Development of Peptide Cyclization Strategies for Their Incorporation into One-Bead-One-Compound Peptide Libraries

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DEVELOPMENT OF PEPTIDE CYCLIZATION STRATEGIES

FOR THEIR INCORPORATION INTO

ONE-BEAD-ONE-COMPOUND PEPTIDE LIBRARIES

A thesis by

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One-Bead-One Compound Peptide Libraries

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Abstract:

Cyclic peptides provide a privileged scaffold when optimizing interactions with various biological targets. Their rigidified structure decreases the entropic cost of binding by preorganizing residues in a fixed conformation, which may enhance binding affinity. These molecules occupy a larger chemical space than typical small molecule drugs and may provide good candidates for inhibiting protein-protein interactions or being able to interact with previously undruggable targets. Given the benefits of these structures we aim to develop a one-bead-one-compound peptide library for screening against relevant biological targets. Herein we describe several routes to achieving cyclic peptides through side chain interactions and head-to tail amide bond linkages. Additional considerations for the development of the on resin library such as linker strategies and sequencing methods will be discussed.

Acknowledgement

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List of Schemes

List of Abbreviations

PS   Phosphatidylserine

SICLOPPS   split-intein circular ligation of peptides

Dap(aoa)   (aminooxyacetyl)-L-2,3-diaminopropionic acid

TBMB   tris(bromomethyl)benzene

TATA   1,3,5-triacryloyl-1,3,5-triazine

TBAB   N,N',N'-(benzene-1,3,5-triyl)-tris(2-bromoacetamide)

Dap   Diaminopropionic acid

2-APBA   2-acetylphenylboronic acid

mRNA   messenger ribonucleic acid

PCR   Polymerase chain reaction

DNA   deoxyribonucleic acid

OBOC   One-bead-one-compound

OBTC   One-bead-two-compound

PyBOP   Benzotriazol-1-yl-oxytripyrrolidinophosphonium

                    hexafluorophosphate

HATU   1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-

                    b]pyridinium 3-oxid hexafluorophosphate

HBTU   2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium

                    hexafluorophosphate

DMF   N,N-Dimethylformamide

TCEP   Tris(2-carboxyethyl)phosphine

DIPEA   Diisopropylethylamine

Boc	tert-butyloxycarbonyl
Fmoc	Fluorenylmethyloxycarbonyl
UV	Ultra violet
LC-MS	Liquid chromatography mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
PBS	Phosphate buffered saline
Lys	Lysine
AOHA	6-aminooxyhexanoic acid
ppm	Parts per million
HBA	4-hydrazinobenzoic acid
THF	Tetrahydrofuran
TEA	Triethylamine
DCM	Dichloromethane
TFA	Trifluoroacetic acid
NMM	N-methylmorpholine
TIS	Triisopropylsilane
CD	Circular dichroism spectroscopy
SDS	Sodium dodecyl sulfate
Mag	Magainin
Mag2-LB1	Magaining2-LB1 mutant
PEG	Polyethylene glycol
ANP	Fmoc-3-amino-3-(2-nitrobenzyl)propanoic acid
PIC	Phenylisocyanate

PITC	Phenylisothiocyanate
TOF	Time of flight
NBD	4-chloro-7-nitrobenzofuran
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine

Chapter 1: Introduction

1.1 Potential of cyclic peptides

Cyclic peptides can provide a rigidified structure that preorganizes residues in a beneficial manner to decrease the entropic cost for target binding. Often cyclization makes these peptides less susceptible to proteolytic degradation than their linear counterparts. As such privileged structures we find them to be an excellent candidate for binding membrane lipids[1], disrupting protein-protein interactions[2,3] and mimicking larger protein surfaces. Cyclic peptides occupy an area of chemical space that is inaccessible to small molecules and large biological polymers and are of particular interest to the scientific community.

1.2 Methods of peptide cyclization through natural peptide functionalities

1.2.1 Backbone cyclization

One of the most commonly used methods for peptide cyclization is forming an amide linkage through the backbone amine and carboxy terminus of the peptide on resin. This method of cyclization relies on having an accessible N-terminus and C-terminus for cyclization to occur. In order to achieve this, there must be side chain immobilization of the peptide to the resin, requiring either the use of a glutamic acid or an aspartic acid on the Wang resin or the use of a glutamine or asparagine on the Rink Amide resin. This method of peptide cyclization was demonstrated within our lab with the synthesis of cLac, a 13 amino acid cyclic peptide.[1] cLac is a cyclic peptide that mimics the functional surface of a larger protein. Its protein mimic, Lactadherin, a 47kDa protein selectively binds phosphatidyl serine. cLac can also bind PS through its rigidified cyclic peptide scaffold that displays the functional

residues for PS binding.[1] One of the main limitations of this method is the reliance on the presence of glutamine, asparagine glutamate or aspartate residues in the peptide sequence.
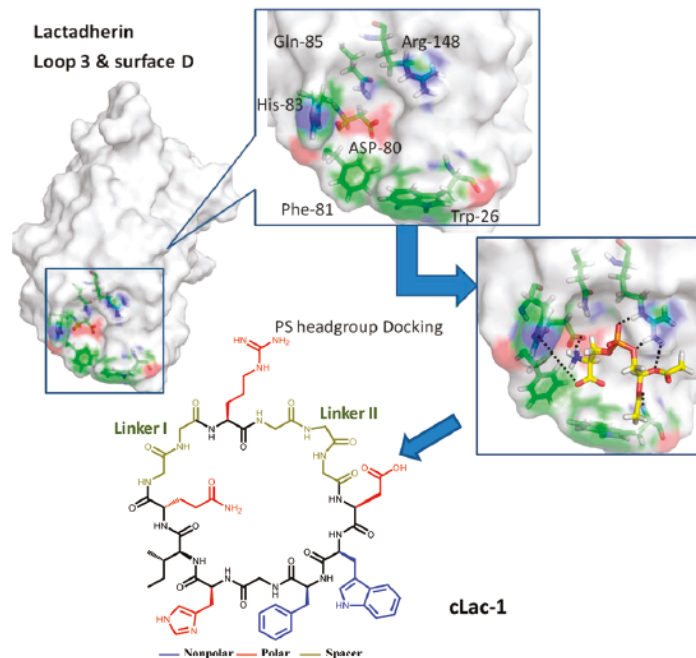
Figure 1. cLac structure (adapted from [1])

Backbone cyclization can also be achieved using the split-intein circular ligation of peptides and proteins or SICLOPPS.[4] Through this methodology the desired cyclic peptide is expressed as a linear fusion protein with a split intein, which can then form the active intein to elicit the thioester intermediate formation.[4] This intermediate can then undergo rearrangement to form the final cyclic peptide with an amide linkage.[4] While this cyclization strategy affords a conformationally rigid cyclic peptide, the mechanism of SICLOPPS requires a cysteine within the sequence, a potential drawback if this residue should not be in the final product.

1.2.2 Disulfide bridges

In addition to backbone cyclization, several other groups have opted to utilize the reactivity of side chains to cyclize peptides. One of the most commonly used side chain cyclization methods is the formation of a disulfide bond between two cysteine residues. The Nilsson group used the disulfide strategy to cyclize the short peptide Ac-C(FKFE)$_2$CG-NH$_2$ to lock the conformation of the peptide.[5] This disulfide linkage provided a switch that could be controlled through reduction to create hydrogels of linear beta sheets.[5] The Schultz group used this disulfide formation to cyclize and staple short alpha helical peptides using an unnatural cysteine homolog.[6] This case presents an example of peptide cyclization to enhance the secondary structure of the peptide[6], in contrast to the work by Nilsson and coworkers that seeks to prevent beta strand and hydrogel formation.[5]

1.2.3 Lysine reactivity for peptide cyclization

Other methods of peptide cyclization take advantage of the reactivity of lysine side chains. Short peptides of varying sequence have shown to be tolerated in the transglutaminase mediated cyclization of glutamic acid and lysine.[7] Lysine can also react with organic linkers to form cyclic peptides through bridging by iodoacetic anhydride[8] or disuccinimidyl glutarate.[9,10]

1.3 Unnatural peptide cyclization strategies

While many groups have taken advantage of these cyclization strategies of natural amino acids, still others have adopted unnatural amino acids to assist in the synthesis of cyclic peptides. Allyl protected homoserine, incorporated into short peptide sequences, undergoes intramolecular ring closing metathesis to afford cyclic

peptide targets.[11] The Fasan group has introduced a mixed approach to peptide cyclization by performing ribosomal synthesis of macrocyclic peptides with unnatural amino acids containing aminothiol moieties. The unnatural linkages make the cyclic peptides highly stable, yet amendable to biological peptide synthesis methods.[12]

Organic crosslinkers have also shown increasing prevalence in cyclic and bicyclic peptide chemistry. The reactivity of cysteine makes it suitable for reacting with organic crosslinkers.[13] The resulting thioether linkages are not susceptible to reductive conditions, making them more stable than their disulfide counterparts.[13] Dichloroacetone can also react with two cysteine residues to cyclize peptides, providing a ketone handle for oxime ligation with various labels.[14] Additionally, thioethers can be generated from disulfide bridged cyclic peptides through desulfurization using base or through beta elimination followed by a Michael addition.[19]

A combination of unnatural amino acids and organic crosslinkers can afford bio orthogonal peptide cyclization. The Horne group has demonstrated that unnatural amino acids presenting oxyamine moieties readily react with dialdehyde linkers of varying orientation.[15] The Cochran group also made use of this methodology with (aminooxyacetyl)-L-2,3-diaminopropionic acid and an extended dialdehyde linker to link two knottin peptides together, showcasing the broad applicability of this chemistry.[16] This strategy has also been demonstrated to work intramolecularly to crosslink aldehyde and oxyamine side chains with the goal of enabling dynamic covalent exchange in water .[17,18, 20] The applicability of this

chemistry to aqueous solutions suggests potential benefit over those requiring organic solvents or cofactors to assist in cyclization.[18,20] Through selection of the nucleophilic residue it is possible to tune the degree of peptide cyclization, which makes this chemistry and similar chemistries suitable for making reversibly cyclic peptides.

1.4 Examples of natural multicyclic peptides

Nature has many examples of rigid peptides such as the cysteine-knot motif, a constrained peptide scaffold with head to tail cyclization bearing several disulfide bonds that are often interwoven throughout the peptide chain.[21] This class of peptides presents broad biological functions including antibiotic, anti-HIV, and antitumor activities.[21] These peptides are achieved in nature through a macrolactam backbone cyclization in addition to multiple of disulfide bridges.[21]

A structural class of knotted peptides is lantibiotics, antibiotic peptides containing lanthionine.[22,23] Lantibiotics contain a thioether bridge formed via post-translational modification.[23] In these peptides, either serine or threonine dehydrate via the LanB enzyme to generate the corresponding α,β unsaturated amino acids, which then undergo cyclization via the enzyme LanC.[23] Their extreme structural rigidity and diverse biological functions have drawn attention to this class of molecules in recent years. Such complex bridging patterns have encouraged chemists to seek multicyclic peptides via various routes that will be discussed below.

1.5 Bicyclic peptide strategies

Structurally rigidified bicyclic peptides have been sought after by many groups in hopes of inhibiting protein-protein interaction[24] or probing various protein targets.[25] The Heinis group thought to incorporate an unnatural amino acids L-4,5-dithiolnorvaline into peptides in order to promote disulfide bridging to free cysteines.[24] This strategy creates bicyclic peptides via oxidation of the unnatural amino acid along with two cysteines.[24] The selected peptide sequences were found to mimic their natural cysteine knot models and the optimized bicyclic ligand was found to inhibit the target protein with 40-fold greater efficiency than that of the original peptide.[24] While the work of the Heinis group provides a way to prepare bicyclic peptides on solid support[24], the Fasan group reported the preparation of bicyclic peptides through recombinant methods.[25] Their work featured the split intein-catalyzed head-to-tail cyclization, described previously as the SICLOPPS method, to form a mono cyclic peptide which they then converted to a bicyclic peptide through inter-side-chain crosslinking of an unnatural O2beY amino acid and a cysteine.[25] This methodology allowed them to access a variety of bicyclic peptides, and proved useful in optimizing peptides for protein binding.[25]

Side chain conjugation has proven to be an effective method for several groups[21-25], but the rigid peptide scaffolds can also be made via intermolecular reactions.[26,27] The Heinis group focused much of their efforts toward achieving bicyclic peptides with cysteine-reactive small molecules such as tris(bromomethyl)benzene[26-28], 1,3,5-triacryloyl-1,3,5-triazinane and N,N',N"-(benzene-1,3,5-triyl)-tris(2-bromoacetamide) (Figure 2).[26] These small molecule

crosslinkers were beneficial to forming structurally rigid peptides first by bicyclizing the peptides through covalent reactions with cysteines and then by forming noncovalent interactions with other amino acid side chains.[26] This bicyclization strategy was found to be applicable to phage-displayed libraries as well as solid phase peptide synthesis.[26] The Pei laboratory used a similar approach to synthesize bicyclic peptides on resin using trimesic acid that conjugates with the N-terminus and two 2,3-diaminopropionic acid residues in the peptide sequence.[29]
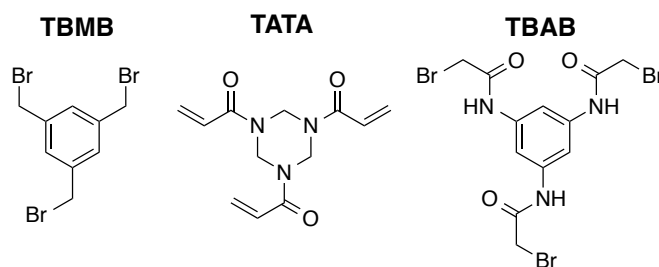

Figure 2. Organic scaffolds for peptide bicyclization

1.6 Routes to reversible peptide cyclization

While there are many routes to cyclic and bicyclic peptides, most of these structural rigidifications come in the form of permanent modifications, with the only exception being the redox reversible disulfide bridge. This leaves much room in the field of peptide chemistry for the development of a reversible methodology to chemically cyclize and bicyclize peptides. One form of chemistry that has become prevalent in our lab recently is iminoboronate chemistry (Figure 3).[30] Bandyopadhyay et. al. showed that installing an ortho boronic acid to acetophenone moieties stabilized Schiff base formation through a dative bond between the nitrogen of the immine and the empty p orbital on boronic acid.[30] While this work showcases the potential of these 2-acetylphenylboronic acid species to conjugate

with small alpha nucleophiles and biological amines[30], we sought to broaden the

scope of this chemistry toward its implementation into peptide cyclization.[30,31]

Importantly, the imino boronate-based peptide cyclization can be reversible by

competing away the iminoboronate through the addition of a stronger alpha

nucleophile. Additionally this methodology provides the ability to tune the degree of

interaction by adjusting the pH of the solution, which makes the iminoboronate

based cyclization reversible. This is highly desirable for developing peptide

cyclization chemistries that would be applicable to both screening and sequencing

protocols.

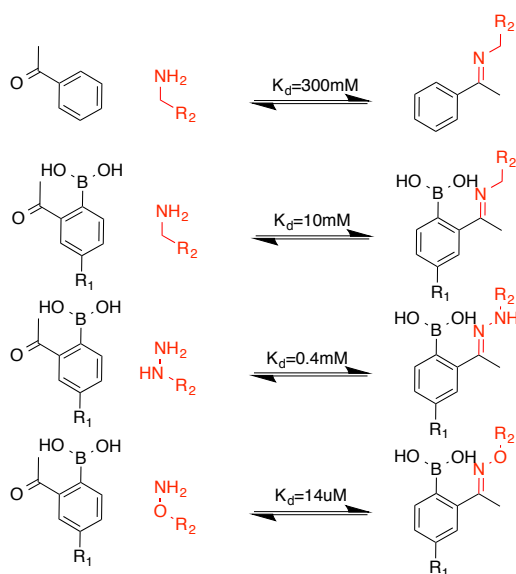**Figure 3.** Iminoboronate formation with various nucleophiles

1.7 Peptide library development

　　While the development of peptide cyclization strategies allows easy access to

rigidified peptide scaffolds, development of peptide sequences that interact in a

favorable way with the target of interest can still take much work. Although

computational approaches has shown promise for the development of a peptide hit[1],

a randomized, unbiased peptide library may offer the best array of molecules when screening against a target of interest.

Three major classes of library development exist in the peptide field. mRNA display utilizing the PURE system is a useful tool for library construction and screening.[33] This method provides a means for covalently linking the genetic information that encodes peptides to the peptides themselves through a Puromycin linker.[33] This approach provides a diversity of $\sim 10^{12}$ accessible compounds for screening.[33] Once compounds have been identified as "hits" the peptide-mRNA complexes can undergo PCR amplification and DNA sequencing to reveal the sequence of the peptide binder.[33]

Another biological method of library synthesis involves the use of phage and their ease of their genome manipulation to express a variety of short peptide variants onto their coat proteins.[34] These libraries have a diversity of $\sim 10^8$ and can be useful for finding low micromolar and nanomolar peptide binding sequences through multiple rounds of panning.[34]

A more chemical approach to peptide libraries involves the use of one-bead-one-compound (OBOC) or one-bead-two-compound (OBTC) peptide libraries.[35.36] Previous work in the Pei laboratory has demonstrated such methods are easily accessible and prove to be good tools for finding inhibitors of protein-protein interactions. Variations in peptide sequence can be achieved through split-pool synthesis method where different amino acids are coupled to portions of resin. The resin portions are then mixed together and re-split into new portions to couple with

new amino acids (Figure 4). This strategy creates a randomized peptide library where each bead presents only one congruent sequence for screening.
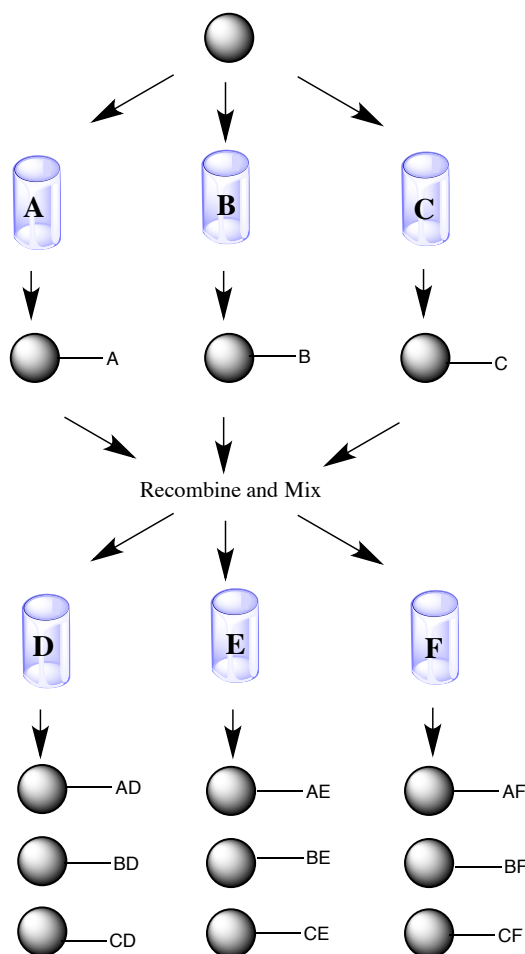

Figure 4. Split-pool synthesis method

1.8 Experimental Goals

Given the benefits provided by cyclic peptides including a rigidified structure, which gives rise to decreased entropic cost of binding and the ability to mimic larger protein structures, our research aims to generate a cyclic peptide library on resin. We propose to generate this library via split-pool synthesis incorporating bioorthogonally reactive groups in order to form reversible iminoboronate cyclized peptides. We aim to generate this library as a means to

screen for sequences that selectively bind targets of biological relevance such as the membrane lipid phosphatidylserine. We then propose to linearize these cyclic peptides for sequencing.

Chapter 2: Experiments and Results

2.1 On resin head to tail cyclization

Initial attempts at the development of an on resin cyclic peptide library focused on identifying an adequate method for peptide cyclization. We sought to utilize the previously developed cLac peptide shown in Figure 5 as a model sequence for optimization of head to tail of cyclization. Screening of coupling reagents proved PyBOP to have more synthetic utility than HATU, and HBTU. This selected coupling agent was taken on for further screening of reaction conditions such as solvent and temperature effects on cyclization efficiency. Data obtained from these screens showed that up to 79% conversion from linear to cyclic peptide could be obtained with heating to 82°C for 4 hours in N, N'-Dimethylformamide (DMF) with only 15% of peptide forming the undesired dimers.
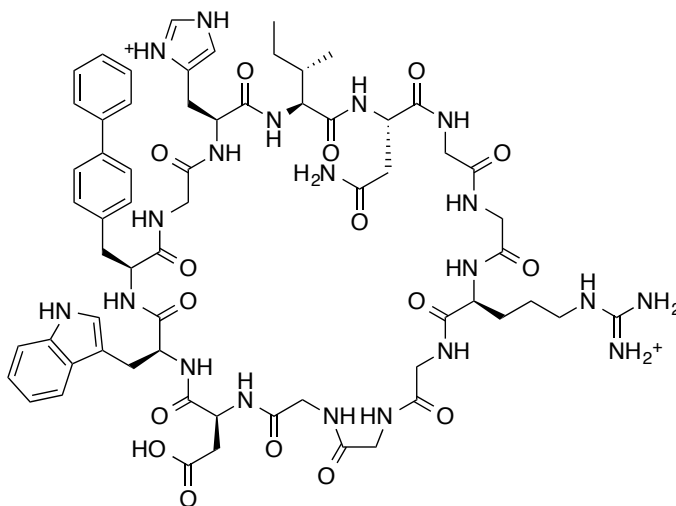

Figure 5. Head to tail cyclization of cLac2 scaffold

After further consideration of this cyclization strategy, we concluded that head to tail cyclization, while efficient enough, would not be a good approach for the development of a one-bead-one-compound (OBOC) library due to the difficulty of sequencing backbone cyclized peptides. Edman degradation would not be accessible due to the lack of a free N-terminus and mass spectrometry would produce random peptide fragments that would be difficult to piece back together. Due to this limitation, other approaches to peptide cyclization and bicyclization were examined through side chain crosslinking.

2.2 Bicyclization through organic crosslinkers

Previous reports from the Heinis group described bicyclic peptide formation on phage for panning against various targets[37]. We believe our on resin library would behave much like the phage in solution so we sought to find optimal conditions to synthesize bicyclic peptides on resin utilizing the organic crosslinker tris-bromomethyl benzene (TBMB) and a reported sequence H_2N-ACVSWHGGCHPQGDICC-OH (Scheme 1).[37]

Solution phase cyclization of the above sequence with TBMB was achieved after incubating for 1 hour at 30°C. The reaction was conducted in an ammonium carbonate buffer to ensure free thiolates for reactivity, and in the presence of acetonitrile to assist in TBMB solubility. With the success of solution phase cyclization, the reaction was further optimized to create bicyclic peptides on resin.
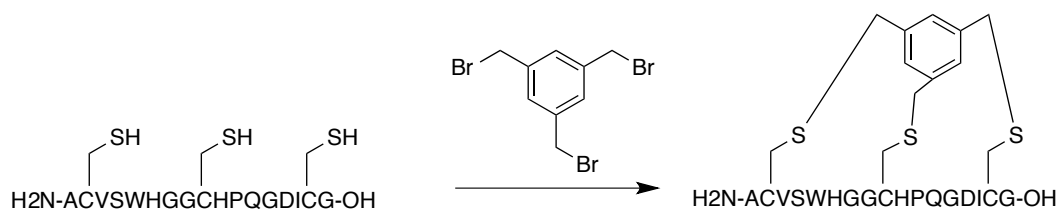
**Scheme 1.** Peptide bicyclization through tris-bromomethylbenzene

The solid phase peptide synthesis of the aforementioned sequence

incorporated an orthogonal cysteine protecting group, (4-

methoxyphenyl)diphenylmethyl. This provided a means to selectively generate free

thiols for reacting with TBMB. The Ellman test identified a small percentage of free

thiols on resin, and when these peptides were further screened for reactivity with

TBMB, only minimal peptide bicyclization was achieved even with optimized

conditions (1.5 equivalents of TBMB, 3 equivalents of TCEP in the presence of 2

equivalents of diisopropylethylamine (DIPEA) for two hours at 30°C).

2.3 Synthesis of unnatural amino acid AB1

With difficulties of peptide cyclization on resin, we sought to develop an

novel strategies to cyclize peptides through the use of unnatural amino acids.

Iminoboronate chemistry has been extensively investigated in our laboratory and

has demonstrated its utility as a conjugation strategy with tunable stability.[30] We

envisioned that incorporating unnatural amino acids containing 2-acetylphenyl

boronic acid (2-APBA) moieties, along with an alpha nucleophile, could provide

intramolecular iminoboronate formation to give a cyclic peptide. This methodology

provides the possibility to form a highly stable iminoboronate or a relatively weak

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iminoboronate that can easily be competed away by small molecule addition or pH

tuning, thus allowing facile control peptide cyclization.

Synthetic approaches to the unnatural amino acid AB1 had been reported by

our laboratory and are described below in Scheme 2. The route to AB1 begins with a

simple Boc protection that affords intermediate 2 in high yields and purities. The

boc protected intermediate 2 was reduced using tri-n-butylphosphine. While this

reaction could be done on large scale, several problems arose with this step. The

first issue came from purification of the free thiol. Column purification with air

caused the reduced cysteine to re-oxidize on column and compromised the product

purity. Furthermore, an unidentifiable side product co-eluted frequently with the

desired product, affecting product purity and potentially affecting the subsequent

reactions.
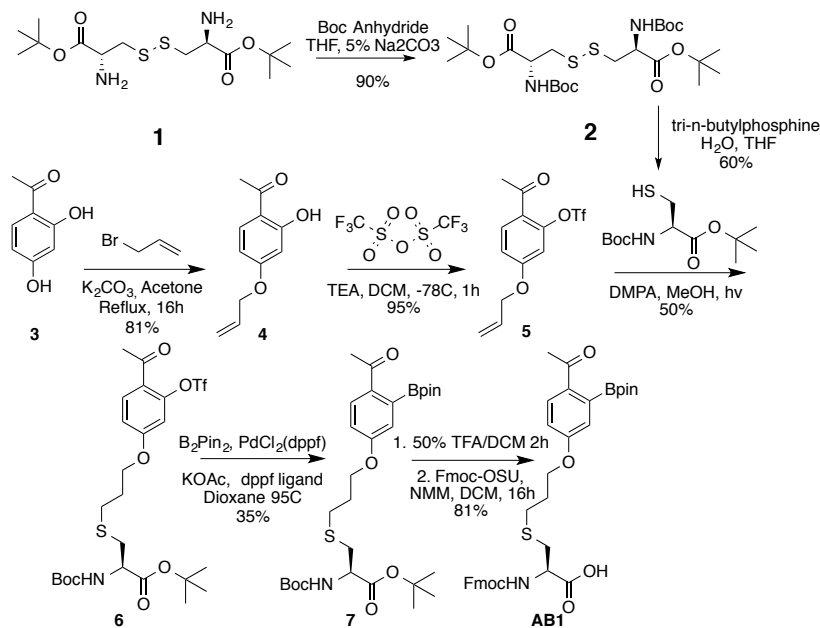
Scheme 2. AB1 Synthetic Route

The UV initiated thiol-ene reaction appeared sensitive to the purity of the cysteine starting material. The residual unidentified compound appeared to compromise the reaction. The percentage of cystine increased over the course of the reaction, further reducing the yield.

Miyaura borylation of intermediate 6 also gave lower than reported yields in my hands with typical yields close to 30%. This appeared to be due to sensitivity to moisture, which we tried to overcome by utilizing molecular sieves and purging of the reaction with argon. Additionally, this reaction appeared to be extremely temperature sensitive. Ultimately this reaction was executed so that enough material could be obtained for peptide synthesis.

2.4 Incorporation of AB1 into model peptide sequences

Once AB1 had been synthesized in sufficient quantities, its potential to induce peptide cyclization was explored using model peptide sequences. We first incorporated these unnatural amino acids into short peptide sequences that included either a Dap(alphahydroxy amino) residue, abbreviated as Dap(aoa), in sequences LB02-132 and LB02-131 or a lysine residue as a nucleophile in sequences SD01-93 and SD01-94 (Figure 6).
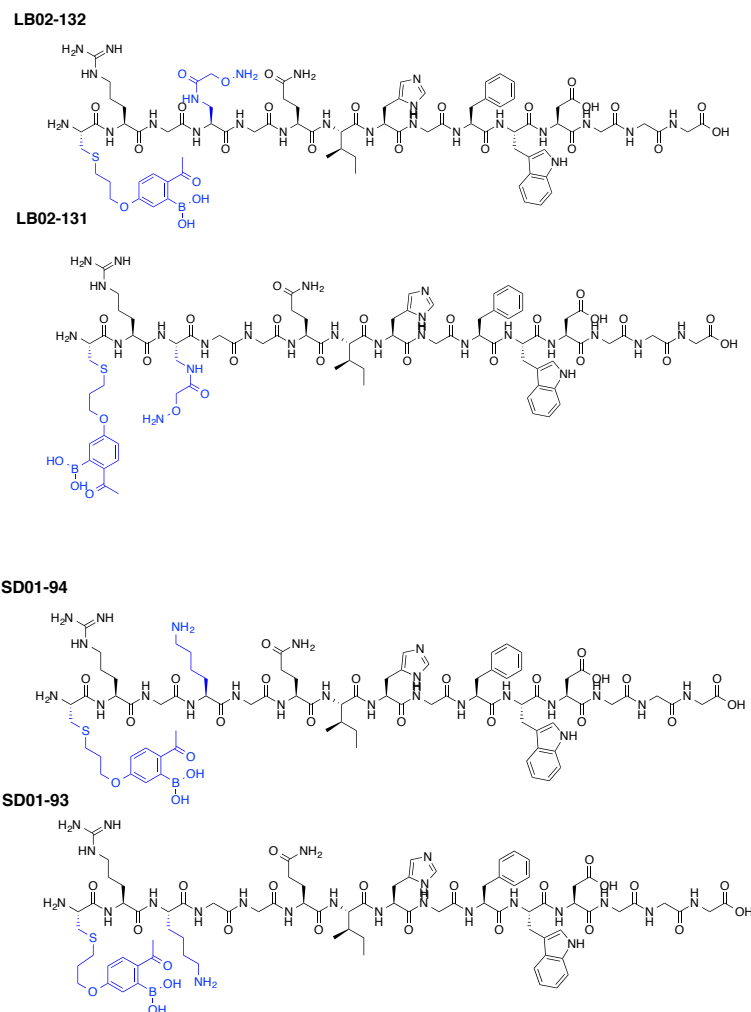
Figure 6. Model peptide sequences with AB1

These sequences provided two different linker lengths between the nucleophile and AB1 in order to probe the optimal distance for intramolecular iminoboronate formation. To observe iminoboronate formation, we examined these peptides by LC-MS, UV-Vis spectroscopy and ^{11}B-NMR experiments.

To develop UV standards for iminoboronate formation we examined the absorption spectra of 2-APBA with small molecule nucleophiles. Specifically, 2-APBA was screened in PBS buffer with varying amounts of either lysine (Lys) or 6-aminooxyhexanoic acid (AOHA) and their UV absorbance profile was measured

(Figure 7). Based on the data obtained, a significant shift in the absorption maximum was observed for lysine and 2-APBA, while a less obvious shift was observed for the conjugation of 2-APBA with AOHA. These initial data provided a basis for our assessment of the intramolecular iminoboronate formation of the model peptides. Peptides containing Dap(aoa) residues appear to have a slight shift in the maximum absorption wavelengths when AB1 is 2 residues away from Dap(aoa) suggesting intramolecular iminoboronate formation (Figure 8).This is comparable to the UV data of the small molecule control experiments presented in Figure 7, where iminoboronate formation is observed as a shift toward 270nm from the free 2-APBA absorption maximum of 250nm. This favored interaction of the i+2 versus the i+3 position may be due to orientation of the residues in space rather than the distance between the 2-APBA head group and the nucleophilic residue.
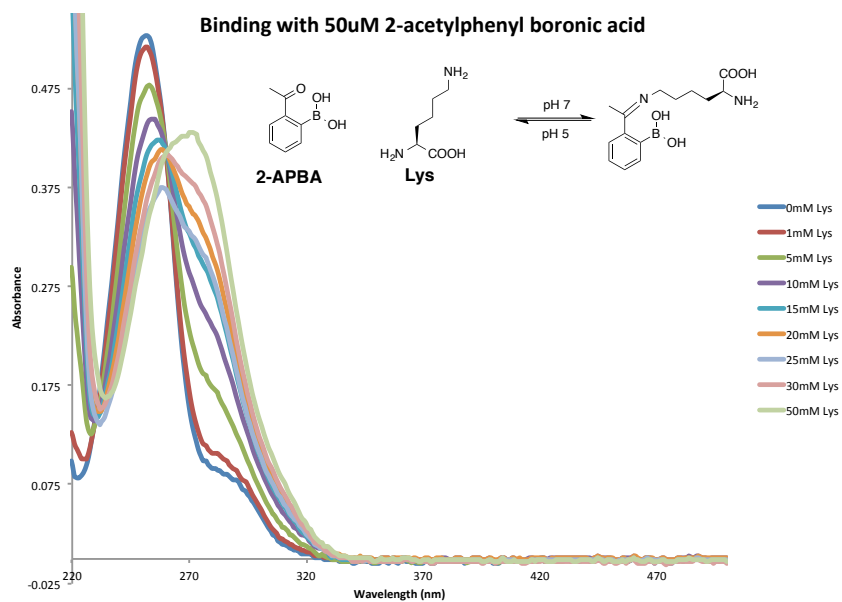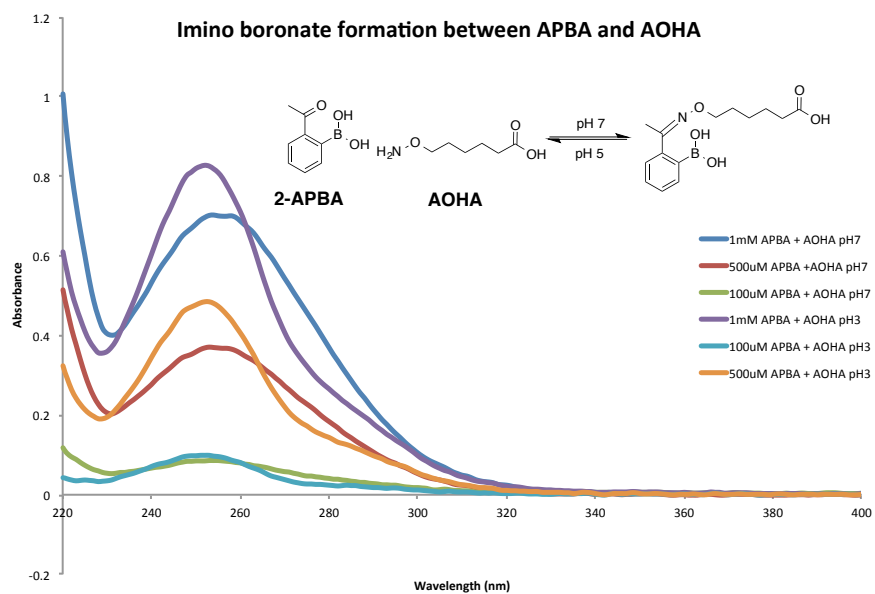
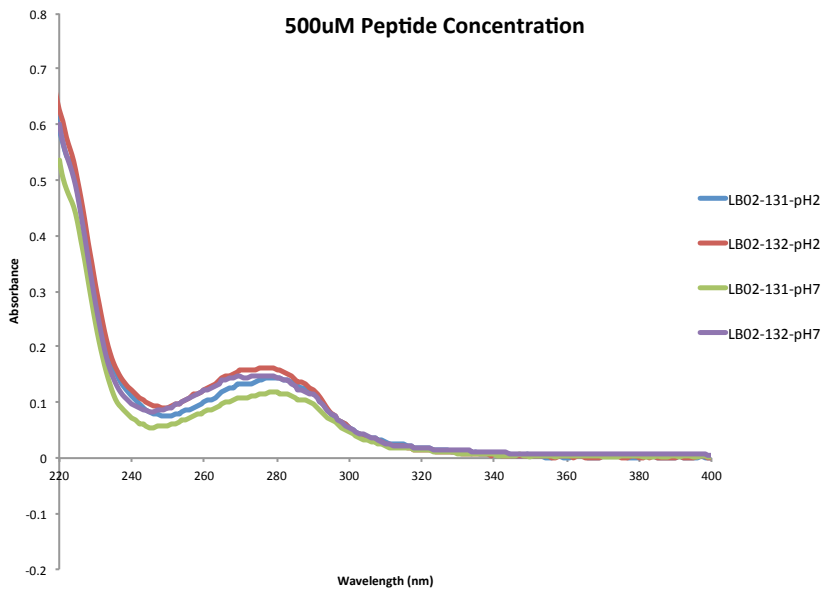**Figure 7.** UV spectra of small molecule iminoboronate formation

Figure 8. UV absorption data for iminoboronate formation in model sequences

In addition to UV observations we examined these peptides using ^{11}B NMR to demonstrate the change in the boron electronic properties. Free boronic acids typically have [11]B chemical shifts around 30ppm whereas iminoboronates typically have peaks near 0-5ppm. As we prepared these peptide series in phosphate buffered saline with 10% D_2O we observed chemical shifts of 19ppm, which corresponds to boric acids. Further investigation showed that these peptides had degraded prior to the NMR experiments, suggesting these peptides may not be as stable as we had hoped.

Given the limitation on peptide stability, we sought to make a smaller model peptide that could be efficiently produced and test tunability of intramolecular iminoboronate formation when presented with an external nucleophile. Previous work from Bandyopadhyay et. al. suggested that, while aminooxy functionalized

molecules may have strong interactions with AB1with K_d's of 14µM, the conjugation of hydrazine functionalized molecules was much stronger with K_d's of 70nM**.** Based on these data we synthesized short peptide H_2N-AB1GGDap(aoa)W-OH. To our delight we observed the expected concentration dependence for the external small molecule competitors the intramolecular iminoboronate. 6-aminooxyhexanoic acid appeared to compete away 40% of the peptide's intramolecular iminoboronates with concentrations greater than 100 µM (Figure 9). This plateau is due to the high reversibility of this conjugation, allowing a rapid exchange between the intramolecular iminoboronate and the intermolecular iminoboronate. We sought to improve upon this competition with a stronger nucleophile 4-hydrazinobenzoic acid. As we predicted, 4-hydrazinobenzoic acid, at greater than 1.5 equivalents, allowed us to linearize the peptide 100% (Figure 10). The ability to tune the degree of peptide cyclization through iminoboronate chemistry could be useful for controlling peptide structure and activity.
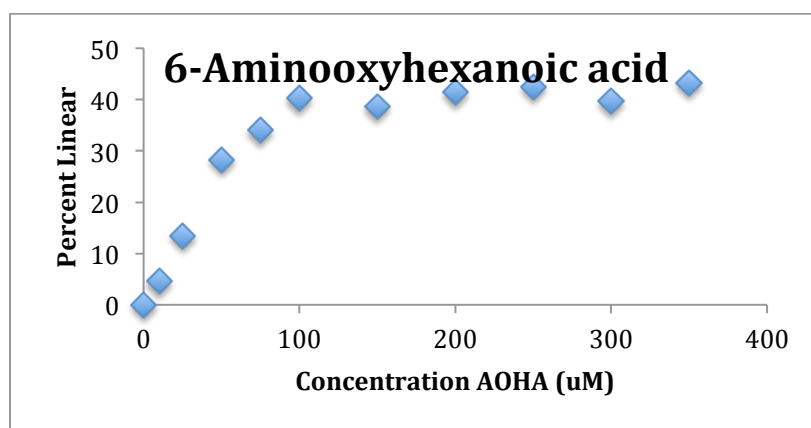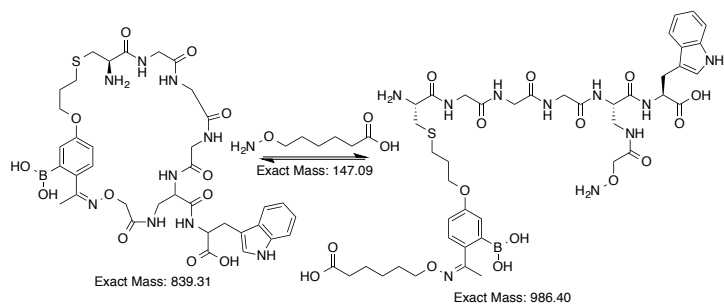
Figure 9. Interaction of 6-aminooxyhexanoic acid with model peptide H$_2$N-AB1GGDap(aoa)W-OH. Peptide concentration was held constant at 50μM.
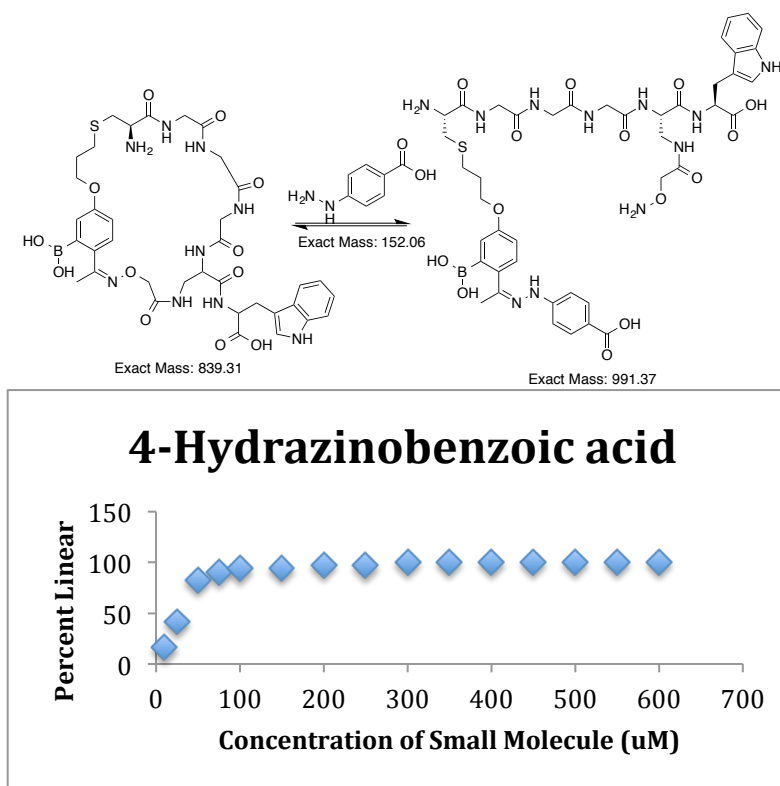
Figure 10. Interaction of 4-hydrazinobenzoic acid with model peptide H_2N-AB1GGDap(aoa)W-OH. Peptide concentration was held constant at 50μM.

2.5 Synthesis of an amino acid modifier LB1

While the model peptides using AB1 allowed us to probe the utility of intramolecular iminoboronate formation for peptide cyclization, the synthesis of AB1 suffered from low yields in my hands. To circumvent these synthetic issues, a new 2-APBA derivative was developed, which involves a shorter synthetic route with improved efficacy for incorporation into peptides. The proposed molecule LB1 could be achieved in four steps with good purities in less than 1 week's time (Scheme 3).
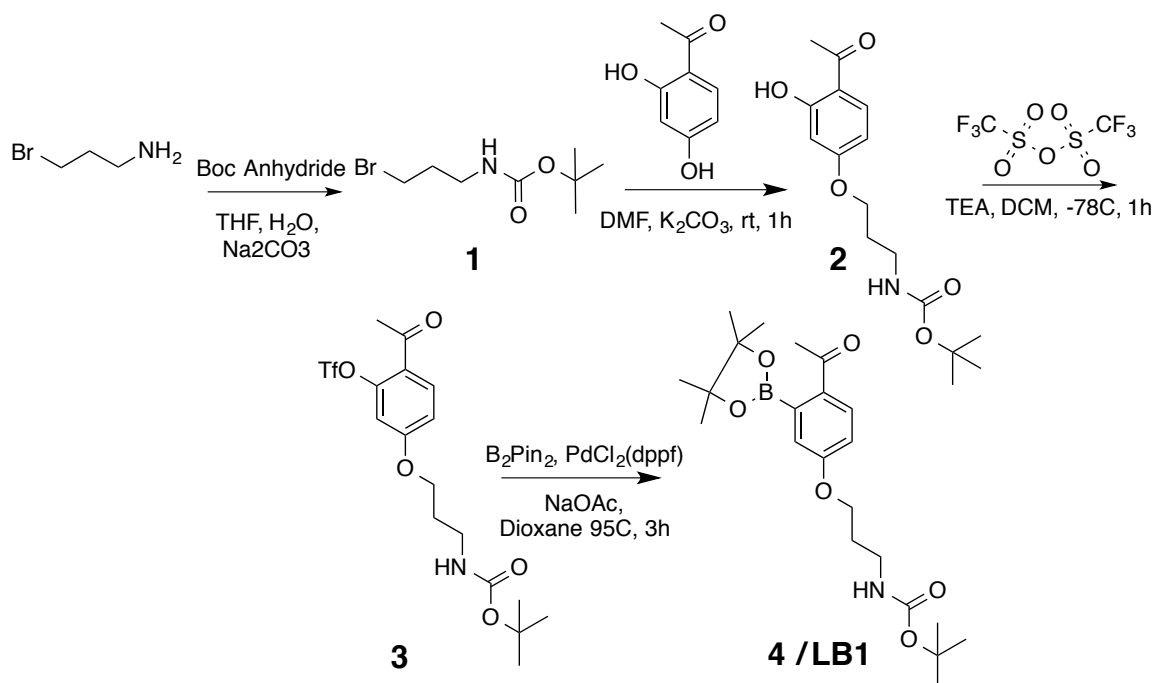
Scheme 3. Synthesis of LB1

  Boc protection of 3-bromopropanamine was achieved in 80-90% yields after reacting overnight with 2.5 equivalents of Boc anhydride and 1 equivalent of sodium carbonate. The resulting compound 1 was then coupled via S_N2 reaction with 2,4-dihydroxyacetophenone in the presence of potassium carbonate in DMF over 4 hours to yield intermediate 2 in modest yields. Triflation of this intermediate could be easily accomplished in 1 hour of reaction followed by easy column purification. Miyaura borylation of intermediate 3 was achieved in 3 hours with no the need for the addition of the dppf ligand. Overall the cost of this synthetic route was reduced by using cheaper starting materials and eliminating much solvent waste by the eliminating several low-yielding steps in AB1 synthesis.

  A benefit of this molecule is its ability to be modified to react with several natural amino acids for incorporation into peptides (Scheme 4). Simple Boc

deprotection of the amine with 80% trifluoroacetic acid in dichloromethane affords

intermediate 5, which can then be coupled to free glutamate and aspartate residues

through amide bond formation (Scheme 4). Additionally, the free amine can be

coupled with iodoacetamide derivatives to provide a reactive handle to couple free

cysteines.
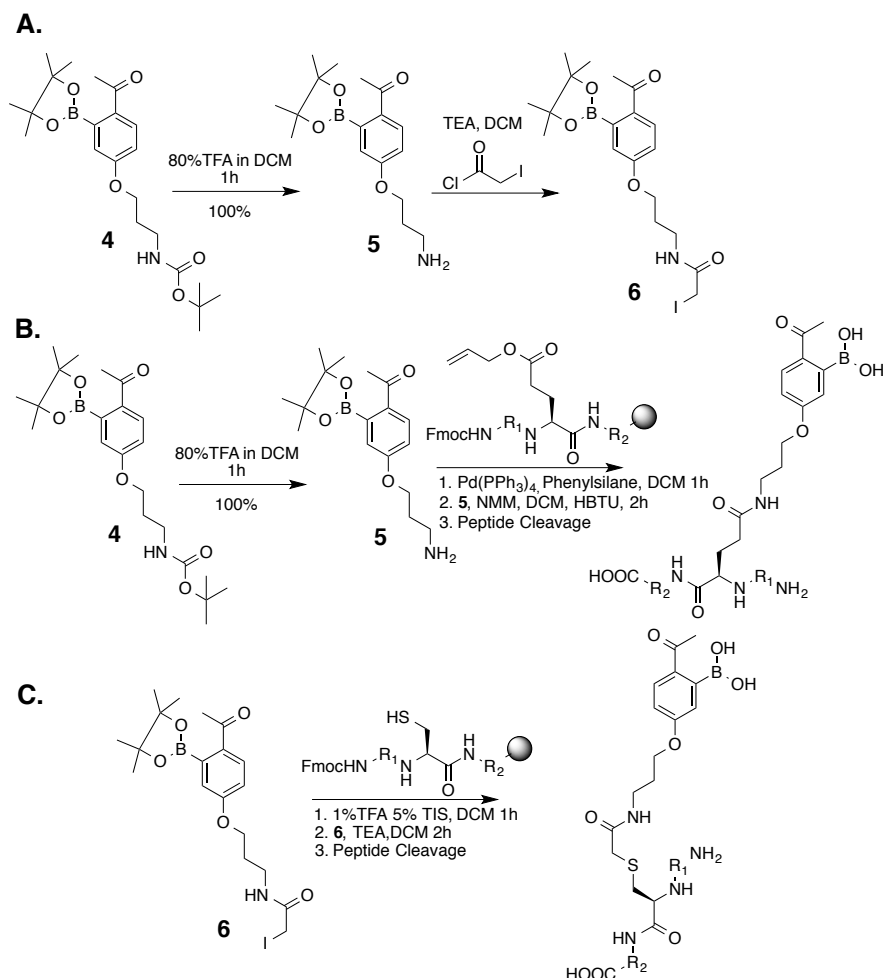
Scheme 4. Peptide modification with LB1

2.6 Application of LB1 into Magainin 2

With LB1 and its derivatives in hand we sought to prove its applicability to

peptides through on resin coupling. Our initial studies of this molecule involved the

utilization of a known 23 amino acid peptide, Magainin 2 (Figure11)[42]. This peptide

presents an alpha helical structure with antibiotic activities[42]. Intramolecular

peptide cyclization through iminoboronate chemistry could prohibit helix

formation, while cleavage of the intramolecular iminoboronate would regenerate
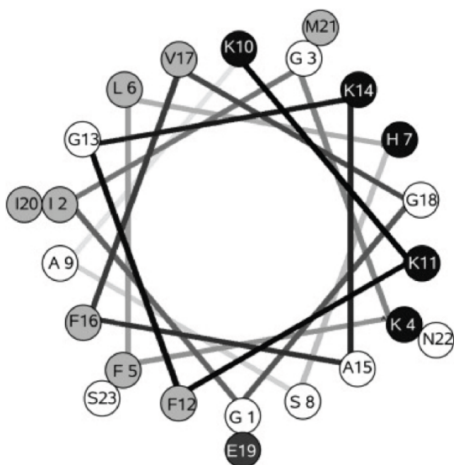
the helical and active peptide.

Figure 11. Helical representation of Magainin 2 (adapted from [42])

Our design of this model peptide included a mutation of glycine 3 to a

glutamate utilizing an allyl protected glutamate residue for orthogonal deprotection

prior to LB1coupling. We also mutated the C-terminal serine to a Dap(aoa) residue

to install an internal alpha nucleophile (Scheme 5). Our hypothesis for this design

proposed that the 2-APBA moiety and alpha nucleophile in the same peptide would

allow us to form intramolecular iminoboronates and cyclize the peptide. We further

postulated that this cyclization would disrupt the alpha helical structure of the

peptide, which could then be recovered by competition with other alpha

nucleophiles such as 4-hydrazinobenzoic acid or 6-aminooxyhexanoic acid as

previously shown with smaller peptide models (Figures 9 & 10).
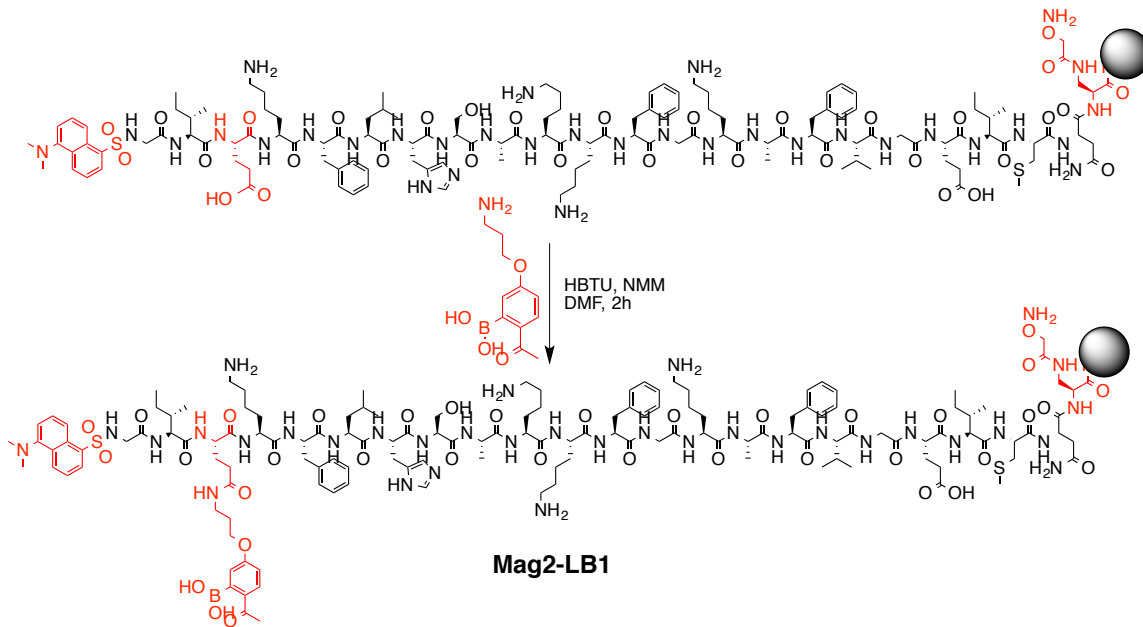
Scheme 5. Installment of LB1 into Magainin 2

To test this hypothesis we prepared the modified peptide Mag2-LB1 (Scheme 5) and confirmed its identity by LC-MS. To characterize the structure of this peptide we performed wavelength scans on a circular dichroism spectrometer, which showed the alpha helical character of the Magainin 2 wild type as well as the Magainin 2 mutant with LB1 modification. The CD data supported our hypothesis as the intensity of the mutant bearing an internal iminoboronate appeared to have a less intense alpha helical CD signature than the Magainin2 wild type at the same concentration.
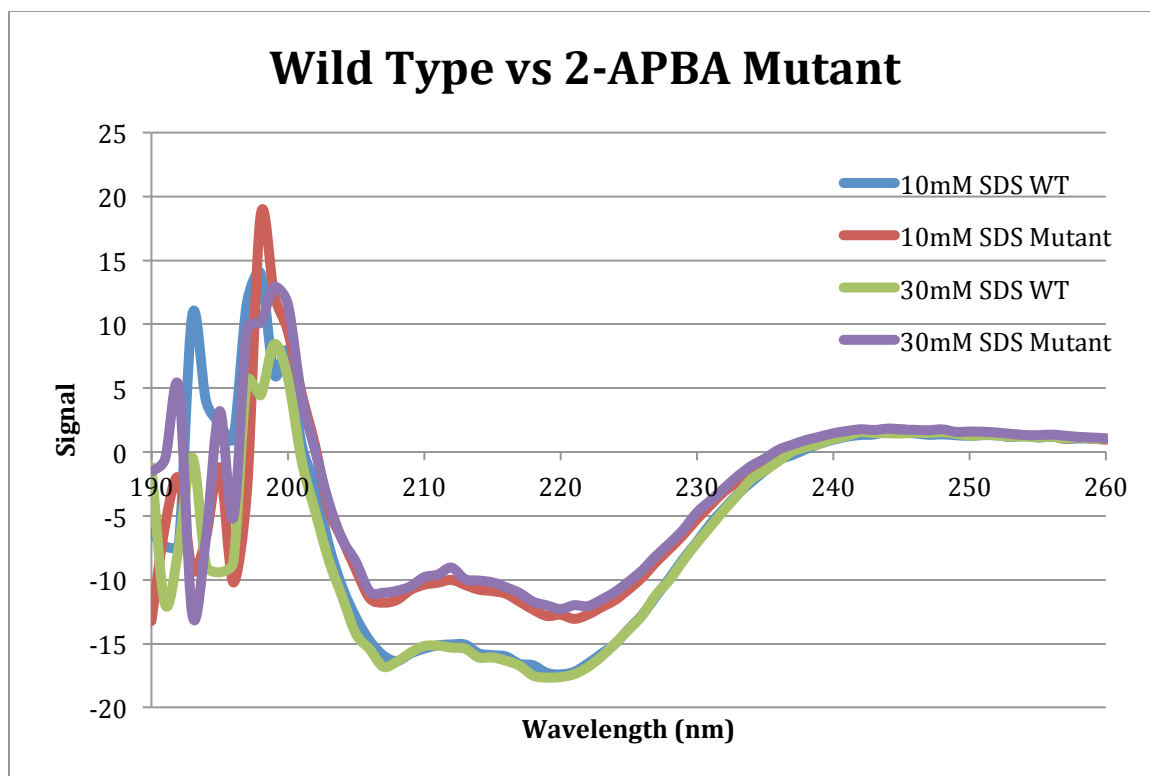
Figure 12. CD signatures of wild type and 2-APBA mutant in varying

concentrations of SDS micelles

In order to determine if we could increase alpha helical character of the

Magainin 2 mutant by adding an alpha nucleophile competitor, we subjected the

above peptide samples to varying concentrations of 4-hydrazinobenzoic acid.

Preliminary data from these experiments suggests the addition of HBA initially

decreases the alpha helical character, shown in blue in Figure 13. Upon further

addition of HBA, the peptide exhibits an increase in alpha helical character, though

not to the extent of the wild type Magainin. These data were somewhat strange and

inconclusive, but we hope to reproduce this data to confirm the trend. Unfortunately

peptide samples degraded before these experiments could be repeated to further

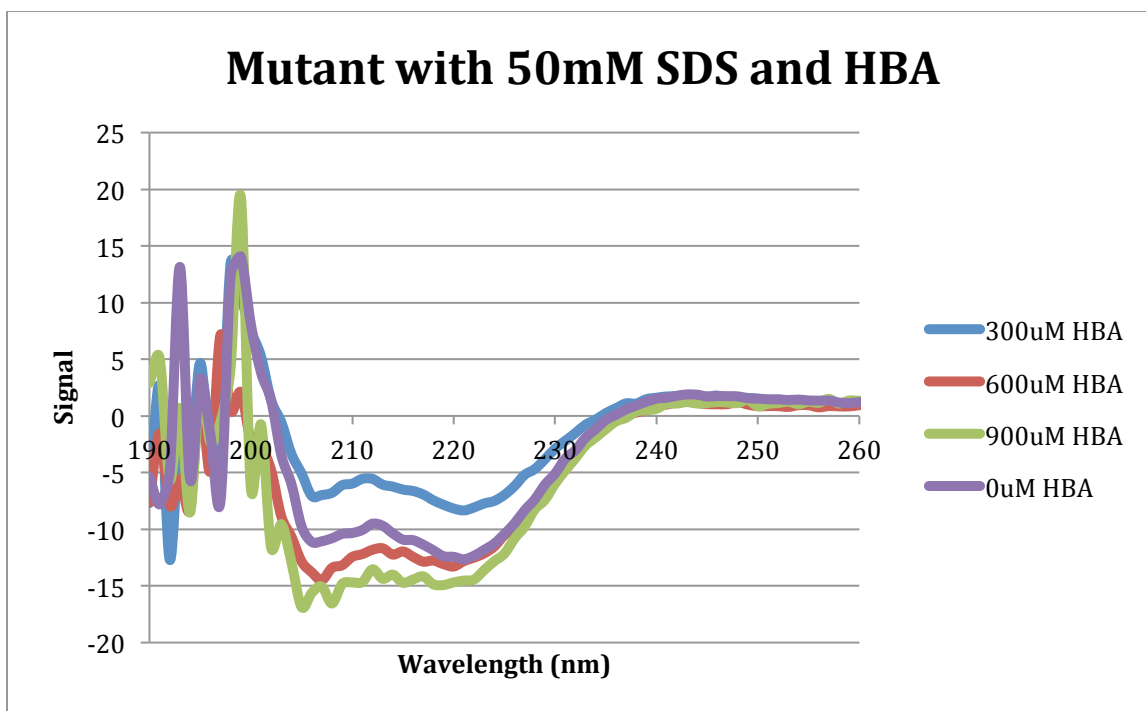confirm the tunability of this structure with small molecules.

Figure 13. Changes in alpha helicity of Mag2-LB1 through conjugation with HBA in the presence of 50mM SDS micelles

2.7 Development of a one-bead-one-compound library

In taking this chemistry of peptide cyclization further into the generation of one-bead-one-compound (OBOC) libraries, there were several other factors that needed to be considered. We needed to determine the optimal linker to attach our peptides to solid supports that would withstand the cyclization chemistry, and screening assays, yet able to provide efficient release of hit compounds from the resin for sequencing.

The first consideration we made when developing our OBOC library was selection of a solid support for the library. Typical resins are comprised of polystyrene, which are not compatible with aqueous screening assays. As we hope to screen our library against various liposomes in aqueous buffers, we needed to

find a resin that would perform in aqueous environments. To circumvent this issue, we chose to incorporate the TentaGel-S-NH$_2$ resin. This resin has a polystyrene core with a polyethyleneglycol shell to aid in interactions with aqueous phases for screening of libraries. It is functionalized with an amine handle and has a low loading (0.26mmol/g) to allow displayed peptides to interact with the target of interest rather than other peptides on resin.

Literature has shown that installing a C-terminal methionine can provide a means for peptide cleavage from the resin that is compatible with side chain protection and peptide sequencing. Unfortunately, treatment with cyanogen bromide not only cleaved the peptides from resin, but severely degraded the resin contaminating the obtained mass spectra with PEG units. While bulk cleavage of the resin still resulted in the observation of the desired peptide mass, single bead cleavage resulted in only PEG units in the mass spectra as their concentration significantly suppressed the signal of the desired peptide mass.

It was clear that the harsh cyanogen bromide treatment was not optimal so we chose to look into more benign approaches to peptide cleavage. One such route was the incorporation of photo labile linkers as shown in Figure 14. To determine the efficiencies and the kinetics of the photo cleavage of these linkers, short model peptide sequences were synthesized on the TentaGel resin. By installing a C-terminal methionine residue alongside the photo labile linkers, we had a positive control for peptide cleavage.
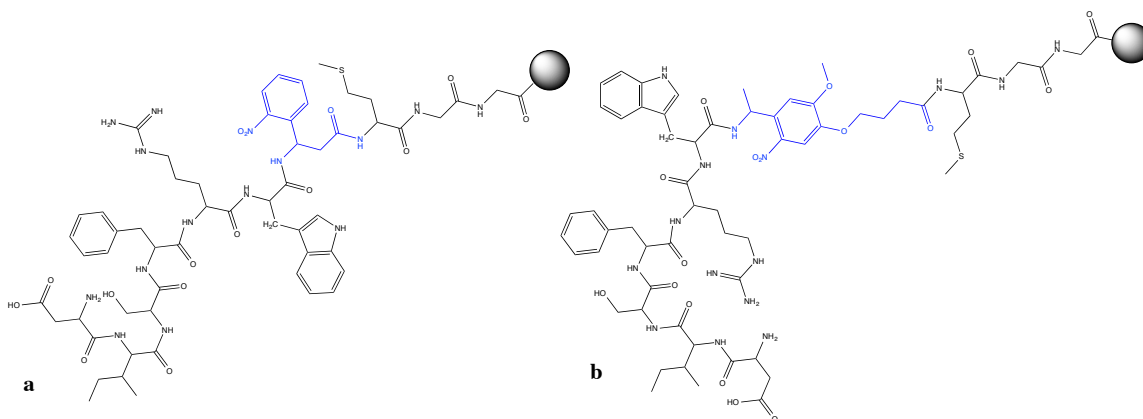
Figure 14. Model peptide synthesis incorporating a) ANP and b) Linker 2

Initially, photo cleavage of peptides bearing the ANP linker produced 5-15% yields when swelled in methanol and irradiated with 4 watt, 365nm UV light. To improve upon these yields we tried various conditions such as lining the platform for photo cleavage with foil to reflect UV light to maximize the cleavage from resin. We also tried cleaving peptides from resin in quartz cuvettes to allow more light to penetrate through to the sample than the previously used glass vials. Unfortunately, neither of these conditions appeared to improve upon the photo cleavage efficiency.

We then thought that perhaps the low photo cleavage yields might be due to slow kinetics. To test this theory we performed kinetic experiments using model peptide sequence H_2N-DISFRW-ANP linker-MGG-TentaGel resin in methanol under 4W UV light. Our results indicate low photo cleavage efficiencies are achieved even after 7 hours of irradiation (Figure 15).
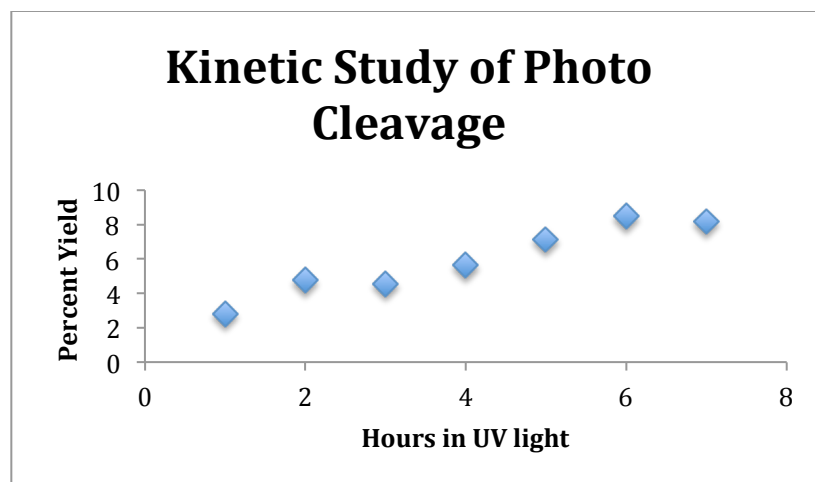
Figure 15. Photo cleavage over time of model peptide with ANP linker under 4W UV irradiation

Knowing the low photo cleavage is not due to slow kinetics, we attempted to utilize more powerful light sources. Specifically, both of the model peptides in Figure 13 were subjected to a 160W 365nm UV lamp. To our delight, we observed 98% photo cleavage from the resin bearing the Linker 2 and 49% photo cleavage from the sequence incorporating the ANP linker. With this information we believe we will be able to incorporate these linkers into our OBOC library to allow efficient release of peptides for sequencing..

2.8 Partial Edman Degradation Optimization

Since finding the optimal resin and linker for our library development, we proceeded to work on development of library sequencing strategies. One commonly used method for sequencing is Partial Edman degradation, which uses phenylisocyante (PIC) and phenylisothiocyanate (PITC) to generate capped and cleaved peptide fragments respectively. These capped and cleaved products generate a peptide ladder over time that can be decoded to reveal the peptide

sequence. As we began to explore this route to sequencing, we observed 3 distinct products from one round of degradation: the original peptide sequence, the PIC capped peptide and the N-terminal truncated product. These findings suggest incomplete capping of the N-terminus of the peptide.

Upon further consideration, we reasoned that the incomplete capping of the original peptide should provide one of the rungs on our peptide sequencing ladder and could eliminate our need for a capping agent. We tested this hypothesis with a model peptide H_2N-DISFRW-ANP-MGG-TentaGel Resin. The sequence was subjected to 30-minute incubation with phenylisothiocyanate, extensive washing and a 10-minute incubation with trifluoroacetic acid to cleave the N-terminal residue. After one round of these conditions approximately 80% of the peptide had degraded to produce the cycle 1 product with the deletion of the N-terminal aspartate residue (Figure 16).
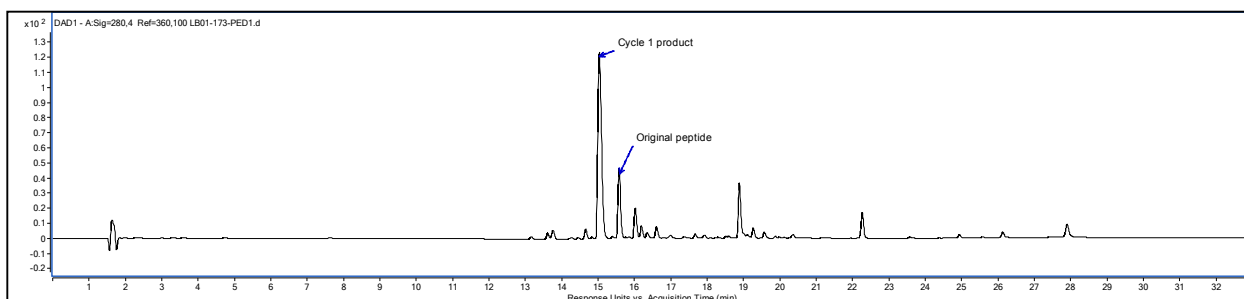

Figure 16. Results from Optimized Partial Edman Degradation conditions of H_2N-DISFRW-ANP-MGG-TentaGel Resin

With these relatively clean LC-MS traces, we sought to test the limits of these optimized degradation conditions without a capping agent. The model peptide sequence was subjected to three cycles of the optimized Edman Degradation protocol and the LC-MS data of the photo cleaved product was analyzed.

Remarkably, we observed all 3 deletion products and the original peptide sequence (Figure 17).
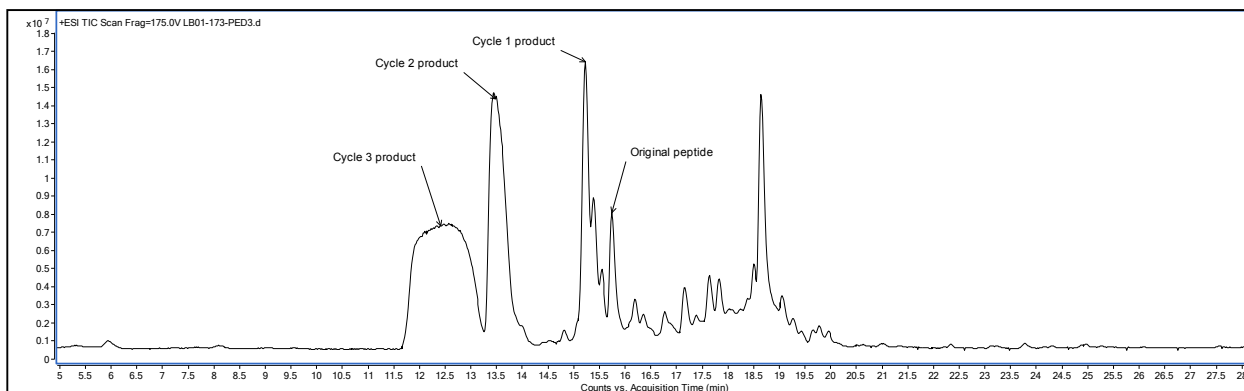

Figure 17. Analysis of the peptide H_2N-DISFRW-ANP-MGG-TentaGel Resin after 3 rounds of Partial Edman Degradation

2.9 Mass Spectrometry Method Optimization

While the optimized Edman Degradation could potentially be a viable route for library sequencing, a serendipitous finding was uncovered that may make sequencing easier. It appeared that further examination of the mass spectra of the individual peaks showed a clear and obvious pattern of y ions (Figure 18). This finding encouraged us to look further into optimizing LC-MS methods to enhance the presence of these for peptide sequencing.
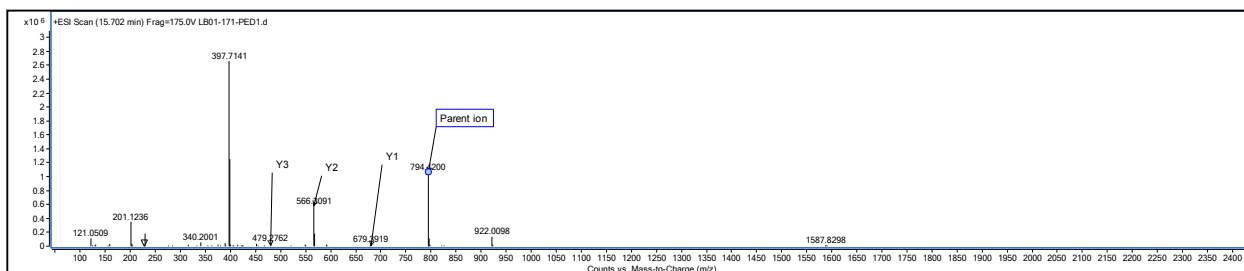

Figure 18. Initial appearance of y ion patterns in mass spectra

To improve sensitivity to detect low quantities of material we tested different parameters within our mass spectrometry method. The default capillary voltage setting is 4000V, which is sufficient for most peptide samples. By increasing this voltage to higher values, there is a stronger attraction between the ions and the capillary, pulling the sample ions in with greater force and increasing the amount of sample that enters the mass spectrometer. Through 100V incremental testing, we found 4500V to be the optimal voltage for detecting small peptide samples and producing enhanced signals.

The skimmer voltage was also a parameter we improved upon from the default setting of 75V. Increasing the voltage of the skimmer produces greater force to send the sample ions up the time of flight (TOF) tube. Increasing this voltage increased the amount of ions that reach the detector, thus increasing the observed signal of the sample. After careful screening it was observed that 115V produced the optimal signal.

Increased signal results from increased amount of ions that reach the detector and further understanding the various parameters that can create more ions led us to investigating the drying gas temperature and flow rate. By increasing the temperature of the drying gas from 300°C to 350°C we were able to dry more of the sample droplets as they left the nebulizer, allowing more sample to enter the instrument. We did dot see improvement of detection with higher temperatures due to what we believe may have been thermal degradation. The flow rate of the drying gas temperature can also be varied, but we opted to disregard this parameter in our method optimization. With increased flow rate, the droplets will become drier, but

may be pushed away from the capillary and will not enter the source. As the

molecular weight of sample ions increases this becomes less of an issue, but with the

small predicted molecular weights of our peptide library, we chose to leave the
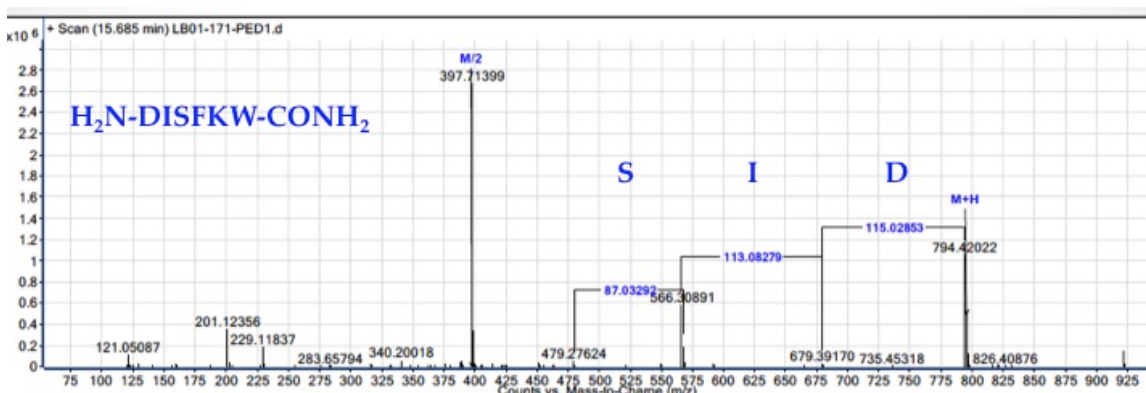
default flow rate of 8L/minute.

Figure 19. Single bead peptide data of model sequence

As shown in Figure 19 the peptide sample produced from photo irradiation

of a single bead can be observed with the optimized parameters described above.

Sequencing data are clearly observed in the mass spectrum of the sample in the

form of the observed y ion pattern.

2.10 Screening of peptide library

With sequencing methods and synthesis methods in place we sought to

develop peptide libraries and screen them against our target of interest.

Phosphatidylserine (PS) is a membrane lipid in mammalian cells that is expressed

exclusively in the inner leaflet of healthy cells (Figure 20). As the cell undergoes

apoptosis phosphatidylserine is translocated to the outer leaflet and is exposed to

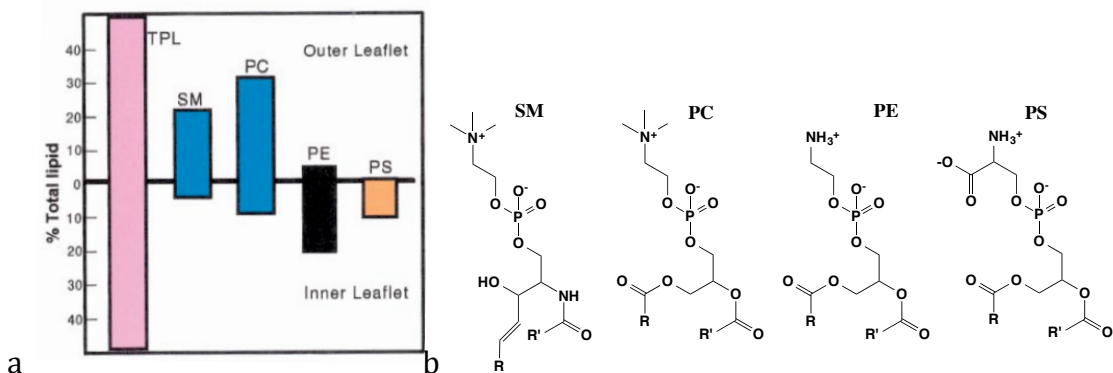the extracellular environment[38-40].

Figure 20. a) Distribution of common membrane lipids b) Structures of common lipid headgroups

As an initial library for screening we chose to use a redox reversible disulfide bridged peptide library made on the TentaGel resin via split-pool synthesis. Two cysteine residues were placed as reactive groups for disulfide formation at the N and C termini of the peptide (Figure 21). We incorporated the ANP linker as a means to photo cleave the library members from the resin, as well as a C-terminal methionine as a control for peptide cleavage from the resin. A GSGS repeat was placed between the photo labile linker and the C terminal cysteine to increase the mass to a detectable range for y ions in our mass spectrometer. The N-terminal tryptophan and phenylalanine residues were incorporated as a hydrophobic anchor to assist library anchoring into liposomes. The residues between the C-terminal cysteine and the N-terminal tryptophan were varied through split-pool synthesis. Post synthesis the library was oxidized with hydrogen peroxide incubation to cyclize the library for screening.
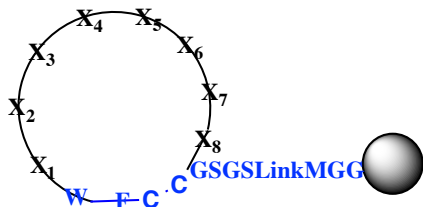
Figure 21. Peptide library design

      Preparation of liposomes is a well-known laboratory technique in our lab so we sought to screen our preliminary library against fluorescently labeled liposomes. We synthesized a NBD labeled 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidyl ethanolamine using (POPE, Scheme 6), which we incorporated as 0.1% of the total lipid composition of our liposomes for screening. 80% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 20% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) comprised the remainder of the liposome lipids.
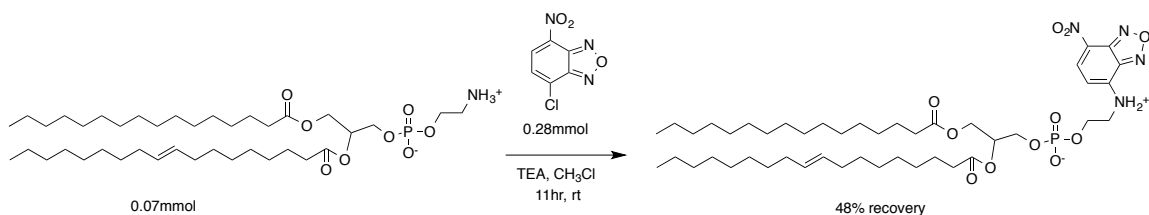
Scheme 6. NBD labeling of POPE

      We attempted to sort the DOPS binding peptides using flow cytometry, but initial screening of the library with our fluorescently labeled liposomes clogged the lines of the flow cytometer. As an alternative method of screening, the library was incubated with 1mM liposome stock solution. The beads were then washed either one, or two times and imaged under a fluorescence microscope. Data from these experiments show that this may be a viable approach to library screening as beads

show varied fluorescence intensities, presumably due to varied degree of binding
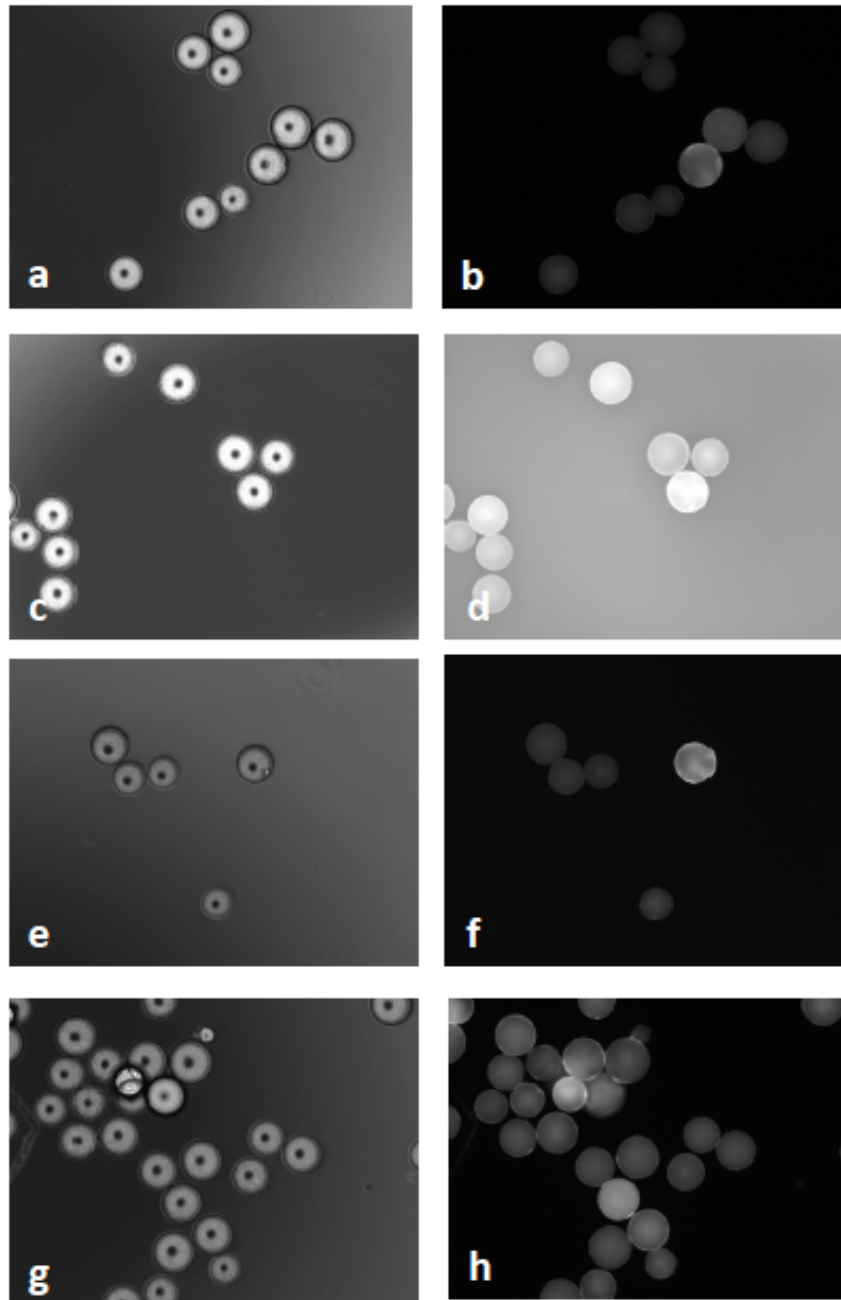
(Figure 22).

Figure 22. Fluorescence images of peptides on resin upon binding to liposomes a) Beads alone bright field b) beads alone NBD fluorescence c)1mM liposome bright field d) 1mM liposome NBD fluorescence e)1mM liposome, 1x wash bright field f) 1mM liposome NBD fluorescence g) 1mM liposome, 2x wash bright field h) 1mM liposome NBD fluorescence

Chapter 3: Conclusions and Future Directions

We have demonstrated the accessibility of cyclic peptide scaffolds on resin through a variety of synthetic strategies. In addition to the head to tail cyclization, we have shown we can cyclize peptides through side chain crosslinking with organic crosslinkers or through incorporation of unnatural amino acids. Furthermore, we have shown the synthesis and utilization of reversibly cyclized of peptides through small molecule cleavage of the intramolecular iminoboronate linkage. This development will allow us to create a cyclic peptide library on resin that can be screened in cyclic forms, while able to revert back to its linear state for sequencing.

We have begun to develop this cyclic peptide library on the TentaGel resin using the optimized photo labile linker chemistry for clean release of the peptides from the resin. Data presented herein supports the use of these UV sensitive linkers over chemically labile linkers, such as methionine, as a way to release desired peptides from the resin. Library members will include a terminal Dap(aoa) residue and an unnatural LB1 moiety to serve as chemical handles for cyclization. Residues between the imino boronate forming residues will be incorporated via split-pool synthesis to ensure diversity in peptide sequences.

We have established preliminary assays to determine peptide sequences that bind lipids of biological relevance. Fluorescently labeled liposomes showed adequate fluorescence in microscopy studies to identify beads containing sequences of interest. Through these methods we will be able to select for sequences that tightly bind phosphatidylserine to label apoptotic cells. We can further modify the

liposome composition to identify library members with selectivity for other lipids of interest or other membrane components.

Optimization of the sequencing method had allowed us to detect a single bead's worth of peptide. Further method optimization through parameter manipulation has provided access to valuable sequencing information through generation of y ion sequences in mass spectra. This ensures the success of our proposal to synthesize, screen, and sequence OBOC peptide libraries.

Chapter 4: Materials and Methods

I. Materials and General Methods

Fmoc-L-Dap(boc-aoa)-OH and L-cystine-bis(tertbutyl ester) were purchased from Chem-Impex International Inc. (Wood Dale, IL). All other Fmoc protected amino acids, Wang resin, Rink Amide MBHA resin and Fmoc-Glu(Wang resin)-OAllyl were purchased from Advanced Chemtech (Louisville, KY). O-Benzotriazole-N,N,N'N'-tertramethyl-uronium hexafluoro phosphate (HBTU), Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from EMD Millipore (Billerica, MA). TentaGel resin was purchased from Anaspec (Freemont, CA). Boc Anyhdride, Trifluoromethanesulfonic anhydride, 2,2,2-trichloroacetimidate, nitrobenzene, dicyclohexylcarbodiiimide, dimethylaminopyridine were purchased from Sigma Aldrich (Milwaukee, WI). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidyl ethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine were purchased from Avanti Polar Lipids (Alabaster, AL). Peptide synthesis was conducted on the Tribute Peptide Synthesizer (Protein Technologies Tuscon, AZ). ^{1}H, ^{13}C, ^{11}B NMR data were collected on the Varian 500MHz spectrometer. LC-MS data was collected on the Agilent 1260 series HPLC and Agilent 6520 LCTOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Circular Dichroism was conducted on the Aviv 420 spectrometer (Biomedical Inc., Lakewood, NJ). LUV's were prepared using the Liposofast Mini Extrusion System (Avanti Polar Lipids, Alabaster, AL). Fluorescence microscopy was conducted on the Zeiss Axio Observer.

A1 (Thornwood, NY). UV experiments were conducted on the Thermo Scientific Nanodrop 2000c (Waltham, MA).

II. Synthesis
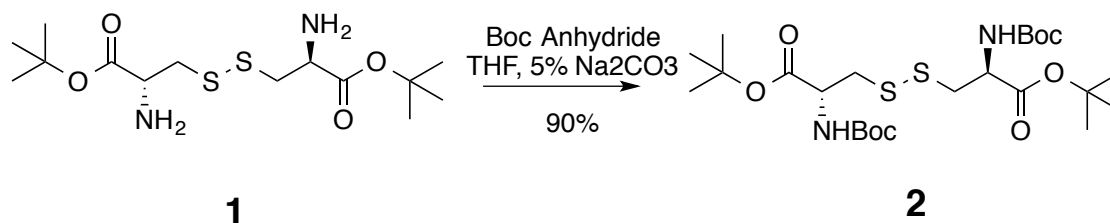
Scheme S1. Preparation of 2

(1) Preparation of *tert*-butyl *S*-(((*R*)-3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)thio)-*N*-(*tert*-butoxycarbonyl)-*D*-cysteinate

L-cystine bis(tert-butyl ester) (5.0062g, 118mmol) was dissolved in 10% Sodium carbonate solution (50mL). Boc anhydride (6.5g, 299mmol) was dissolved in tetrahydrofuran (50mL) and added to the reaction solution and allowed to stir at room temperature for 24 hours. The reaction solution was acidified to pH2 with 2N hydrochloric acid, extracted with ethyl acetate (3x 100mL), washed once with brine and concentrated to give a white solid (7.2g, 91%).
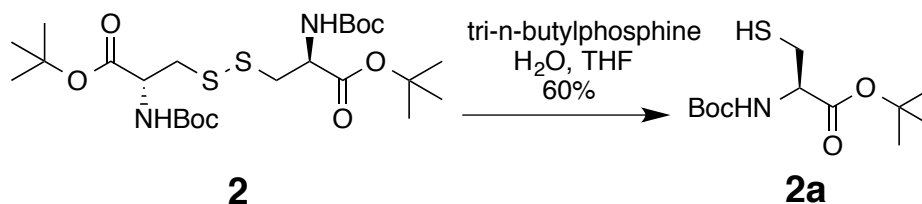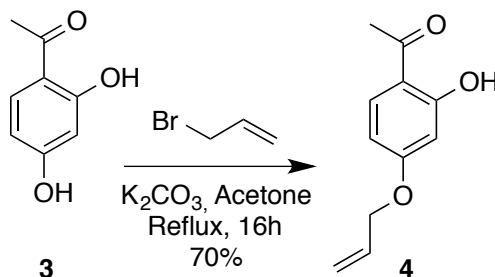

Scheme S2. Preparation of 2a

(2) Preparation of *tert*-butyl (*tert*-butoxycarbonyl)-*L*-cysteinate

tert-butyl *S*-(((*R*)-3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-

oxopropyl)thio)-*N*-(*tert*-butoxycarbonyl)-*D*-cysteinate (7.2g, 13mmol) was

dissolved in tetrahydrofuran (65mL) with tributylphosphine (7mL, 26mmol) and

water (2.7mL). After reacting overnight, the solution was concentrated and column

purified on silica gel (hexanes/ethyl acetate 10:0-9:1) to yield white solid (7.1g,

99%).

Scheme S3. Preparation of 4

**(3) Preparation of 1-(4-(allyloxy)-2-hydroxyphenyl)ethan-1-one**

Sodium iodoide (7.28g, 49 mmol) and allyl bromide (4.2mL, 49 mmol) were refluxed

in acetone (75mL) for one hour. 2,4-dihydroxyacetophenone (5.00g, 33 mmol) was

then added with potassium carbonate (4.14g, 30 mmol) in acetone (70mL) and

allowed to react for 22 hours at 65°C. Reaction was concentrated in vacuo and

resuspended in water (50mL). Extracted with ethyl acetate (3x50mL) and washed

once with brine (50mL). Purified with silica gel chromatography (hexanes/ethyl

acetate 4:1) to produce a white solid (4.4g, 70%).
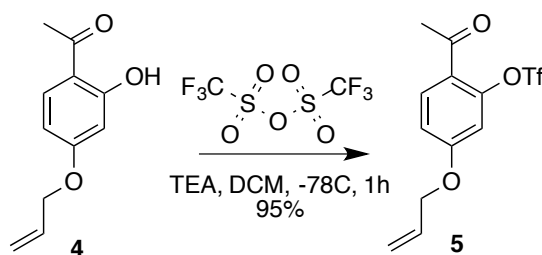
Scheme S4. Preparation of 5

(4) Preparation of 2-acetyl-5-(allyloxy)phenyl trifluoromethanesulfonate

1-(4-(allyloxy)-2-hydroxyphenyl)ethan-1-one (4.55g, 24 mmol) was dissolved in

dichloromethane (182mL) and chilled to -78°C. Triethyl amine (13.4mL, 96 mmol)

and trifluoromethanesulfonic anhydride (4.2mL, 25) were added drop wise to the

solution. After stirring for 1 hour, saturated sodium bicarbonate (90mL) was added.

This was extracted with dichloromethane (2x 90mL) and washed once with brine

(80mL). Concentrated to give a black oil, which was then purified with sicila gel

chromatography (hexanes/ethyl acetate 8:2) to afford an off white solid (4.5g)
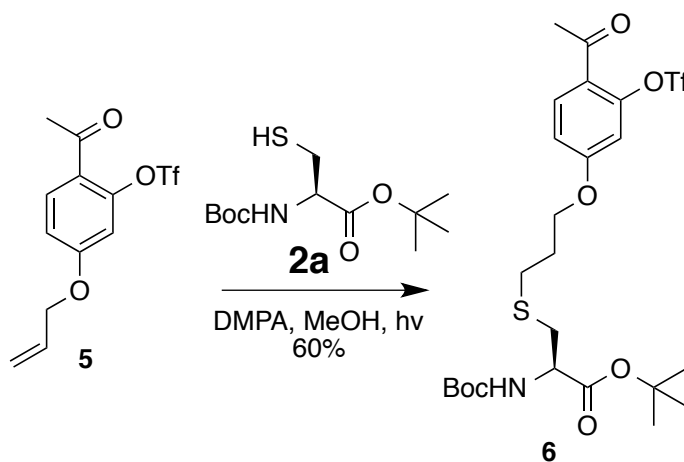
Scheme S5. Preparation of 6

(5) Preparation of *tert*-butyl *S*-(3-(4-acetyl-3-

(((trifluoromethyl)sulfonyl)oxy)phenoxy)propyl)-*N*-(*tert*-butoxycarbonyl)-*L*-

cysteinate

2-acetyl-5-(allyloxy)phenyl trifluoromethanesulfonate (773mg, 2.4mmol) and *tert*-

butyl (*tert*-butoxycarbonyl)-*L*-cysteinate (725mg, 2.6mmol) were dissolved in

methanol (1.65mL) with 2,2-dimethoxy-2-phenylacetophenone (104mg, 0.4mmol).

The reaction mixture was placed under 160W, 365nm UV light for 3 hours,

concentrated and purified by silica gel chromatography (hexanes/ethyl acetate 85:15) to give an off white solid (2.652g, 60%).
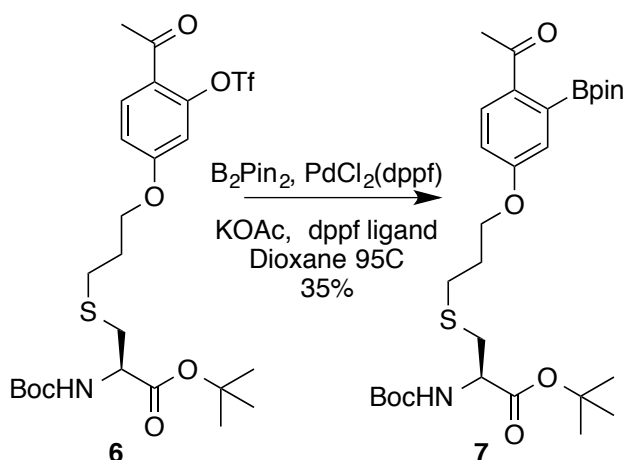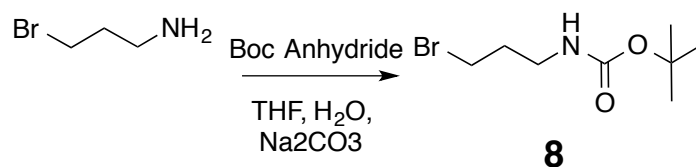

Scheme S6. Preparation of 7

(6) Preparation of *tert*-butyl *S*-(3-(4-acetyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)propyl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate

tert-butyl *S*-(3-(4-acetyl-3-((((trifluoromethyl)sulfonyl)oxy)phenoxy)propyl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (1.87g, 3.3mmol), bis(pinacolato)diboron (1.967, 8.3mmol), 1,1′-Bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane (201mg, 0.264mmol), 1,1′-Ferrocenediyl-bis(diphenylphosphine)(136mg, 0.264mmol), potassium acetate (604mg, 6.6mmol), 4Å molecular sieves and dioxane (21 mL) were purged with argon for 10 minutes in a round bottomed flask with stirring. The reaction was then allowed at 95°C to proceed for 2 hours and 20 minutes and then 160mL of water was added. The solution was extracted with ethyl acetate (3x80mL) and washed once with brine (80mL). The concentrated organic layer was column purified on silica gel (hexanes/ethyl acetate 7:3-5:5) to produce a yellow oil (455mg, 25%).
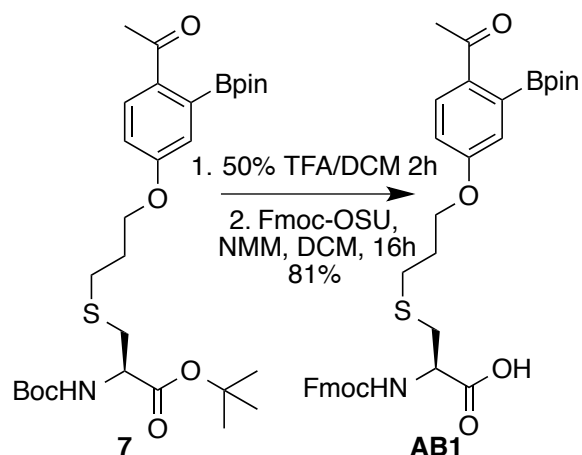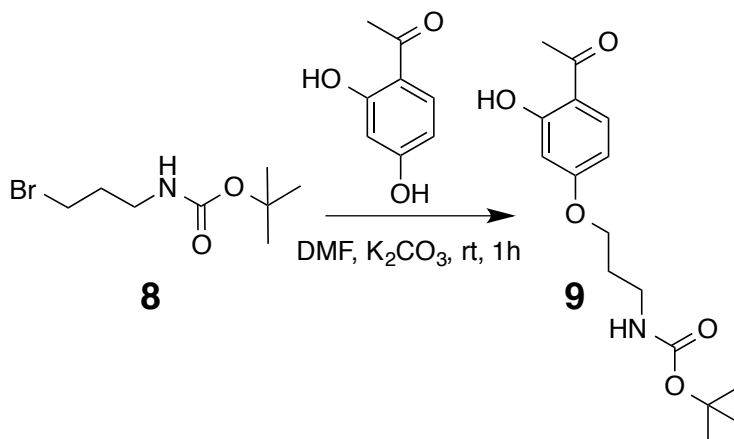
Scheme S7. Preparation of AB1

(7) Preparation of *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*S*-(3-(4-acetyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)propyl)-*L*-cysteine

tert-butyl *S*-(3-(4-acetyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)propyl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (455mg, 0.8mmol), trifluoroacetic acid (6.76mg, 8.8mmol) were allowed to react for 1 hour in dichloromethane (1.69mL). After 1 hour the reaction was concentrated and TFA was removed by azeotroping with DCM five times. 9-Fluorenylmethyl *N*-succinimidyl carbonate (186mg, 0.6mmol) and N-methylmorpholine (434uL, 4mmol) were added to the vial with dichloromethane (15mL) and allowed to stir for 1 hour. After 1 hour 2N hydrochloric acid (150mL) was added and the solution was extracted with ethyl acetate (3x50mL) and washed once with brine (50mL). The organic layer was dried over anhydrous sodium sulfate and concentrated. The product precipitated as a yellow-orange solid from 5% Ethyl acetate, 95% hexanes solution overnight (206mg, 80%).

Scheme S8. Preparation of 8

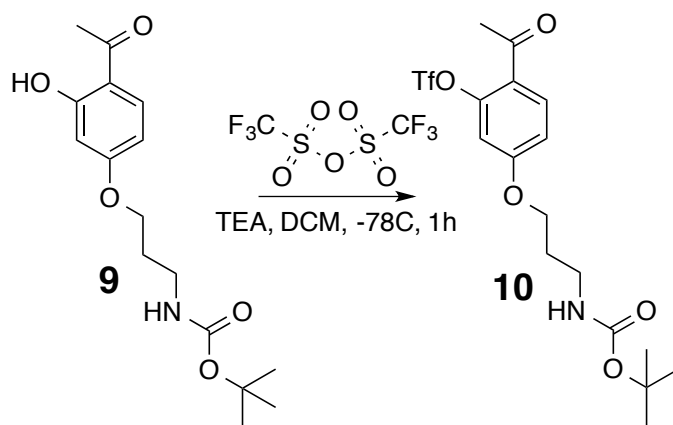## (8) Preparation of *tert*-butyl (3-bromopropyl)carbamate

3-bromopropanamine hydrobromide (1.0g, 4.6mmol), boc anhydride (2.5g, 11mmol) and sodium carbonate (488mg, 4.6mmol) were dissolved in tetrahydrofuran (19mL) and water (19mL). After reacting 24 hours the reaction was acidified to pH 2 using 2N hydrochloric acid and extracted with ethyl acetate (3x 30mL). The organic layer was concentrated and purified on silica gel chromatography (hexanes/ethyl acetate 7:3) to give the product as a white solid (889mg, 82%). ^{1}H NMR (500MHz, chloroform-d$_3$) δ: 4.62(broad, 1H), 3.40 (t, 2H), 3.22 (q, 2H), 2.01 (m, 2H), 1.42 (s, 12H).

Scheme S9. Preparation of 9

(9) Preparation of *tert*-butyl (3-(4-acetyl-3-hydroxyphenoxy)propyl) carbamate

tert-butyl (3-bromopropyl)carbamate (1.389g, 6mmol), 2,4-dihydroxyacetophenone (713mg, 5mmol), potassium carbonate (2.6g, 19mmol) were dissolved in N,N-dimethylformamide (9.4mL). After stirring at room temperature for 4 hours the reaction as acidified to pH 2 with 2N hydrochloric acid and extracted with ethyl ether (5x 10mL). The ether layer was washed once with water (10mL) and was then dried over anhydrous sodium sulfate. Purified on silica gel column (hexanes/ethyl acetate 7:3-5:5) to give the product as a white solid (441mg, 24%). ESI+: MH+: calculated 209.11, observed 210.11. ^{1}H NMR (500MHz, chloroform-d$_1$) δ: 7.8(d, 1H), 6.54 (d, 1H), 6.41(s, 1H), 4.1 (t, 2H), 3.25 (q, 2H), 2.57 (s, 3H), 2.15 (m, 2H), 1.42 (s, 9H).

Scheme S10. Preparation of 10

(10) Preparation of 2-acetyl-5-(3-((*tert*-butoxycarbonyl)amino)propoxy)phenyl trifluoromethanesulfonate

tert-butyl (3-(4-acetyl-3-hydroxyphenoxy)propyl) carbamate (500mg, 2mmol) was dissolved in dichloromethane (17mL) and was chilled to -78°C. Triethylamine (1.17mL, 8mmol) and trifluoromethane sulfonic anhydride (531uL, 3.2mmol) were added dropwise. The reaction was allowed to stir for 1 hour and then saturated

sodium bicarbonate was added (8.4mL) and extracted with ethyl acetate (3x 20mL).

The organic layer was concentrated and purified on a silica gel column

(hexanes/ethyl acetate 8:2-7:3) to yield the product as yellow oil (609mg, 71%).

ESI+:MH+: calculated 427.09. ^{1}H NMR (500MHz, chloroform-d_1) δ: 7.82 (d, 1H), 6.95

(d, 1H), 6.81 (d, 1H), 4.7 (broad, 1H), 4.1 (t, 2H), 3.35 (q, 2H), 2.6 (s, 3H), 2.05 (p,
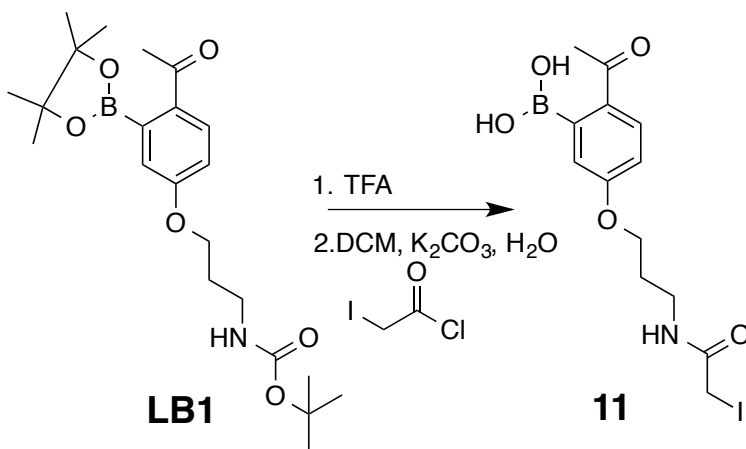
2H), 1.42 (s, 9H).
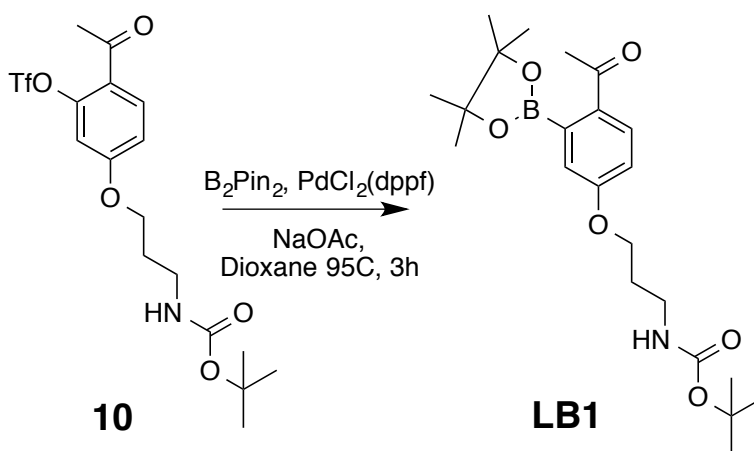
Scheme S11. Preparation of LB1

(11) Preparation of *tert*-butyl (3-(4-acetyl-3-(4,4,5,5-tetramethyl-1,3,2-

dioxaborolan-2-yl)phenoxy)propyl)carbamate (LB1)

2-acetyl-5-(3-((*tert*-butoxycarbonyl)amino)propoxy)phenyl

trifluoromethanesulfonate (586mg, 1.4mmol), bis(pinacolato) diboron (713mg,

2.8mmol), 1,1′-Bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex

with dichloromethane (102mg, 0.14mmol), sodium acetate (344mg, 4.2mmol) were

placed under vacuum and allowed to stir for 30 minutes. Dioxane (1mL) was added

and the reaction was purged with argon for 10 minutes. The reaction was allowed to

proceed at 95°C for 3 hours. Added diethyl ether (20mL) and concentrated. The

concentrate was purified on silica gel chromatography (dichloromethane/ether 10:0-9:1) to afford an orange gel (162mg, 28%). ESI+:MH+: calculated 419.25, observed 420.26. ^{1}H NMR (500MHz, chloroform-d$_1$) δ:7.9 (d, 1H), 6.9 (s, 1H), 6.85 (d, 1H), 4.7 (t, 1H), 4.08 (t, 2H), 3.3 (q, 2H), 2.55 (s, 3H), 1.99 (p, 2H), 1.42 (s, 21H).


Scheme S12. Preparation of 11

(12) Preparation of 1-(4-(3-aminopropoxy)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one

tert-butyl (3-(4-acetyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)propyl)carbamate (20mg, 0.024mmol) was dissolved in 80% trifluoroacetic acid in dichloromethane (1mL) and allowed to stir for 1 hour. Trifluoroacetic acid was azeotroped off using dichloromethane three times and the resulting material was resuspended in 80% trifluoroacetic acid in dichloromethane (1mL) and allowed to stir for 1 hour. Trifluoroacetic acid was azeotroped off with dichloromethane four times. Iodoacetyl chloride (4.9mg, 0.024mmol), potassium carbonate (4mg, 0.030mmol) were added to the resulting material at 0°C in chloroform (100uL) and water (18uL) and allowed to warm to room temperature

and react for 5 hours. The reaction was then dried and purified by reversed phase preparatory HPLC (C-18 column, water/acetonitrile 95:5-5:95) to give the desired product as an oil (3.16mg). ESI+:MH+: calculated 319.20, observed 320.20.
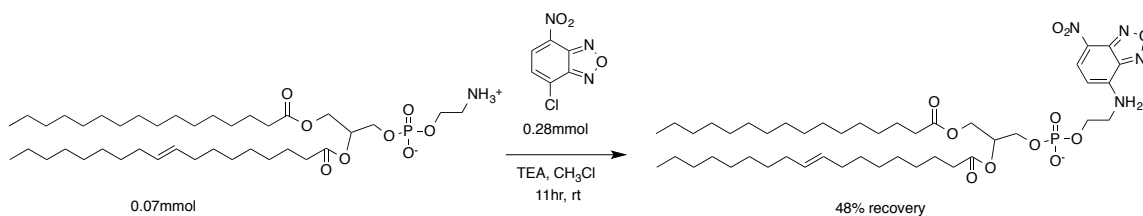
(13) Preparation of NBD-POPE

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (51.82mg, mmol) and 4-chloro-7-nitrobenzofuran (62mg, mmol) were dissolved in triethylamine (100uL) and chloroform (1mL) and allowed to react in the dark for 6 hours. The reaction was then diluted with chloroform and extracted with 10% sodium carbonate solution. Purified on silica gel (dichloromethane/methanol 95:5-90:10) to give product (29mg, 48%).

(14) Preparation of cLac on resin

Peptides were synthesized using Fmoc/tbutyl peptide chemistry on Fmoc-Glu(Wang resin)-O-allyl as the solid support. Synthesis was carried out on a 0.1mmol scale with 5 equivalents of commercially available amino acid and HBTU for each coupling step. Peptides were cyclized on resin by deprotecting the resin for 1 hour with palladium tetrakis(triphenylphosphine) (60mg, mmol), and phenylsilane (0.3mL, mmol) in dichloromethane (2mL). Post deprotection the peptides were washed with dichloromethane (9x 2mL). Peptides were cyclized using 1 equivalent of PyBOP (4mg, mmol) in N,N'-dimethylformamide (500uL) at 82°C for 4 hours.

Peptides were cleaved from the resin using Reagent K (80% TFA, 5% water, 2.5% ethanedithiol, 5% thioanisole and 7.5% phenol).

(15) Preparation of H$_2$N-ACVSWHGGCHPQGDICG-OH

The peptide was prepared using standard Fmoc/t-butyl chemistry with 5 equivalents of commercially available amino acids and HBTU on the Fmoc-Gly-Wang resin solid support. Peptides were cleaved from resin using Reagent K (80% TFA, 5% water, 2.5% ethanedithiol, 5% thioanisole and 7.5% phenol) and purified by reversed phase preparatory HPLC (Waters prep LC, Jupiter 10u, C4, 300A column).

(16) Bicyclization of H$_2$N-ACVSWHGGCHPQGDICG-OH

Purified H$_2$N-ACVSWHGGCHPQGDICG-OH was cyclized by incubating the peptide with 1 equivalent of tris(bromomethyl benzene)for 1 hour at 30°C in 60mM ammonium carbonate solution (500uL) and acetonitrile (500uL).

(17) Preparation of H$_2$N-AB1-RG-Dap(aoa)-GQIHGFWDGGG-OH and H$_2$N-AB1-RKGGQIHGFWDGGG-OH H$_2$N-AB1-RGKGQIHGFWDGGG-OH and H$_2$N-AB1-R-Dap(aoa)-GGQIHGFWDGGG-OH

Peptides were synthesized on 0.05mmol scale by standard Fmoc/tbu chemistry with 5 equivalents of commercially available amino acids and HBTU. AB1 was coupled to the sequence using 3 equivalents of the unnatural amino acid and 3 equivalents of HBTU. The peptides were cleaved from resin using Reagent K (80% TFA, 5% water, 2.5% ethanedithiol, 5% thioanisole and 7.5% phenol) and purified by reversed phase preparatory HPLC (Waters prep LC, Jupiter 10u, C4, 300A column).

(18) Preparation of H$_2$N-AB1-RG-Dap(aoa)-GGG-Dap(aoa)-W-OH

Peptides were synthesized on 0.05mmol scale by standard Fmoc/tbu chemistry with 5 equivalents of commercially available amino acids and HBTU. AB1 was coupled to the sequence using 3 equivalents of the unnatural amino acid and 3 equivalents of HBTU. The peptides were cleaved from resin using Reagent K (80% TFA, 5% water, 2.5% ethanedithiol, 5% thioanisole and 7.5% phenol) and purified by reversed phase preparatory HPLC (Waters prep LC, Jupiter 10u, C4, 300A column).

(19) Preparation of Magainin 2

Peptides were synthesized on 0.1mmol scale by standard Fmoc/tbutyl chemistry with 5 equivalents of commercially available amino acids and HBTU on the Rink Amide MBHA resin solid support. Peptides were deprotected on resin then incubated overnight with triethylamine (40uL, mmol), DMF (2.96mL) and dansyl chloride (40mg, 0.15mmol, 3 equivalent to resin) in the dark. The peptides were cleaved from resin using Reagent K (80% TFA, 5% water, 2.5% ethanedithiol, 5% thioanisole and 7.5% phenol) and purified by reversed phase preparatory HPLC (Waters prep LC, Jupiter 10u, C4, 300A column).

(20) Preparation of Magainin 2 Mutant with LB1

Peptides were synthesized on 0.05mmol scale by standard Fmoc/tbutyl chemistry with 5 equivalents of commercially available amino acids and HBTU on the Rink Amide MBHA resin solid support. The glutamate residue was deprotected with palladium tetrakis(triphenylphosphine) (60mg, mmol), phenyl silane (0.300mL, mmol) and dichloromethane (2mL) for 1 hour. The resin was then washed with dichloromethane (6x 2mL) and deptrotected LB1 (60mg, 0.15mmol) was coupled

using HBTU (55mg, 0.15mmol) in 0.4M NMM in DMF. Peptides were deprotected on resin then incubated overnight with triethylamine (40uL, mmol), DMF (2.96mL) and dansyl chloride (40mg, 0.15mmol, 3 equivalent to resin) in the dark. The peptides were cleaved from resin using Reagent K (80% TFA, 5% water, 2.5% ethanedithiol, 5% thioanisole and 7.5% phenol) and purified by reversed phase preparatory HPLC (Waters prep LC, Jupiter 10u, C4, 300A column).

III. Measurements

(1) Model iminoboronate formation studies

50uM 2-acetylphenylboronic acid was prepared in 1x PBS buffer at pH 7. 6-aminooxyhexanoic acid or lysine each prepared in 1x PBS buffer was titrated into the 2-APBA stock and the UV spectra was recorded.

(2) Peptide iminoboronate formation studies

500uM peptide was prepared in 1x PBS buffer and the UV absorbance was recorded.

(3) Small molecule competition assay with model peptides

H_2N-AB1-GG-Dap(aoa)W-OH was prepared as a 50uM stock solution and varying concentrations of 6-aminooxyhexanoic acid or 4-hydrazinobenzoic acid were tritrated into samples. The samples were then analyzed by LC-MS and percent linear and cyclic peptides were calculated by integrating the UV trace at 254nm.

(4) Circular dichroism of Magainin 2 and Magainin 2 LB1 mutant

Peptides were prepared in 50uM concentration 1x PBS buffer in the presence of SDS micelles. For each spectrum two scans were obtained and averaged and plotted.

(5) Circular dichroisim with HBA and Magainin 2 LB1 mutant

50uM mutant peptide in the presence of 50mM SDS micelles in 1x PBS buffer and 4-hydrazinobenzoic acid prepared in 1x PBS buffer was titrated in prior to scans. Wavelength scans were obtained in duplicate and the trials were averaged and plotted.

(6) Cyanogen bromide cleavage of peptides from TentaGel resin

peptides on the TentaGel resin were placed in 40mg/mL cyanogen bromide in 70% trifluoroacetic acid in water solution in the dark and were incubated at room temperature overnight.

(7) Photo cleavage

Resin was placed in clear glass vials with methanol and placed under 365nm UV light. Optimal results came from light sources with 160W power for 1 hour.

(8) Partial Edman degradation

20mg Resin was swelled for 1 hour in 500uL 1:1 pyridine/water and filtered. Equal volumes of 1:1 pyridine/water (250uL) and 20:780 phenylisocyanate/pyridine (250uL) were added to the resin and were allowed to incubate for 30 minutes in the dark. Resin was then filtered and incubated with 500uL of trifluoroacetic acid for 10 minutes in the dark. Resin was then filtered, washed thoroughly with dichloromethane (3x), and methanol (3x). Process was repeated for subsequent degradation.

(9) Optimized LC-MS methods for peptide sequencing

Default settings for the Agilent 6520 were manipulated to an optimal capillary voltage of 4500V, skimmer voltage of 115V, drying gas temperature of 350°C and flow rate of 8L/minute.

(10) Liposome preparation

POPC, POPS and NBD labeled POPE were placed in a vial in chloroform in desired ratio and left on vacuum to remove all chloroform for 2 days. Resuspended lipids in 1x PBS buffer at pH 7.4. Liposomes were subjected to 20 freeze thaw cycles and extruded through a 100nm filter 20 times.

(11) Library preparation

Linker from the TentaGel resin was synthesized using standard Fmoc solid phase peptide synthesis with dichloromethane as the primary solvent in place of DMF. 6 rounds of split-pool synthesis were then performed on the linker using commercially available Fmoc-Arg (pbf)-OH, Fmoc-Asp(otbu)-OH, Fmoc-Gln(trt)-OH, Fmoc-His(trt)-OH, Fmoc-Ile-OH and Fmoc-Gly-OH with HBTU. After 6 rounds of split and pool synthesis Fmoc-Cys(trt)-OH was coupled to the resin. The resin was then deprotected in bulk in the dark with reagent K for 1 hour. Peptides were oxidized in water with air bubbling overnight.

(12) Fluorescence imaging studies

TentaGel resin bearing the oxidized peptide library was incubated for 10 minutes in the dark with 1mM liposome stock of either 99.9% POPC 0.1% NBD-POPE vesicles or 80% POPC 20% POPS with 0.1% NBD-POPE vesicles. Beads were washed with 1x PBS buffer and resuspended in 1xPBS buffer for imaging under bright field and PITC channel for fluorescence.
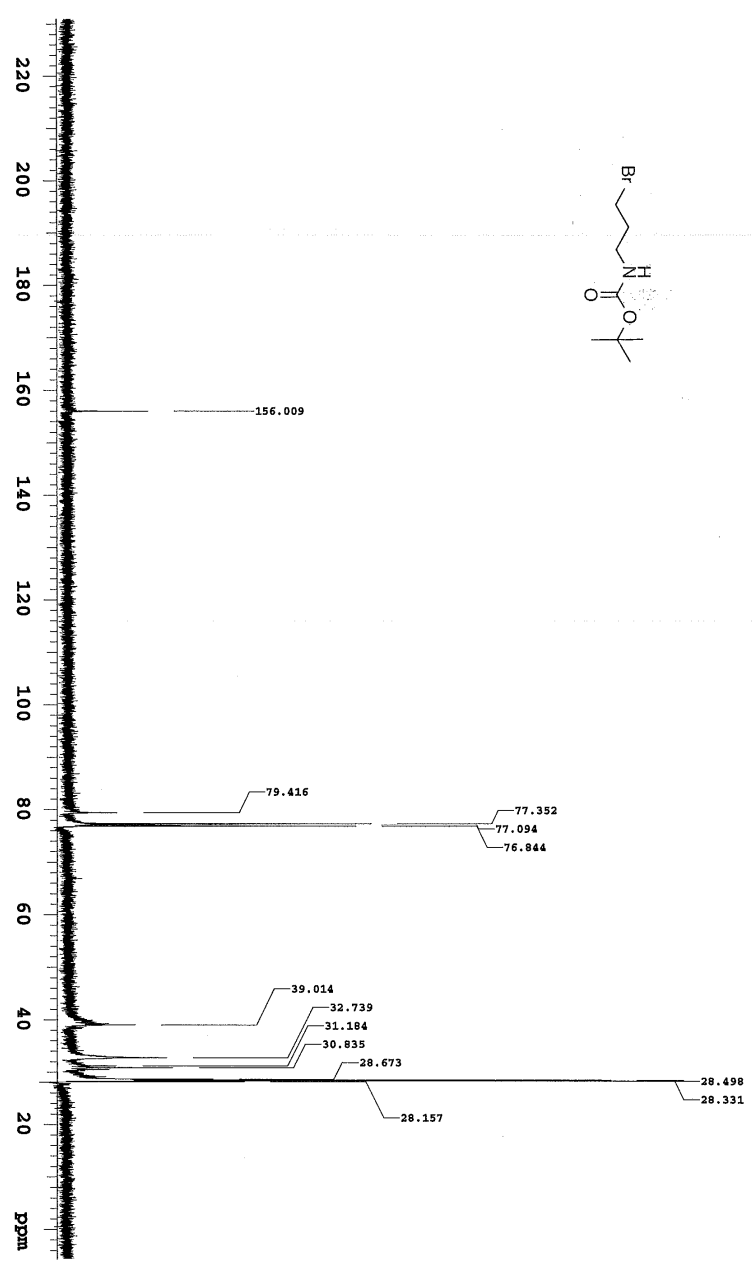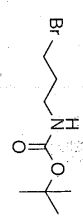
Appendix: NMR Data
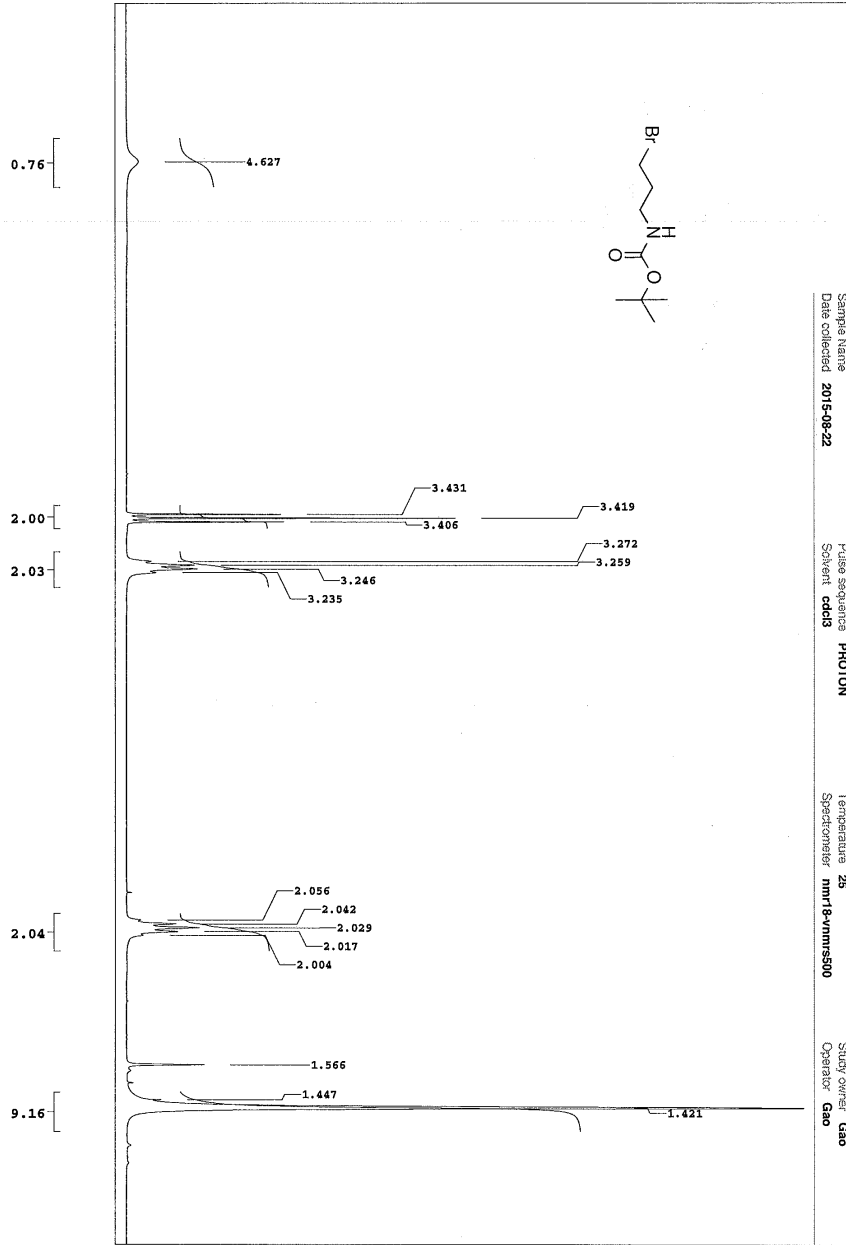
LB02-222-13C

Sample Name **LB02-222-13C**
Date collected **2015-11-21**

Pulse sequence **CARBON**
Solvent **cdcl3**

Temperature **25**
Spectrometer **nmr18-vnmrs500**

Study owner **Gao**
Operator **Gao**

156.009

79.416

77.352
77.094
76.844

39.014
32.739
31.184
30.835
28.673
28.498
28.331
28.157

220 200 180 160 140 120 100 80 60 40 20 ppm

58

LB02-217-column1prod

| Sample Name | LB02-217-column1prod | Pulse sequence | PROTON | Temperature | 25 | Study owner | Gao |
| Date collected | 2015-10-14 | Solvent | cdcl3 | Spectrometer | nmr18-vnmrs500 | Operator | Gao |

1.00 — 7.634
— 7.616

7.258

6.441
6.436
1.97 — 6.424
6.419
6.404
6.399

0.79 — 4.682

4.063
2.05 — 4.051
4.039

3.323
2.10 — 3.311
3.299

3.04 — 2.555

2.016
2.003
2.18 — 1.992
1.979
1.966

4.85 — 1.547
1.441

LB02-218-product

Sample Name	**LB02-218-product**
Date collected	**2015-10-15**

Pulse sequence	**PROTON**
Solvent	**cdcl3**

Temperature	**25**
Spectrometer	**nmr18-vnmrs500**

Study owner	**Gao**
Operator	**Gao**

1.02 — 7.836
7.819

0.68 — 7.258

1.06 — 6.952
6.947
6.934
6.929

1.00 — 6.809
6.804

0.81 — 4.682

2.24 — 4.094
4.081
4.070

2.21 — 3.345
3.332
3.319
3.307

3.04 — 2.590

2.25 — 2.040
2.030
2.017
2.004
1.992

1.579

9.78 — 1.435
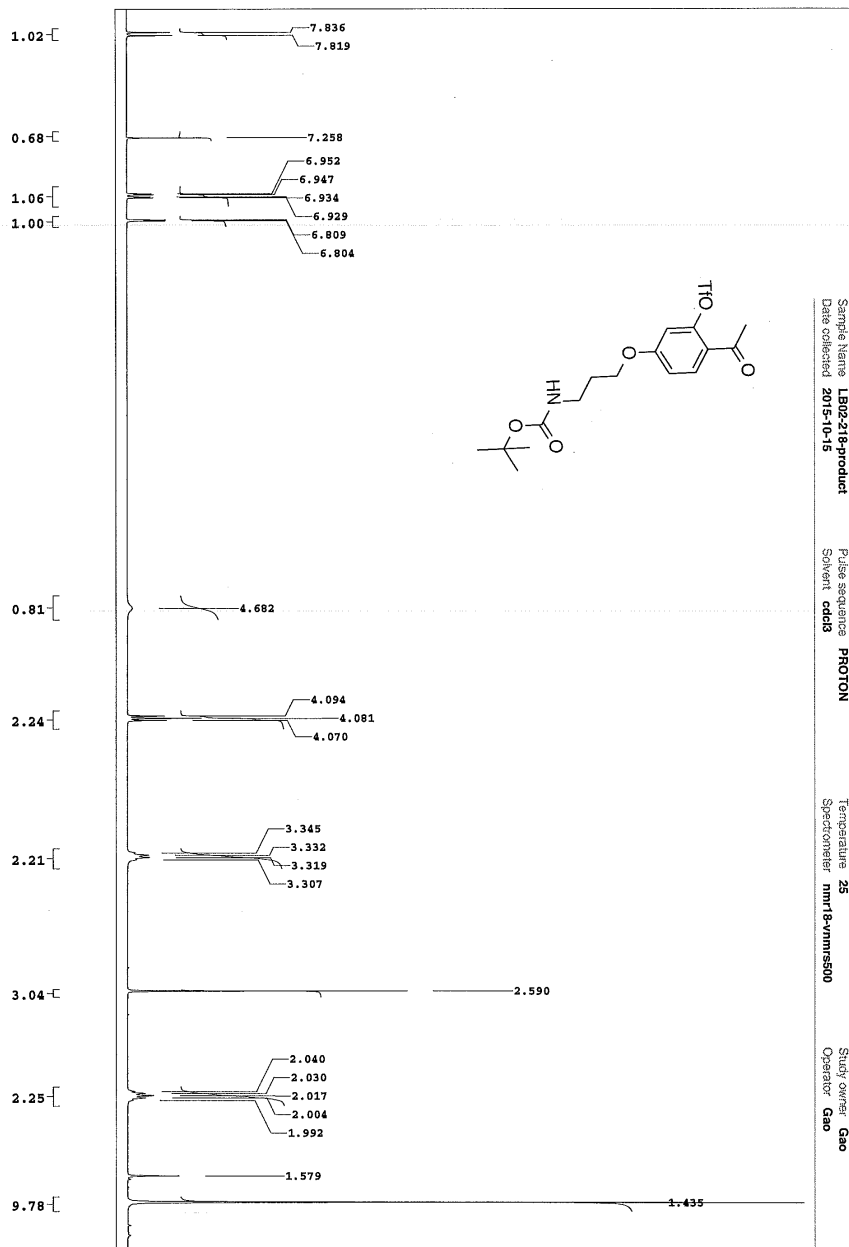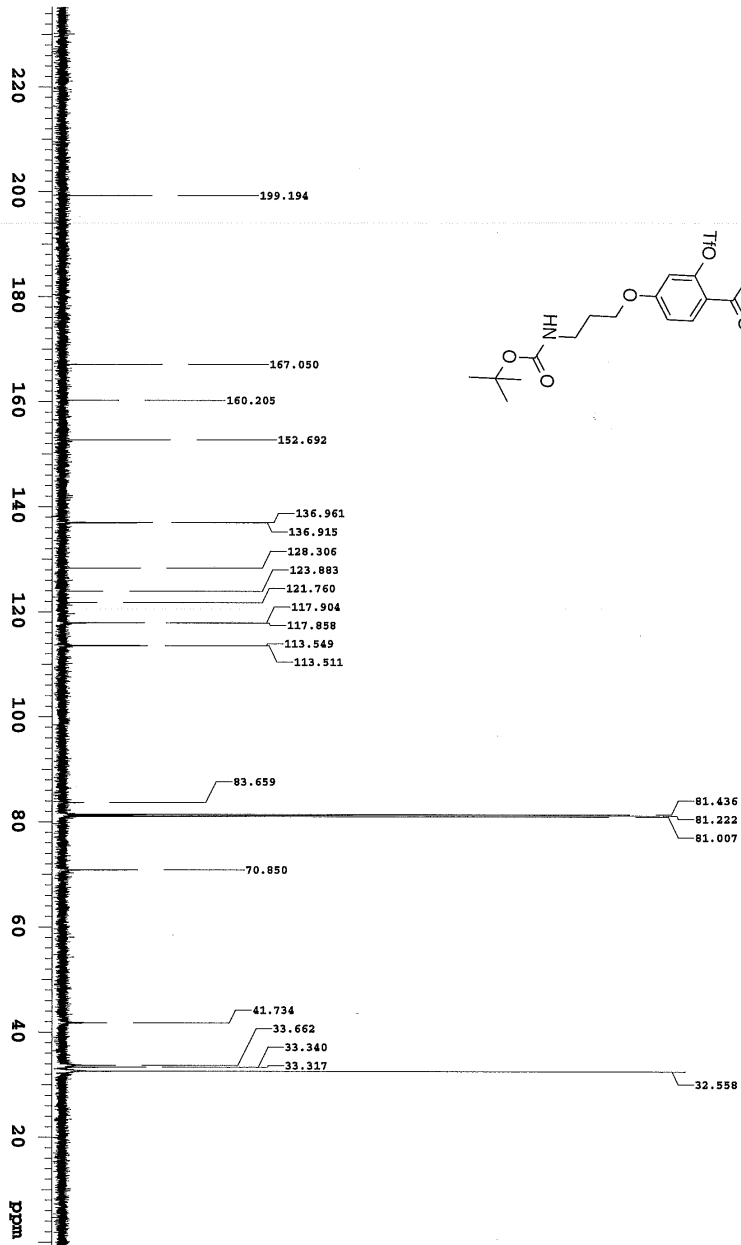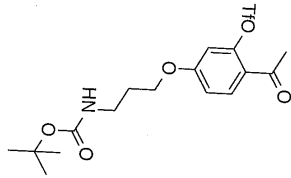
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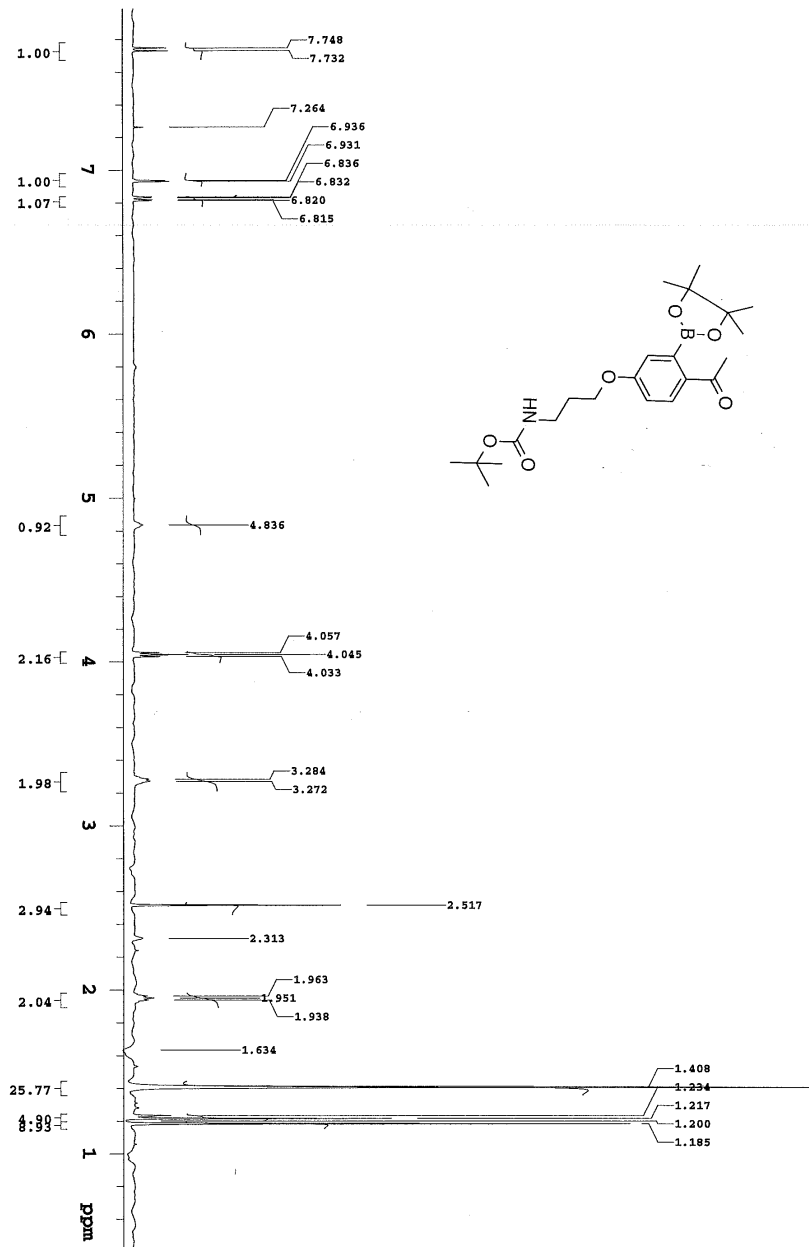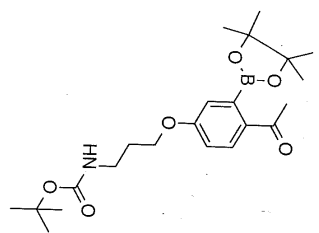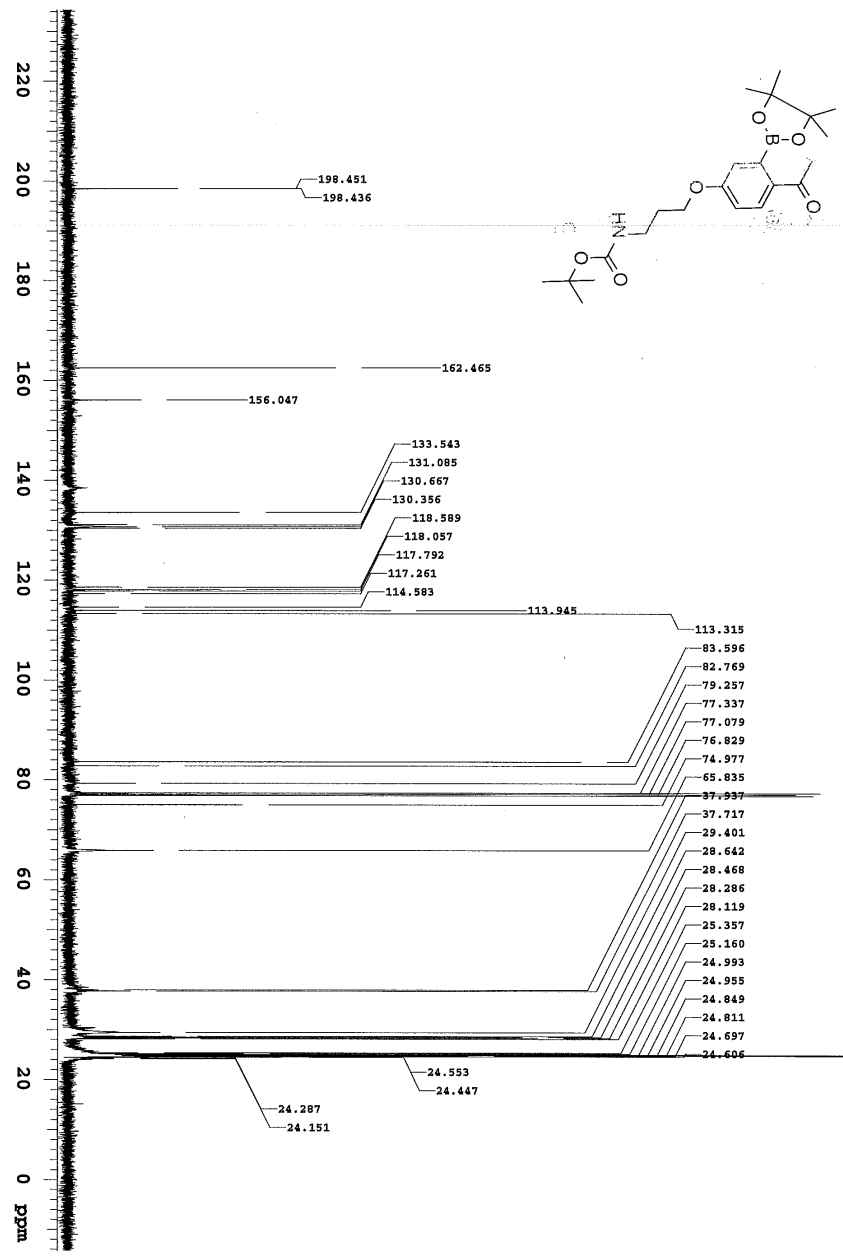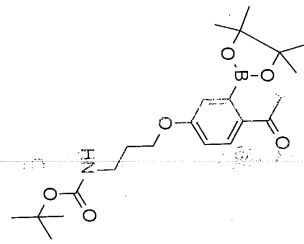
LB02-219-F2

Sample Name LB02-219-F2
Date collected 2015-11-19

Pulse sequence PROTON
Solvent cdcl3

Temperature 25
Spectrometer nmr18-vnmrs500

Study owner Gao
Operator Gao

7.748
7.732

7.264
6.936
6.931
6.836
6.832
6.820
6.815

4.836

4.057
4.045
4.033

3.284
3.272

2.517

2.313

1.963
1.951
1.938

1.634

1.408
1.234
1.217
1.200
1.185

1.00
1.00
1.07
0.92
2.16
1.98
2.94
2.04
25.77
4.00
8.99

198.451
198.436

162.465

156.047

133.543
131.085
130.667
130.356
118.589
118.057
117.792
117.261
114.583

113.945
113.315
83.596
82.769
79.257
77.337
77.079
76.829
74.977
65.835
37.937
37.717
29.401
28.642
28.468
28.286
28.119
25.357
25.160
24.993
24.955
24.849
24.811
24.697
24.606
24.553
24.447
24.287
24.151

220 200 180 160 140 120 100 80 60 40 20 0 ppm

64

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