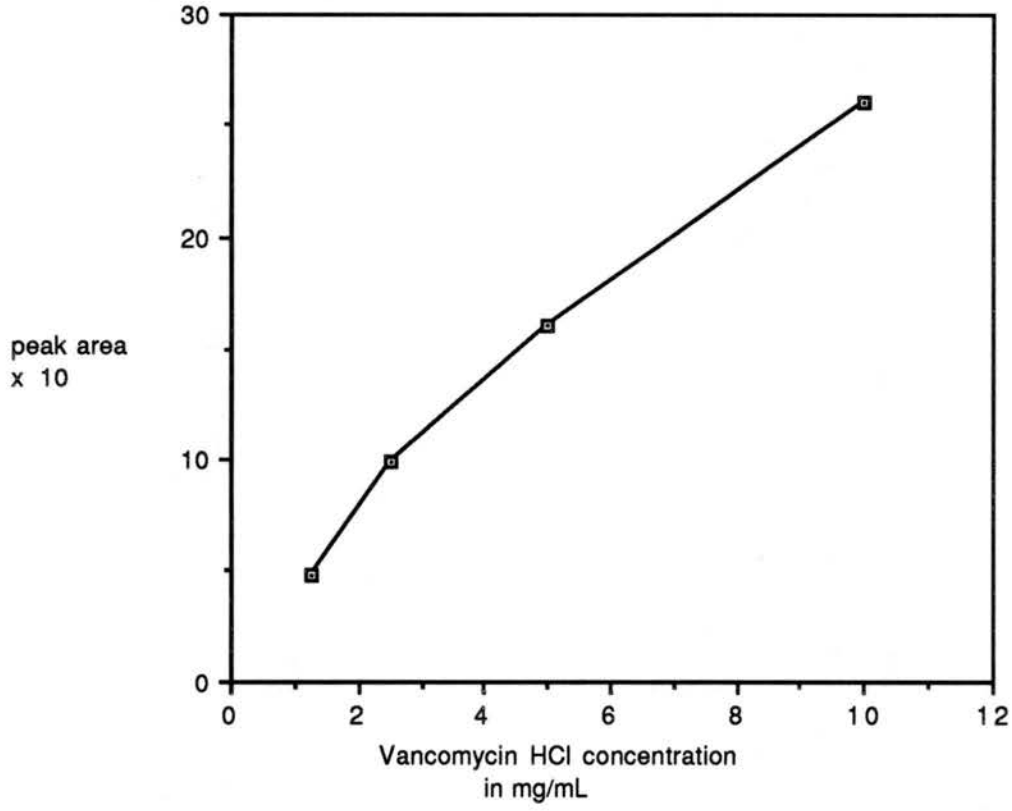
Derivatization of Affigel-10 with CBZ-L-lys-D-ala-D-ala

A solution of CBZ-L-lys-D-ala-D-ala(**16**) in 0.1M K_2HPO_4 (pH 8.2) was prepared. The concentration was 4.9 mg/mL. The OD_{280} was measured to be .575 OD units at this concentration, or 1 OD unit = 8.52 mg/mL.

1mL (15-20 μmol according to manufacturer) of Affigel-10 was washed with H_2O, then 0.1M K_2HPO4 pH 8.2 buffer. The washed Affigel was placed in a sterile tube, and 2mL(20umol or 9.8 mg) of the peptide solution prepared above was added. The tube was shaken at 4°C for 4 hours. The supernatant was removed and the OD_{280} reading was measured to be .176(equivalent to 1.49mg/mL or 3mg in 2 mL). 2 mL of buffer solution was added, and the slurry was shaken for one hour, then stored overnight at 4°C. The supernatant was removed and the OD_{280} was measured to be .045 (= .38 mg/mL, or .76 mg in 2 mL). 2 mL more buffer was added, and after several hours the supernatant was removed, and measured to have an OD_{280} of .006. By subtracting out the peptide removed from the three supernatants, it was calculated that approximately 6 mg or 14μmol of peptide had been coupled to the gel. 100 μL of ethanolamine in 2 mL buffer was added to cap any remaining underviatized sites on the affigel. The gel was stored in buffer at 4°C for further use.

B. Reversible Binding of Vancomycin to Derivatized Affigel-10.

10mg of vancomycin HCl (donated by Eli Lilly corporation) was dissolved in 1 mL 0.1M K_2HPO_4 (pH8.2). From this solution, sequential dilutions were made to give 5, 2.5, and 1.25 mg/mL concentrations. 10 μL of each of these solutions were injected in the HPLC equipped with a C-18 reverse phase column and eluted with 10% CH_3CN/.1% TFA using a flow rate of 2 mL/min, and monitoring absorbance at 254nm. The area of the vancomycin peaks were plotted versus their respective concentrations.



10 mg of vancomycin in 1 mL of buffer was added to 1 mL of derivatized Affigel, and the slurry was agitated at room temperature for 20 minutes. The supernatant was removed and the concentration was measured with HPLC injection and shown to be

5% of the original concentration. 5 mg more vancomycin(in 1 mL buffer) was added to the gel, and after an hour, no vancomycin remained in the solution as measured by HPLC. Again 5 mg vancomycin in 1 mL buffer was added to the gel. The HPLC concentration of vancomycin is measured to be 2 mg/mL after shaking for one hour.. A total of 18mg or (12 μmol) of vancomycin had bound to the Affigel. This measurement approximates the OD readings during the derivatization of the gel to have 14 μmol of derivatized sites available per mL of gel. Excess free peptide was used to sequentially elute the vancomycin off the Affigel.

Panning of the First Library on Derivatized Affigel

1. 5μL of derivatized gel, 10μL of fd/tet-apamin library phage, and 10μL of PBS/EDTA buffer were combined in 75μL H_2O in an ependorf tube and shaken gently on the vortex for 30 minutes at room temperature.

2. The tubes were centrifuged to pellet the gel to the bottom of the tube and the supernatant was removed.

3. The gel was washed four times with buffer by adding PBS/EDTA buffer, agitating for a minute then centrifuging and removing the supernatant.

4. 50 μL of a 0.5mg/mL solution of α-CBZ-L-lys-D-ala-D-ala(**16**) was added to the gel. The tube was agitated for five minutes, and the supernant removed and saved. This step was repeated, and the two supernatants were combined as eluted phage.

5. 100μL of eluted phage were combined with 100μL of log-phase K91 cells and incubated for 15 minutes at 37°C. 10μL of this was removed for serial dilutions and titering. The remainder was added to 25mL LB broth supplemented with tetracycline, conc= 18μg/mL and amplified overnight.

Derivatization of Covalink Plates

A. Fluorodinitrobenzene

1.100 µL of the following dilutions of fluorodinitrobenzene (FDNB) were placed in each column of a 96 well NUNC plate as follows:

1	2	3	4	5	6
1mg/L	10 µg/mL	500 ng/mL	400 ng/mL	350 ng/mL	300ng/ml

7	8	9	10	11	12
250ng/ml	200ng/ml	150ng/ml	100ng/ml	50ng/ml	blank

2. 30µL of 10% $NaHCO_3$ was added and the plate was vortexed overnight on setting #1 at room temperature.

3. The plates were washed as follows:

4 quick rinses with PBS

1 15 minute incubation with PBS at 37°C

2 quick rinses with PBS

4. Wells were blocked with either 1% BSA or .05% Tween 20 in PBS, shaking at room temperature for 1 hour.

5. The wells were washed as in step three, and stored at 4°C in PBS until needed.

B. DNP-L-lys-D-ala-D-ala (**16**)

1.The wells were first treated with100 uL of disuccinimidyl suberate solution (12.5 mg in DMSO/carbonate buffer) for one hour vortexing at room temperature.

2. The wells were rinsed five times with PBS

3. 100μL of tripeptide solution in carbonate buffer was added to each well in the following concentrations:

1	2	3	4	5	6
10ug/mL	1.5ug/mL	1ug/mL	800ng/mL	700ng/mL	600ng/mL
7	8	9	10	11	12
500ng/mL	400ng/ml	300ng/mL	200ng/mL	100ng/ml	blank

4. The plate was shaken overnight at room temperature.

5. The supernatant was removed and the wells were washed with PBS five times.

6. The plates were then blocked by treating with either 1% BSA in PBS, or .05% Tween-20 in PBS for one hour, then rinsing five times with PBS.

ELISA ASSAYS

1. A fresh dilution of rabbit anti-DNP antibody from Sigma Chemical Co.was prepared (1:3000 in PBS) and 100μL was added to each well, and shaked for one hour at room temperature.
2. The wash was performed by rinsing five times quickly with PBS, then one 15 minute incubation at 37°C, followed by two more quick rinses.
3. A 1:5000 dilution in PBS of goat anti-rabbit alkaline phosphatase was prepared, and 100μL was added to each well for one hour.
4. The wash in step 2 was repeated.
5. 100 μL of the substrate *p*-nitrophenyl phosphate (5 mg/mL) was added to each well, and the plate was incubated at 37°C for 30 minutes.
6. 50 μL of stop solution (3M NaOH) was added to each well.
7. The absorbance was measured at 405 nm on an ELISA plate reader.

Derivatization of Dynabeads

1. 50μL of tosyl activated M-450 Dynabeads were placed into each of two tubes.
2. The beads were washed twice with 1mL of ddH_20 using the magnetic particle concentrator (MPC) to adhere the beads to the wall of the tube.
3. Tube 1 was derivatized with 1mL of 150μg/mL of Ac-L-lys-D-ala-D-ala(**22**). Tube 2 was derivatized with 150μg/mL of ethanolamine. The tubes were mixed by slow rotation over a 24 hour period.
4. The supernatant was removed and the beads were washed with PBS buffer by vortexing for one minute, the using the MPC to immobilize the beads while drawing off the wash solution.
5.The beads are stored at 4°C in PBS until used for panning.

Panning on Dynabeads

1. 240μL of unpanned library phage was added to 10μL of Dynabeads in each of two tubes. Tube 1: Dynabeads derivatized with tripeptide **22**, Tube 2: Control Dynabeads derivatized with ethanolamine.

2. The tubes were rotated at room temperature for two hours.

3. The supernatant was removed and saved and each tube was washed twice with PBS for 15 minutes rotation.

4. 8 quick rinses followed by vortexing in PBS for 10 seconds, then removing the supernatant with the MPC.

5. The phage from tube 1 was removed by eluting with tripeptide (0.5mg/mL) and then a 1mg/mL solution of tripeptide. The eluants were amplifed using the plate method and subjected to another round of panning under the same conditions.

Derivatization of ECH Sepharose 4B Beads

1. 2mL of the gel is washed several times over a sintered glass funnel with 0.5M NaCl.
2. 500µl of each gel is aliquotted into each of two tubes.
3. One tube is derivatized with a 2mg/mL solution of Ac-L-lys-d-ala-D-ala(**22**), the other with ethanolamine.
4. 20mg of EDC is added to each tube.
5. The tubes are rotated end over end for 24 hours.
6. The supernatnat is removed and the gel is washed with 1mL 0.1M NaOAc, followed by 1mL of 0.1M Tris Cl, this series is repeated three times.
7. The gel is stored in PBS at 4°C until used for panning.

Amplification of the LX_4 Library

This library was obtained from the laboratories of Dr. Jamie Scott at Simon Fraser University. It contains a randomized insert in the pVIII gene with the sequence XCX_4CX where X= randomized codon NNK, and C= cystyeine. This amplification procedure was obtained from Dr. Scott.

1. Inoculate 30 mL of LB broth with a single K91 colony and incubate with shaking overnight at 37°C.
2. Prepare 2L of Superbroth and autoclave, also 4 X 1L flasks and 2 X 250 mL flasks.
3. Inoculate 110 mL of superbroth in a sterile 250mL flask with 7mL of the overnight culture prepared in step 1. Shake at 250rpm until the OD reading is 1.8 (about 3 hours).
4. Slow the shaker down to 50 rpm fpr 10 minutes to regenerate pili.
5. Aliquot remaining superbroth to give 550 mL in each of three 1L flasks. Add 23uL of 5mg/mL tetracycline to make the concentration .2ug/mL.
6. Add 50 uL of phage (10^{12} phage/mL) to the 110 mL cells from step 3. The solution was allowed to stand at room temperature, swirling occasionally.
7. Add 35 mLs of the infected cells to each of the three 1L flasks of superbroth, shake at 220rpm for 30 minutes at 37°C for induction.
8. Increase tetracycline concentration to 15ug/mL and shake overnight at 37°C.
9. Pellet the bacterial cells by centrifuging: 500rpm for 20 minutes, then 7000 rpm for 20 minutes.
10. Pour cleared supernatant into six centrifuge tubes: 270mLs each. Add .15 vol of 16.7% PEG/4.4 M NaCl to each. Shake 100 times and store at 4°C overnight.
11. Centrifuge at 30,000 rpm for one hour at 4°C.
12. Pour off supernatant and take each pellet up in 2mL PBS, total = 50 mL.
13. Repellet any nondissolving material by centrifuging in the microfuge.

14. Take 1mL of supernatnat and transfer to a new Eppendorf tube and add 150μL of 16.7% PEG/4.4M NaCl. Store at 4°C overnight.

15. Pellet by centrifuging in the microfuge for 30 minutes.

16. Pour off supernatnat, recentrifuge for for 1 minute then pipette off the remaining supernatant.

17. Take each pellet up in 300uL of PBS, store at -20°C. 50 x 300uL = 15mL.

The same procedure was used to amplify f88.4 phage which is the parent phage vector from which the LX4 library is derived. It has no randomized region on the pVIII gene.

Panning the LX4 library

1. Strip 1 is from an underivatized covalink plate and strip 2 is from a tripeptide derivatized covalink plate blocked with SDS.

2. Add 50μL of LX4 phage to each well in strip 1 and 2.

3. The strips were shaken for two hours at room temperature.

4. The supernatant was removed, and each strip was washed by adding 100μL of PBS to each well, shaking for several minutes, then aspirating. This wash step was performed three times.

5. 100μL of log phase K91 cells were added to each well and the strips were incubated at 37°C for 30 minutes.

6. The infected phage were removed from each well and the added to 30mL of LB broth-1 and 2, shaking for 10 minutes at 37°C.

7. 108μL of 5mg/mL tetracycline was added and the flasks were shaken overnight at room temperature.

8. The cells were pelleted, and the phage precipitated from the supernatant to carry on to the next round of panning.

Competition Experiments on High Affinity Phage from the LX4 Library

1. The high affinity phage from the LX4 library were diluted to approximately 1000 phage per mL.

2. 50μL of the diluted phage were combined with 100μl of log phase K-91 *E.coli* cells. The solution was incubated for 30 minutes at 37°C.

3. The infected cells were spread on LB/tet plates and incubated overnight at 37°C.

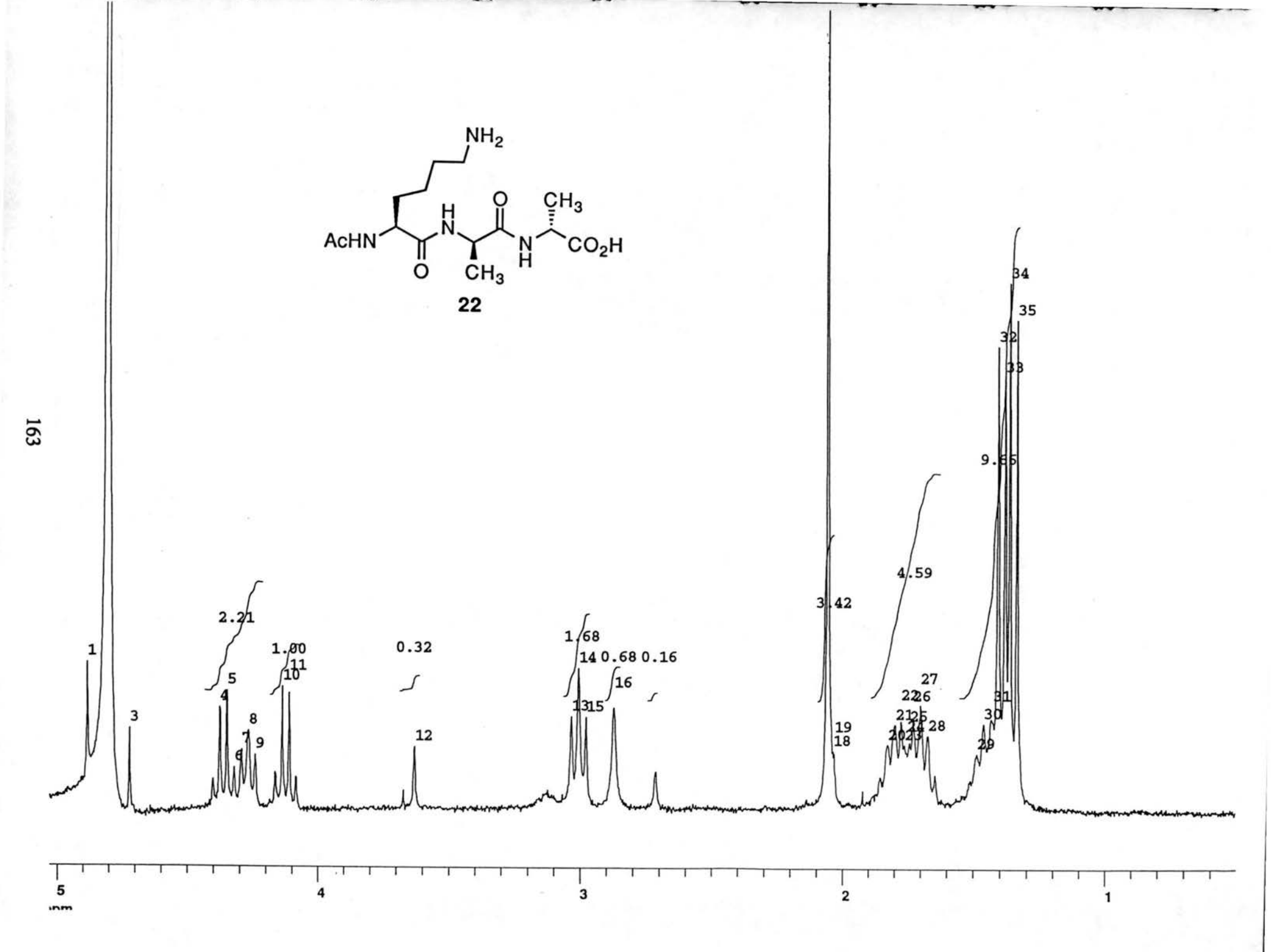
4. 96 single colonies were picked and each placed in a separate eppendorf tubecontaining 500μl of LB broth. The tubes were labelled A1 through H12 corresponding to each well in a 96 well plate. The tubes were incubated for 30 minutes at 37°C.

5. For plate #1- the positive control plate: 50μL of phage are added to each well and incubated for an hour. The phage solutions are removed and the plates are washed several times with PBS. 100μL of a 1:5000 dilution of rabbit anti-phage antibody is added and incubated on the plate for one hour, then removed and subjected to rigorous washing-see step 2 page 152. 100μL of a 1:3000 dilution of anti-rabbit antibody-alkaline phosphatase is added and incubated on the plate for one hout, then washed as above. 50μL of 5mg/mL p-nitrophenylphospate is added and incubated on the plate at 37°C for 30 minutes, then a stop solution of 3M NaCl is added and the absorbance at 405nm is determined with a microtiter plate reader.

6. For plate #2- the first competition experiment. Before being added to the plate , 100μL of each phage clone is preincubated for 30 minutes with 50 μL of antiphage antibody. This solution is added to each well, and the ELISA is continued as described above.

7. For plate #3, the second competition experiment. The wells of the plate are treated with a 1 mg/mL solution of vancomycin for 30 minutes. The solution is removed, and the phage are added and the ELISA performed as in step 5.

APPENDIX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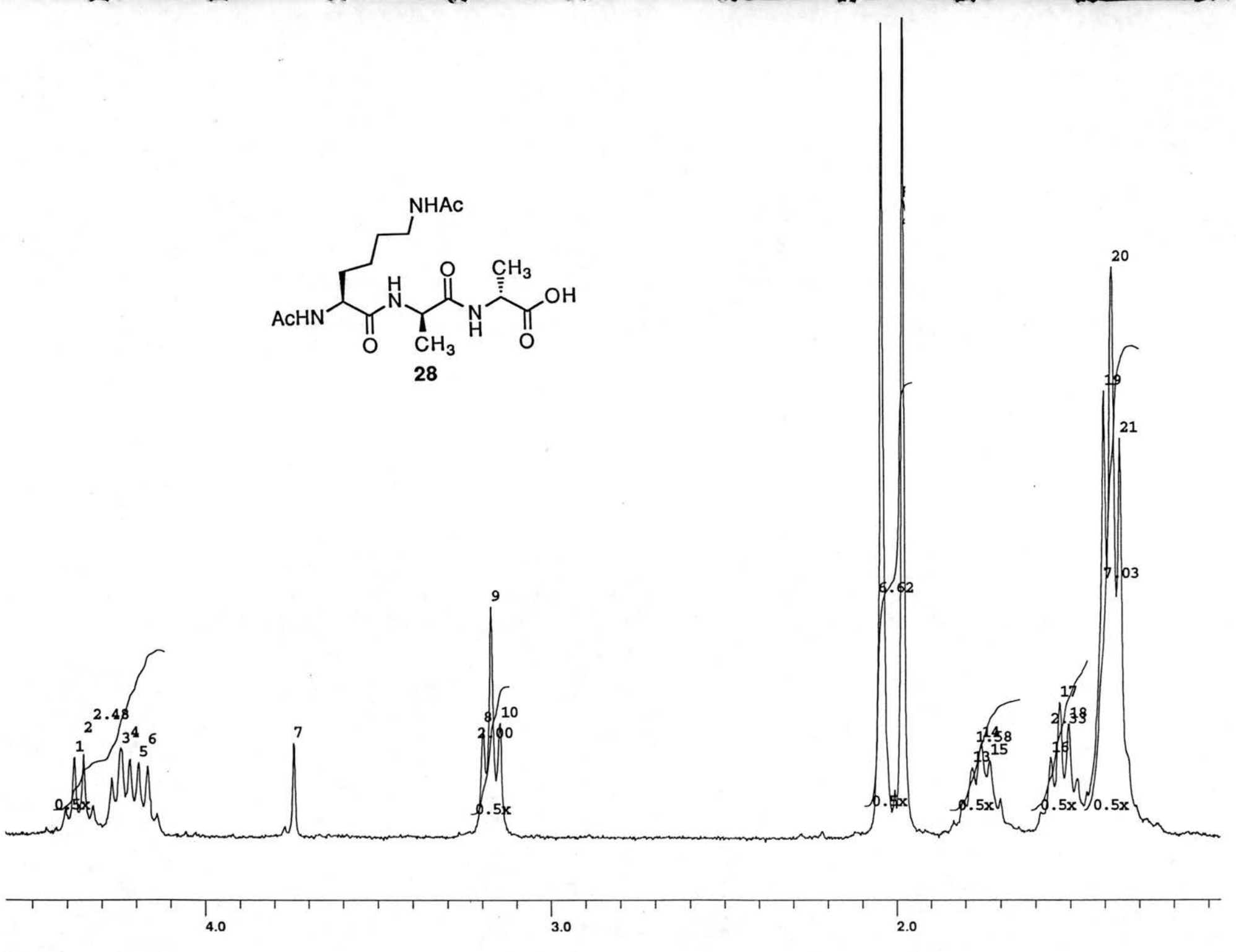
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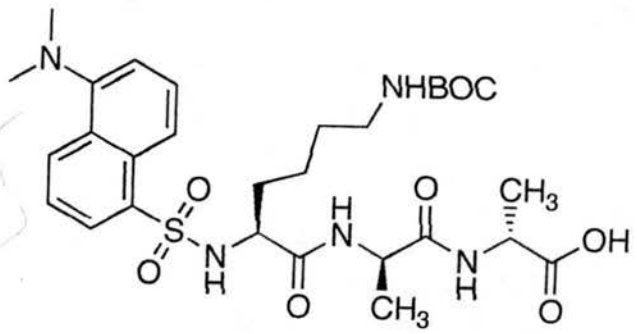
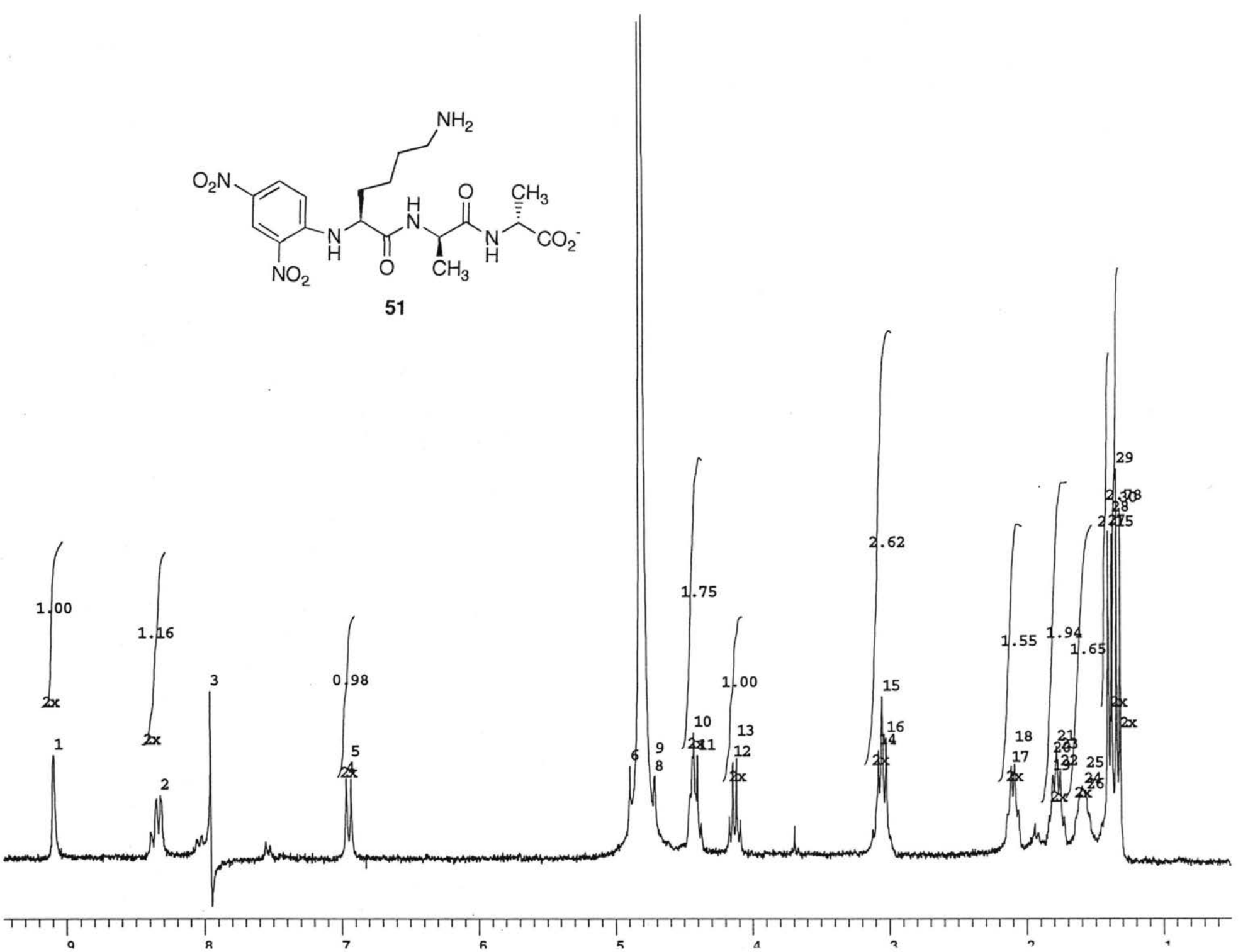


39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