

Applications of *bis*-amino acid oligomers

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The ability to precisely position functionality in three-dimensional space is a long term goal for our group. Some progress has been made so far. Using oligomers of our *bis*-amino acids, we are able to control the distance between two groups, ranging from 2-4nm. Also we are able to control shape and curvature by incorporating monomers that make bends or kinks. There are many potential applications. Some of the most powerful are described in the following pages. We have made a bivalent ligand for Cholera Toxin varying the number of monomers and found that all bivalent molecules that bind more tightly than the natural ligand GM1. The oligomeric scaffold is rigid and the linker contains about 5 rotatable bonds. We have made bivalent vancomycins and bivalent D-Ala-D-Ala, each consisting of linkers of between 1-6 building blocks. These molecules should be capable of associating into matched or mismatched bivalent pairs. In addition, we have made large macrocycles capable of forming a binding pocket. Ligands for these receptors will be identified using phage display. Also, our rigid chiral scaffolds have been studied to determine the influence of chirality on the efficiency of electron transfer. Finally, a scaffold detergent was synthesized, with the application of solubilization of membrane proteins in mind. These are all promising applications of our unique technology and will be explored further in the future.

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1. INTRODUCTION

1.1 PRECISION ENGINEERED NANOSTRUCTURES

Nanoscience is an emerging field of study of objects ranging in size from a few nanometers to less than 100 nanometers. Advances and discoveries in this area are likely to be part of the engine that drives the future economy of this country and the world, much like the Internet and information technology spurred the productivity and efficiency of the economy of the 1990s.

Opportunities for chemists are numerous as chemists have the most expertise in making molecules by joining together smaller fragments. The safe, efficient synthesis of sub-nanometer scale molecules on huge scales is a major part of the plastics and pharmaceutical industries. All of the marketing and manufacturing support systems are in place for a vibrant industry to be based on the coming advances in nanoscience made possible by the discoveries of chemists and scientists.

There are two approaches for making nanoscale molecules. The first is a top-down approach and uses bulk patterning similar to that employed by the semi-conductor industry, but on a smaller scale.[1] The top-down approach best lends itself towards the fabrication of nanoscale semiconductors, as well as microfluidic devices used as sensors[2] and analytical tools for the emerging concept of lab-on-a-chip approaches,[3, 4] because there is a lack of atomic-level control. On the other hand, a bottom-up approach builds nanoscale structures by arranging atoms and molecules, either through bonds or self-association. This ensures that the nanoscale molecule has a uniform and atomically defined constitution.

A bottom-up approach gives the chemist more control over the length and dimension of the nanostructure. The chemist often employs a segment condensation approach, where units of shorter length are grafted together to give larger molecules. Large molecules are not necessarily nanostructures. Nanostructured molecules require design and control on the nanoscale. Nature

is the preeminent nanoscale designer and chemists would do well to mimic the approach that nature takes in designing and assembling molecules on the nanoscale, which are ordered and adopt a structure or shape.

These “foldamers” could potentially have functions similar to biomolecules, including molecular recognition, information storage and catalysis. Moore has defined a foldamer “any oligomer that folds into a conformationally ordered state in solution, the structures of which are stabilized by a collection of noncovalent interactions between nonadjacent monomer units. There are two major classes of foldamers: single-stranded foldamers that only fold (peptide-like) and multiple-stranded foldamers that both associate and fold (nucleotide-like).”[5]

Many varieties of foldamers exist as seen in Figure 1, including β -peptides,[6, 7] peptoids,[8] carbopeptoids,[9] polypyrrolinones,[10] vinyllogous peptides[11] and sulfamidepeptides,[12] oligoanthranalamide,[13] oligoureas,[14] and phenylene-ethynylene oligomers.[15] Foldamers employ non-covalent interactions such as hydrogen bonding, steric repulsions, or π -stacking, in order to self-associate into helices, turns or sheet-like structures.

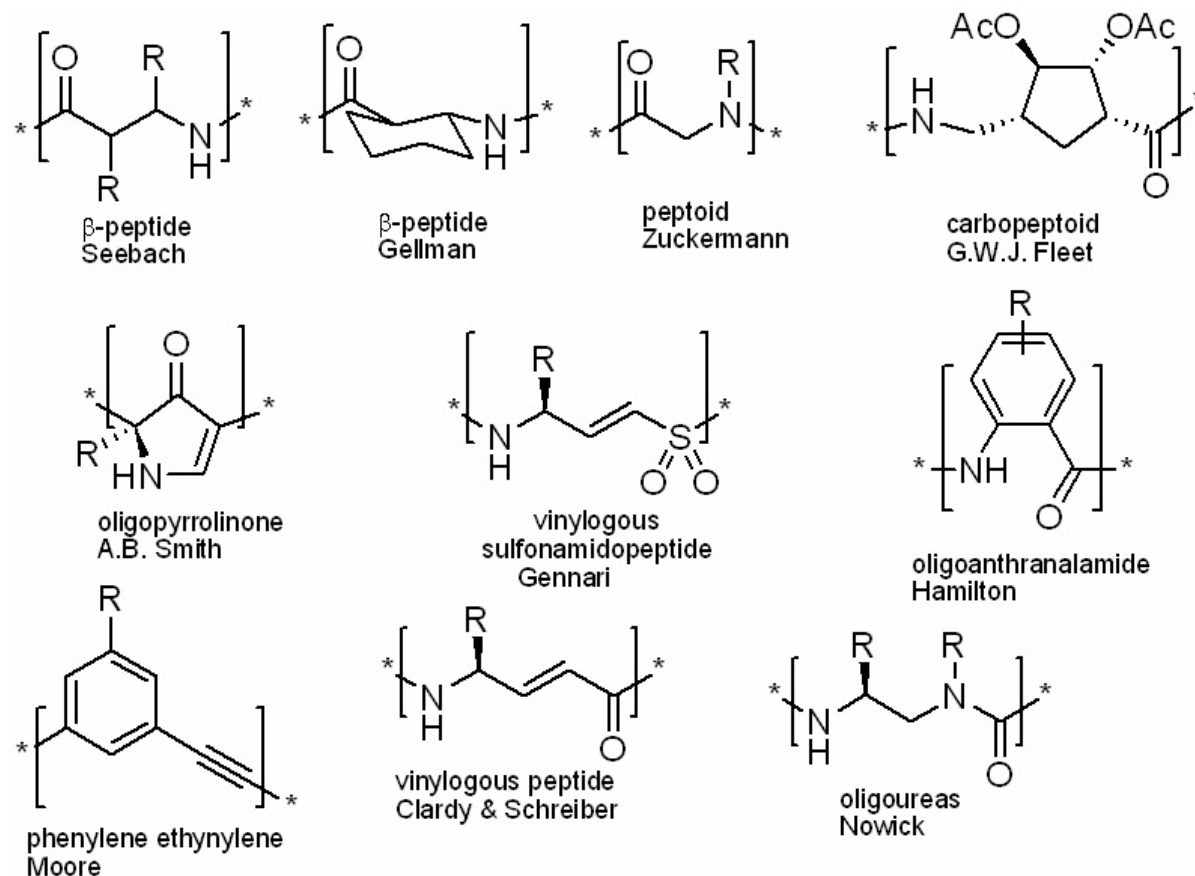


Figure 1 Selected examples of foldamers.

To date most foldamers form simple helices, but no foldamers have been created that fold back on themselves to create complex functionalized cavities like those seen in proteins. The most important capability that nature has and we need to develop is the ability to create functional macromolecules. Proteins are linear chains of amino acids that fold into functional three-dimensional structures. The process of folding is complex and the folded conformation of a protein is not predictable. Chemists have worked to create monomers that can be assembled into oligomers and then fold into predictable folded conformations.

Our laboratory has developed an approach to macromolecules with defined structures (Figure 2). The macromolecules are assembled from our collection of monomers which are connected through pairs of bonds to create spiro ladder oligomers.

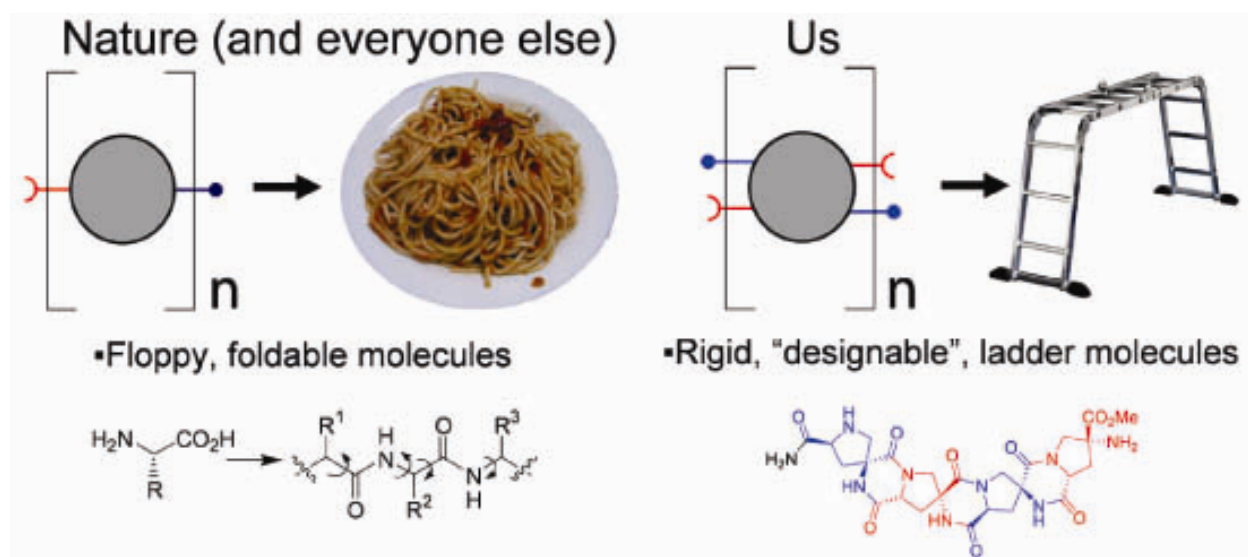


Figure 2 A comparison of monomers linked through single-bonds and pairs-of-bonds.

The chemistry of the amide bond is well developed, as are ways to make them with efficiency and control.[16] Cyclic monomers linked together through pairs of amide bonds will form structured oligomers dictated by the stereochemistry of the monomers, as well as the conformations of the rings (Figure 3). Predicting the conformations of the rings is a manageable task that molecular modeling programs can handle, allowing for the design and prediction of oligomeric structures. By incorporating monomers of different stereochemistry, regiochemistry, and ring size, nanostructures with any controlled shape or length are possible.

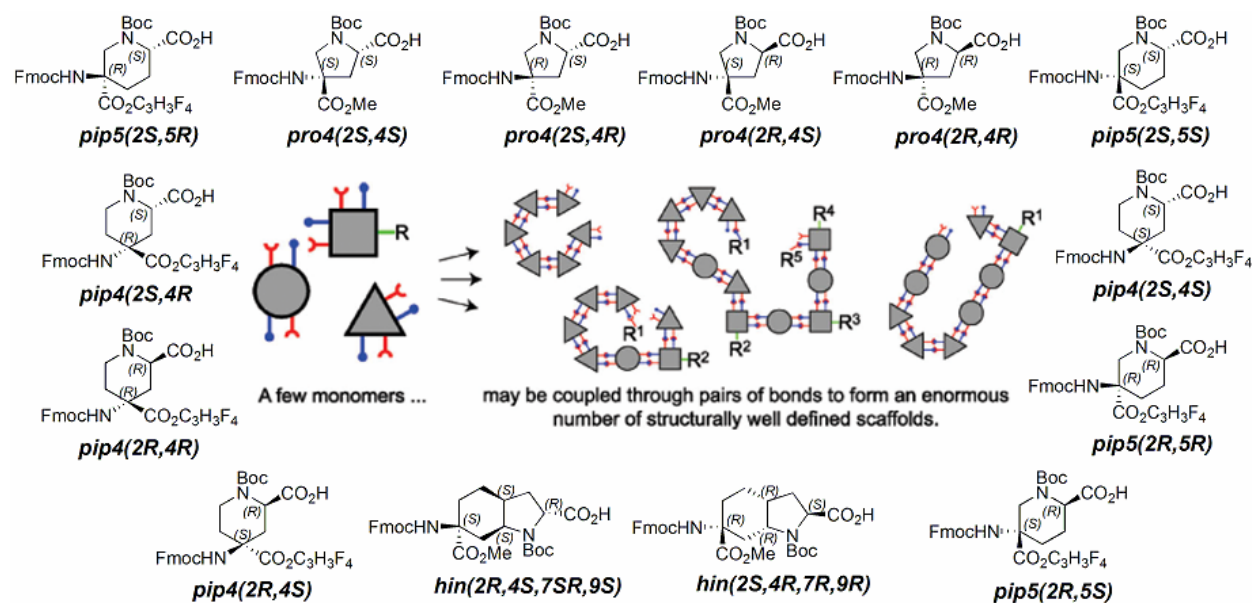


Figure 3 Toolbox of *bis*-amino acid building blocks that we have developed synthetic access to.

The syntheses of these *bis*-amino acid monomers have been published and used to make short oligomers.[17-20] These syntheses employ about 10 steps which transform cheap, amino acids into the desired products, in multigram scales (Figure 4). The nomenclature used here is derived from the building block name (*pro4* (1) – proline with substitution at position 4, *pip4* (2) or *pip5* (3) – pipercolic acid with substitution at position 4 or 5, and *hin* (4) – derived from hydroindole) and the code in parenthesis represents the absolute configuration at each stereocenter.

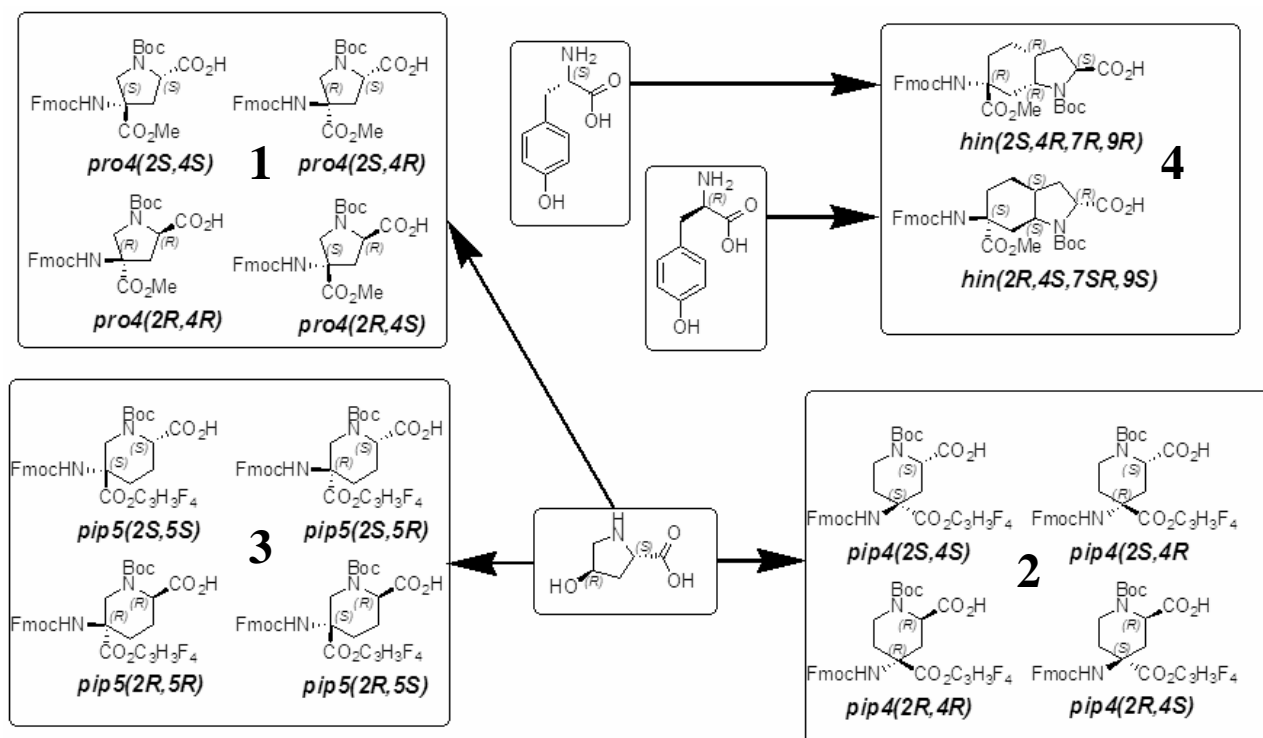


Figure 4 Building blocks are made from readily available amino acid starting material.

Our building blocks are *bis*-amino acids and require orthogonal protecting groups which can be removed at different stages in the solid-phase synthesis of oligomers (Figure 5). The Fmoc- group is the temporary protecting group which is removed in order to elongate the oligomer chain and allows the implementation of all traditional Fmoc based solid-phase peptides synthesis methods and commercially available reagents. The Boc group remains intact throughout the synthesis being removed using various conditions, but most often with the strong acid used to cleave the oligomer from the solid support (**5**). Treatment with base catalyzes multiple DKP ring closures when the liberated amine of one building block (or amino acid) attacks the methyl ester of the preceding building block, ejecting methanol as a leaving group to form a 6-membered diketopiperazine ring via an amide bond, resulting in a rigid “molecular scaffold” (**6**).

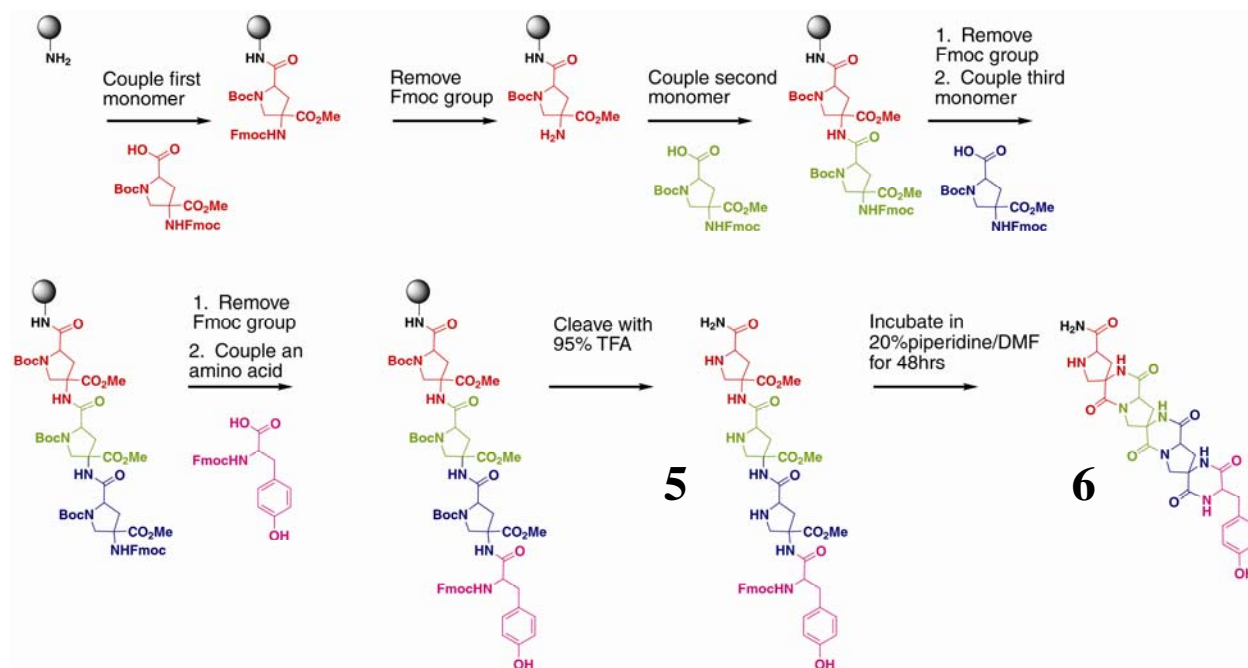


Figure 5 General scheme illustrating oligomer synthesis, cleavage, and DKP closure to form a rigidified scaffold.

The three-dimensional structure of these oligomeric structures have been solved using 2-D NMR in order to determine the conformational preferences of the rings, as these are some of the key determinants of the global oligomeric shape (Figure 6). For the *pro4* class of monomers, the diketopiperazine (DKP) between two building blocks adopts a shallow boat conformation, such that the substituent, R_1 , is in a pseudo-equatorial position. The 5-membered ring adopts an envelope conformation so that the 1-3 strain and steric clash between the indicated carbonyl and NH is avoided.[17, 20]

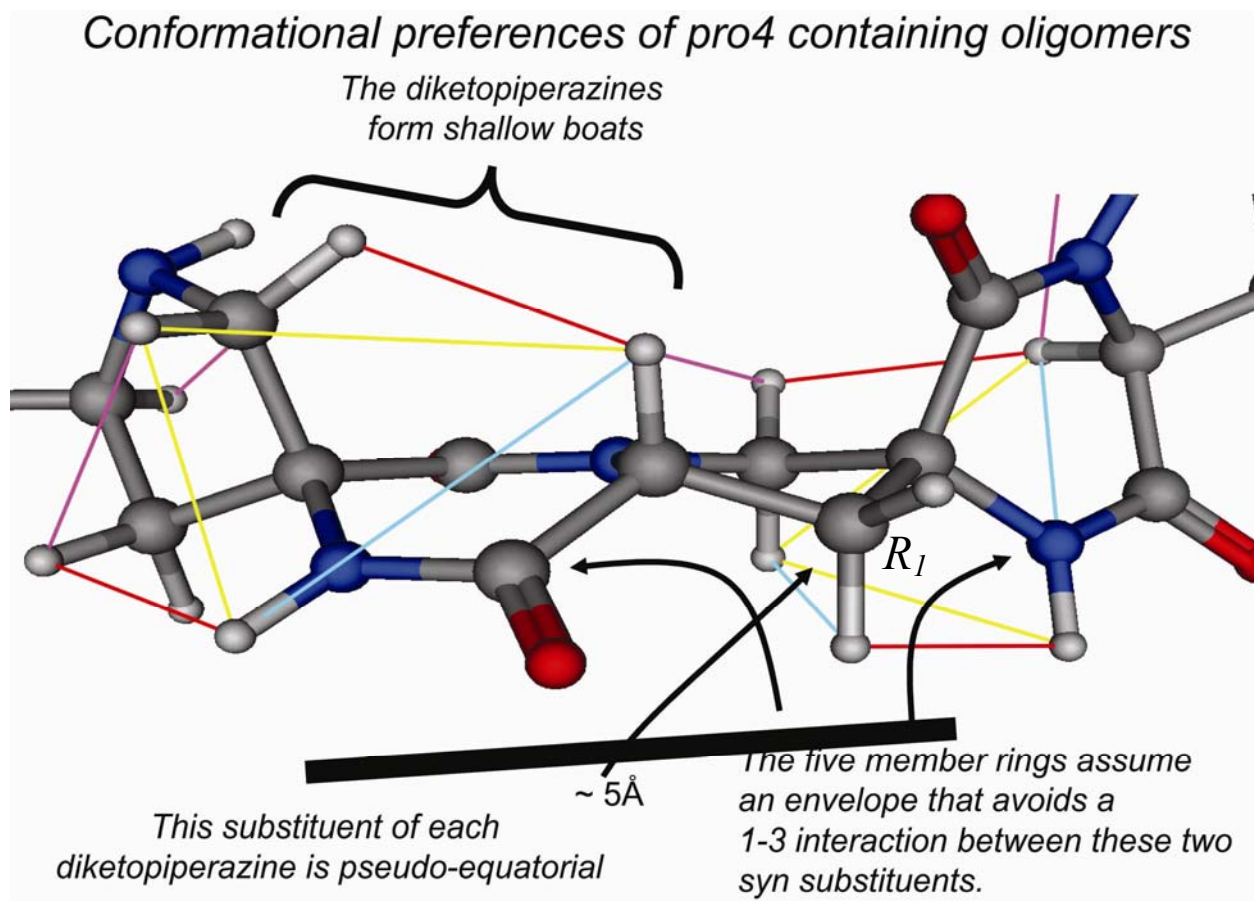


Figure 6 The conformational preference of a *pro4* monomer within the context of an oligomer

The bulk of the research contained in the following chapters of this thesis deals with the design and synthesis of functionalized nanoscale molecular scaffolds and exploring their potential applications, including bivalent ligands and bivalent interactions, artificial antibodies, nanoelectronics, and designer detergents for membrane proteins. An additional project yielded structural insights into these molecular scaffolds by high-resolution measurements of the distance between the two ends.

The toolbox of building blocks used for all projects consisted of the four stereoisomers of the *pro4*-class pictured in Figure 7. A simple two-letter abbreviation system will therefore be sufficient to identify exactly which building blocks are used in the proceeding chapters.

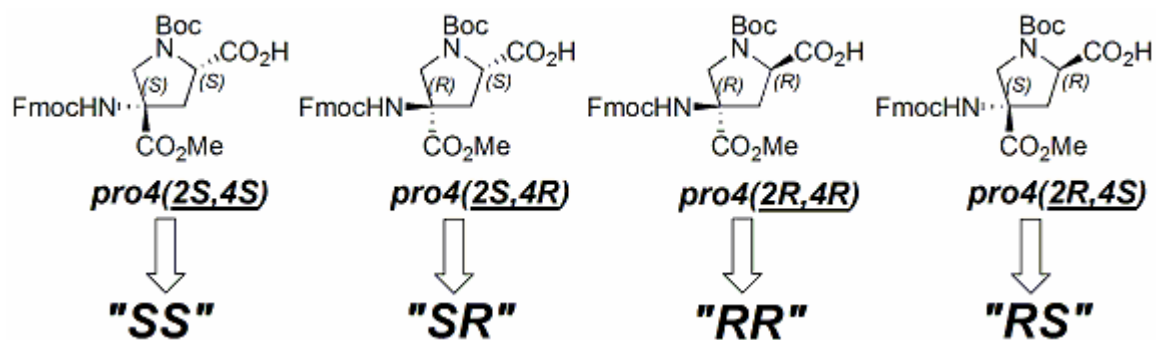


Figure 7 Toolbox of building blocks used for all projects and discussion in this thesis, and the corresponding two-letter abbreviations.

2. A BIVALENT LIGAND FOR CHOLERA TOXIN

2.1 INTRODUCTION

Multivalent interactions are comprised of recognition and binding events between multiple ligands and multiple receptors. Multivalent interactions form the basis of virus-host cell recognition and the pathogenic features of some bacteria. Multivalent interactions form the basis of the immune system and its ability to recognize non-self, and have importance in DNA recognition and intercalation as well as in cellular communication and signal transduction pathways. The binding affinity of a multivalent interaction is larger than the sum of its monovalent binding affinities. These interactions can act to iteratively agonize or antagonize a biological target, which is a property excluded from monovalent interactions.

Multivalent interactions, relative to monovalent interactions can be positively cooperative, additive or negatively cooperative, depending on the value of α , a ratio of free energies. The classical example of positive cooperativity is that of hemoglobin, where binding of each oxygen makes the next bind easier. Another metric of multivalent enhancement is β , the enhancement factor, which is the ratio of association constants.

$$\alpha = \text{degree of cooperativity} = \frac{\Delta G_N^{\text{multi}}}{N\Delta G^{\text{mono}}} \quad \beta = \text{enhancement factor} = \frac{K_a^{\text{multi}}}{K_a^{\text{mono}}}$$

In terms of synthetic multivalent molecules, the structural features of the linker are most important. The linker physically holds the ligands at distances suitable for simultaneous interactions with the multivalent target. If two linkers, one flexible and one rigid, both hold two

ligands the same distance apart, the rigid linker will be energetically more favorable. The more flexible the linker is, the more conformations that will exist in the unbound state. All unbound conformations must coalesce into a handful of conformations upon binding and rotatable bonds must be restricted upon binding, which costs energy at the expense of binding affinity. This entropic cost has been estimated by Jencks to be ~ 1.2 kcal/mol per bond.[21] Whitesides suggests that the actual value is less than this, and is highly dependant on the type of bond being restricted.[22] Choi groups linker types into three relative categories: rigid (polyaromatic or polyolefinic), intermediate (polyamide or polyether) or flexible (polymethylene or polythioether).[23]

$$\Delta G^{\text{mono}} = \Delta H^{\text{mono}} - T\Delta S^{\text{mono}}$$

$$\Delta G^{\text{di}} = \Delta H^{\text{di}} - T\Delta S^{\text{di}}$$

$$\Delta S^{\text{mono}} \approx \Delta S^{\text{mono}} (\text{translational}) + \Delta S^{\text{mono}} (\text{rotational})$$

$$\Delta H^{\text{di}} \approx 2\Delta H^{\text{mono}}$$

$$\Delta S^{\text{di}} \approx \Delta S^{\text{mono}} (\text{translational}) + \Delta S^{\text{mono}} (\text{rotational}) + \Delta S^{\text{di}} (\text{conformational, linker})$$

Simply tethering multiple ligands together with a flexible linker will reduce the translational and rotational entropies relative to monovalent, because in both monovalent and bivalent binding, two molecules become one. In the bivalent system, it is hypothesized that an extra entropic term results from freezing the rotatable bonds found in the linker. The degree of multivalent binding enhancement depends on this balance of added vs. withdrawn entropy. Whitesides states that “multivalent systems joined by flexible linkers (i.e. PEG) are almost guaranteed to fail for entropic reasons.”[24] Many examples, nonetheless, exist where a modest multivalent enhancement is seen, but nothing like the exponential enhancements seen for the *tris*-D-Ala-D-Ala and *tris*-vancomycin system consisting of minimal linker flexibility.[25] In this system, the driving force of the association is the enthalpic term which is more than three times that of the monovalent system. Based on the definition of the entropic contributions for a multivalent interaction, it is apparent that the ΔS^{tri} (conformational, linker) term corresponding to the flexibility in the linker is disfavoring in this multivalent association. The number of rotatable bonds in the linkers is about ~ 25 , which is much less than is found in typical polyamide or PEG linkers. However, the net result is a $K_d^{\text{tri}} = 4 \times 10^{-17}$, which is an incredible enhancement over the $K_d^{\text{mono}} = 1.6 \times 10^{-6}$.

tris-Vancomycin

$$\Delta G^{\text{tri}} = \Delta H^{\text{tri}} - T\Delta S^{\text{tri}}$$

$$\Delta G^{\text{tri}} = -94\text{kJ/mol}$$

$$\Delta H^{\text{tri}} = -167\text{kJ/mol}$$

$$T\Delta S^{\text{tri}} = -73\text{kJ/mol}$$

mono-Vancomycin

$$\Delta G^{\text{mono}} = \Delta H^{\text{mono}} - T\Delta S^{\text{mono}}$$

$$\Delta G^{\text{mono}} = -33\text{kJ/mol}$$

$$\Delta H^{\text{mono}} = -50\text{kJ/mol}$$

$$T\Delta S^{\text{mono}} = -17\text{kJ/mol}$$

A model used for the estimation of the binding constant of a multivalent system lends insight into the most important factors for making high affinity multivalent interactions.[23]

$$K^{\text{multi}} = F(s \times 10^{-2})^{n-1} (K^{\text{mono}})^n$$

F=statistical factor for the particular system
 s=30/(interceptor distance in Å)
 n=valency number

The strength of an interaction can increase exponentially with respect to valency. Also, a strong monovalent interaction to start with will increase the strength of the multivalent interaction. Interestingly, because the distance between two sites is important in this model, one can infer that this a correction based on the number of rotatable bonds in the linker.

The kinetic aspects of multivalent interactions are also unique. A bivalent ligand dissociates from its two binding sites in two steps, whereby the off-rate of the second dissociation closely mirrors the off-rate of the monovalent dissociation.[26]

$$k_{\text{off}}^{\text{di}} = \frac{2k_{\text{off}} K_d}{C_{\text{eff}} + K_d}$$

k_{off} = monovalent off-rate
 K_d = monovalent dissociation constant
 C_{eff} = [effective] at the second binding site

Thus the off-rate of a bivalent ligand is equal to the monovalent off-rate, times 2 because there are two sites where dissociation can initiate from, times the likelihood that the second site will dissociate as well ($K_d/(C_{\text{eff}}+K_d)$). Since effective molarities are often several orders of magnitude greater than the actual molarity, a bivalent off-rate can also be drastically slower than a monovalent off-rate.

The first synthesis of a bivalent molecule was designed by Knowles as a potential inhibitor of influenza, shown in Figure 8.[27, 28] Attempts to measure binding to non-membrane bound hemagglutinin (HA) using NMR showed that all bivalent molecules, irrespective of linker length or type, were binding with equal affinity to the monovalent control. However, measurements of the bivalent molecules' ability to inhibit the adhesion of influenza virus to red blood cells showed an enhancement of up to 100x relative to monovalent K_d of ~2mM, dependant on linker length. One interpretation of these results is that the bivalent

molecules are reaching between pairs of trimers of HA, instead of within the trimers, or intermolecular vs. intramolecular.

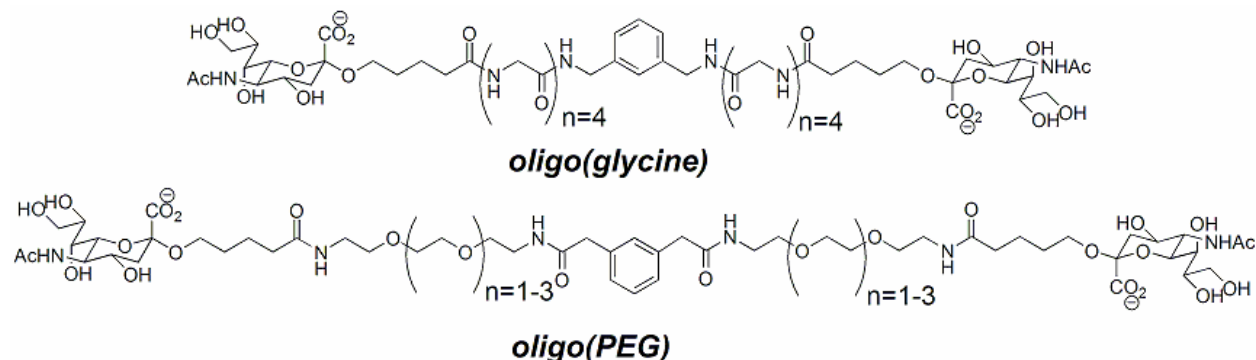


Figure 8 Two series of bivalent sialosides inhibitors of hemagglutinin.

Cholera toxin, β -subunit (CTB) is a model multivalent system, which has a low nM affinity for its natural ligand, the human cell ganglioside GM1. This ligand is displayed in moderate density on the surface on small-intestine epithelial cells. CTB is an AB₅ protein excreted by the pathogenic organism *vibrio cholera*. The B₅ portion is involved in recognition and binding to GM1, while the A portion is responsible for the pathologic effect. Because the cure for cholera is clean drinking water, a better understanding of multivalency is the practical goal in developing multivalent ligands for CTB.

Several groups have developed multivalent ligands for CTB. The first and most prolific group of them is Erkang Fan. Fan and coworkers have synthesized three classes of inhibitors, all with varying linker lengths, including a pentavalent star-shaped ligand capable of binding to all five sites at once,[29] a *bis*-pentavalent or decaivalent ligand, capable of binding to two pentameric CTBs simultaneously,[30] and short, bivalent ligands with linker lengths unable to span the distance between two sites.[31]

When the authors vary the distance between the core and the ligand from shorter to longer, they see an increase in inhibition in an ELLA assay (Figure 9). The assay is similar in principle to an ELISA, where the natural, GM1 ligand is adhered to a polystyrene microtiter plate. A CTB-horseradish peroxidase (CTB-HRP) conjugate is then incubated in a parallel microplate with increasing concentrations of synthetic ligand. The ligand-CTB-HRP solutions are then added to GM1 coated plate, allowed to sit for ~30min, and then washed. Upon addition of a chromogenic solution, the amount of color that forms is proportional to the amount of CTB-

HRP bound to the plate, and by inference, proportional to the relative affinity of the synthetic ligand for CTB. This assay is, in essence, a competition and is a non-equilibrium binding measurement. Substantial gains in inhibition over monovalent ligand were attained, which the authors claim is due to binding affinity and not aggregation, as the dynamic light scattering measurements suggested a particle size on the order of a pentameric CTB plus pentavalent ligand.[32] Similar trends were seen when a branch was added to each arm of the pentavalent core, resulting in a branched pentavalent or decavalent ligand.[30] More insight into this interaction could have been achieved by increasing the length of the PEG linker further, to see if the multivalent enhancement decreased.

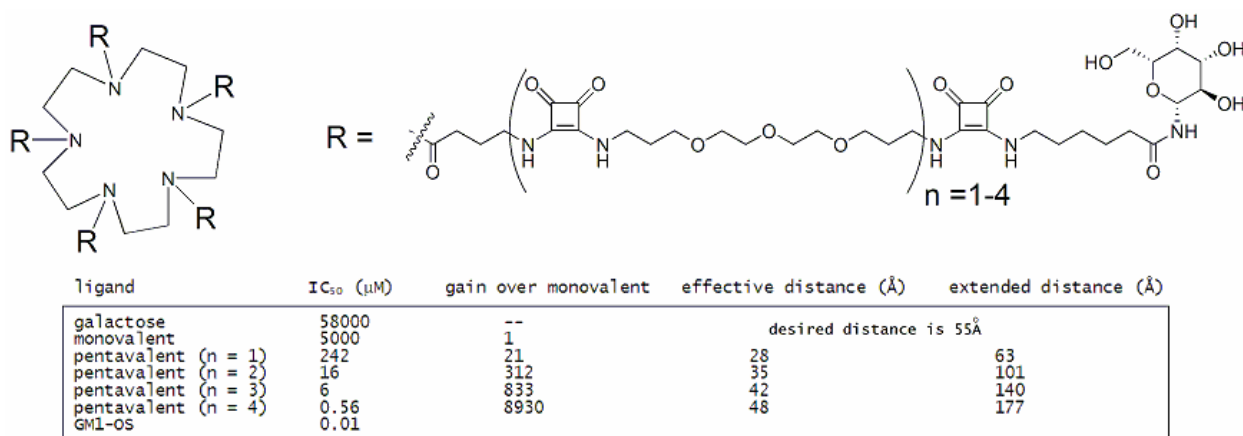


Figure 9 Fan's branched pentavalent CTB inhibitors.

In the case of the non-spanning bivalent ligands, a contradictory result emerged, namely when the linker was too short to span the distance, a bivalent enhancement was still seen (Figure 10). In all cases the “effective distance” was less than the desired distance, and in the case of the shortest linker, even the “extended distance” was too short to span. Nonetheless, all bivalent ligands showed an enhancement over monovalent of between 5-15, after normalizing for the number of ligands. Again, the authors refute the aggregational hypothesis by saying that the DLS measurements ruled out higher-order aggregates and that the concentration of CTB in solution is so low (~100pM). The author’s explanation for this observation was that of steric blocking. When the binding sites of CTB are partially saturated, the dangling arm of a bivalent ligand which is bound monovalently could prevent access of the CTB to the surface of the GM1-microtiter plate. This hypothesis would be best tested by synthesizing a bivalent linker, but only attaching one ligand to it, which the author states is currently ongoing.

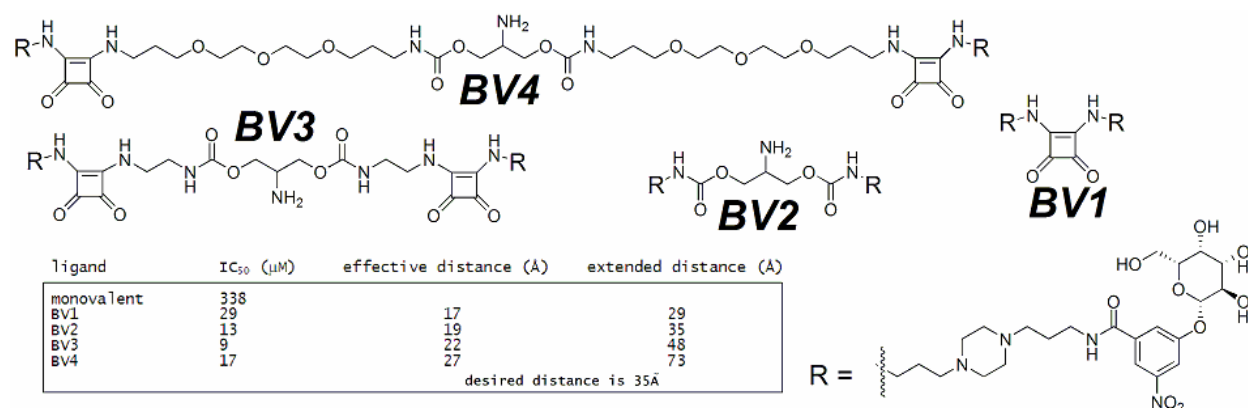


Figure 10 Fan's non-spanning bivalent ligands.

Two more examples of multivalent ligands for CTB are the bivalent truncated GM1-analog containing a PEG linker with a calixarene core (Figure 11) and multivalent dendrimers (Figure 12) containing a lactose thiourea derivative. In both cases, binding constants were measured using a Trp fluorescence intensity shift. The bivalent GM1 analog produces an enhancement of 2000 over monovalent, making this interaction stronger than the natural GM1 ligand K_d of 200nM.[33] The dendrimers do not show large bivalent enhancements, especially when considering the density of ligands. The D-2Lac has twice the ligands as the monovalent, but no difference in binding affinity. Likewise, the D-4Lac has four ligands, but only twice the binding affinity of the monovalent. Finally, the D-8Lac has eight times the ligands, but only 8 times the binding affinity. This dendrimer work illustrates the point that multivalent interactions require a careful organization of ligands. It is not sufficient to just present a cluster of ligands and expect to get enhancement. On the contrary, a dense cluster of ligands can cause steric clash which can result in a multivalent interaction much less than the sum of its parts.

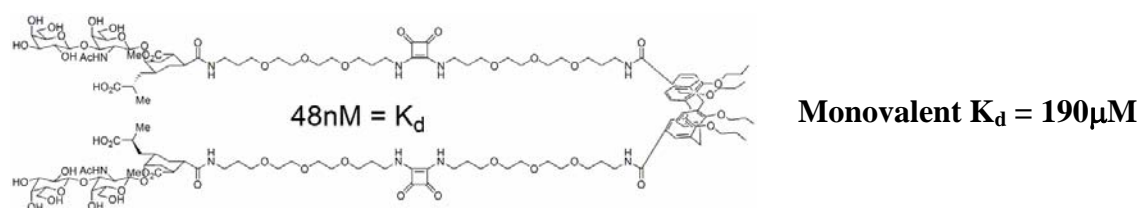


Figure 11 GM1 analog with PEG linker and calixarene core.

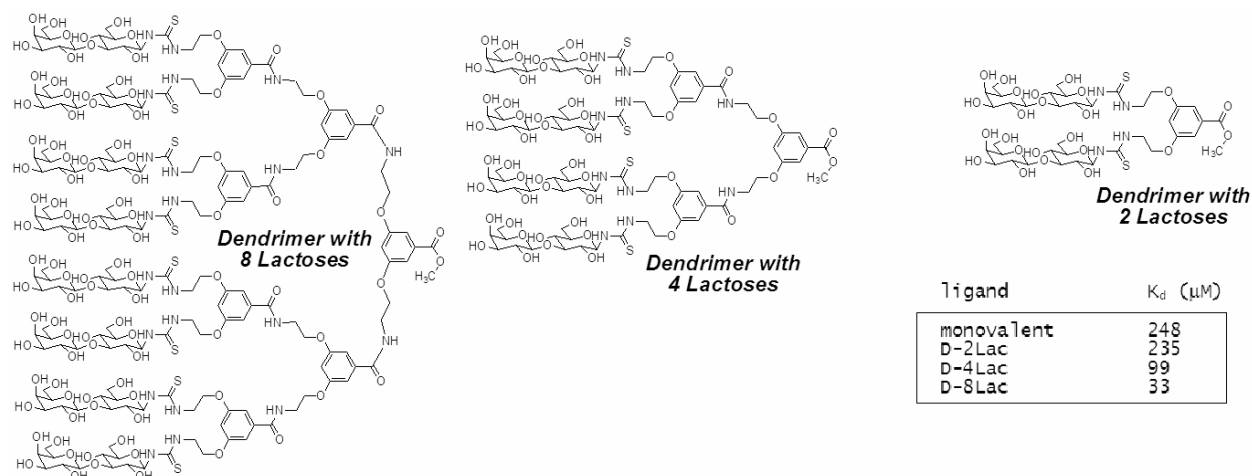


Figure 12 Lactose-thiourea dendrimers.

The bis-amino acid technology being developed in our lab is ideal for furthering the understanding of bivalency. By varying the number of building blocks, we can change the length of the rigid linker (Figure 13).

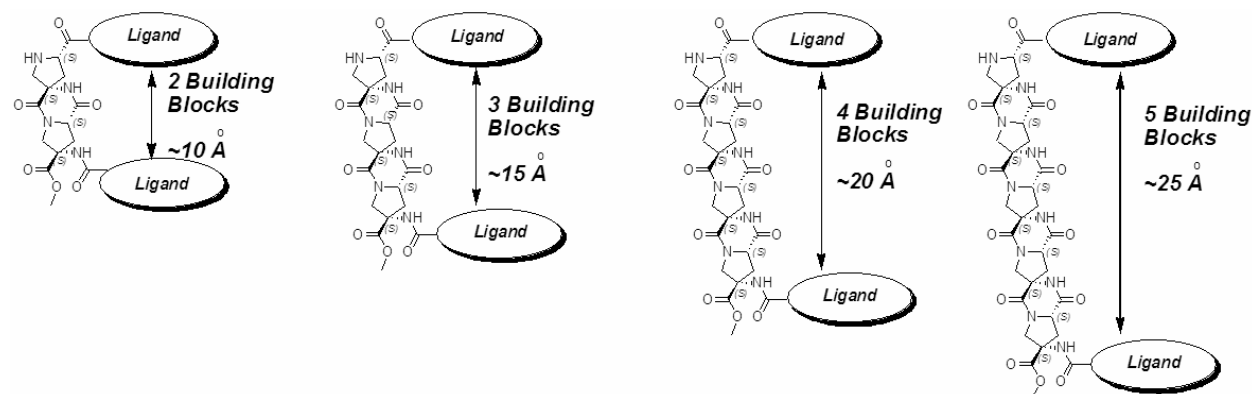


Figure 13 Bivalent scaffolds of varying lengths.

The influence of the length and flexibility of the linker on binding affinity can also be evaluated by varying the type of linker from PEG to β Ala, as well as the number of β Ala. One goal along these lines is to synthesize a series of lengths of bivalent ligands that differ in binding affinity. Ideally, the series would have a transition from low affinity when the linker was too short, to high affinity when the linker was length was ideal, to low affinity again when the linker was too long. This “Goldilocks” effect has been demonstrated before with varying lengths of PEG linkers, from 18 atoms to over 1300 atoms in length (Figure 14).[26]

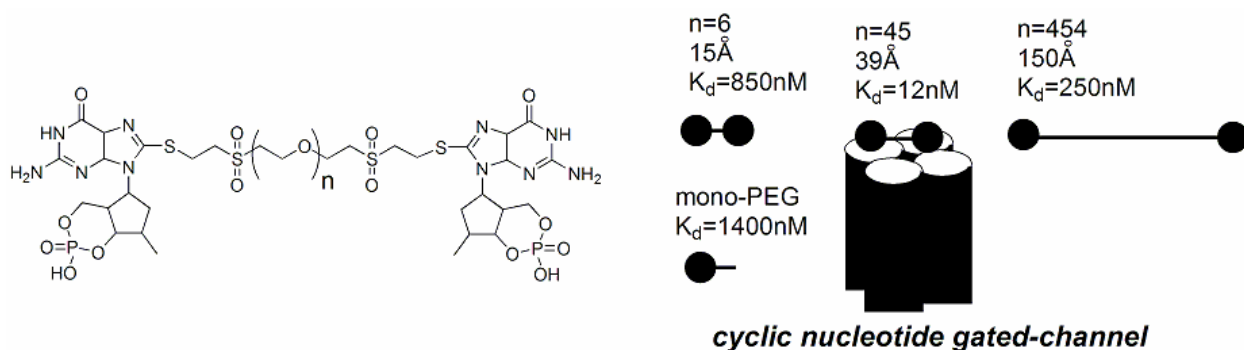


Figure 14 Bivalent ligands with varying linker lengths.

Controversy in this field exists when considering the mechanism of bivalent enhancement. Fan and coworkers believe that chelation binding or intramolecular complexation between one multivalent ligand and one protein is the best explanation for their data.[29-32] The other opinion, championed by Toone, advocates that multivalent enhancements are primarily the result of aggregation, or intermolecular complexation involving multiple ligands interacting with multiple proteins.[34, 35] By controlling the shape of a linker and the orientation by which two ligands are presented, it is conceivable that we could synthesize a bivalent ligand that could only bind by chelation and not aggregation (Figure 15).

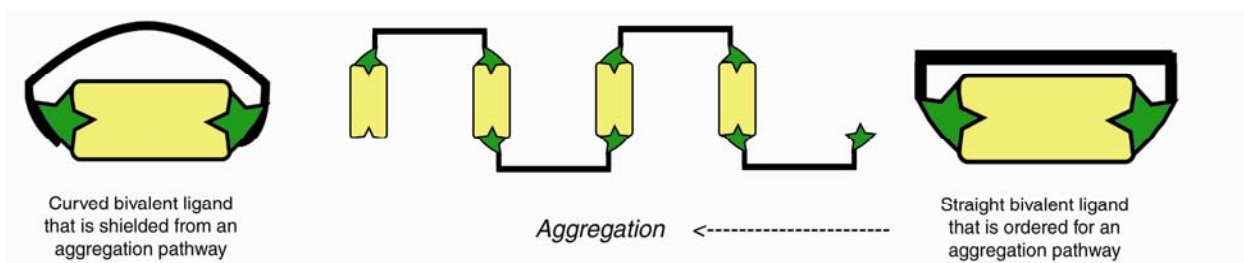


Figure 15 Cartoon depiction of a potential molecule that would disfavor aggregation, and a molecule that would be indifferent toward aggregation.

Another contribution that we are uniquely poised to achieve is bivalent selectivity. This principle is based on observations from pharmaceutical companies that bivalent molecules that show promise *in vitro*, always fail *in vivo* (personal communication with Eric Toone). It is likely that most bivalent ligands are indiscriminate in binding to receptors. Any receptor with binding sites spaced within a large distance window would be targeted by a flexible bivalent ligand. By precisely and exactly controlling not just spacing, but orientation of the two ligands, a specific,

desired target can be selectively inhibited without side effects or sequestering of material in undesired targets.

By addressing these two critical areas of deficiency in multivalent interactions, our group is striving to open up a new area of biological targets to medicinal chemistry. Bivalent ligands that interact through complexation and not aggregation, along with a rigid, designed linker that arranges two ligands in an ideal binding geometry may lead to a renaissance in therapeutics. No longer will the medicinal chemist have to spend long hours trying achieve binding constants in the low nM. The result of attaching two 100 μ M binders together with a custom-tailored rigid linker will be a 10nM inhibitor with inherent specificity, while devoid of the toxic functionality sometimes needed to maximize binding affinity.

2.2 RESULTS AND DISCUSSION

2.2.1 Galactose Ligand

Initial attempts to synthesize a bivalent ligand employed the nitrophenyl galactoside ligand and utilized fluorescence polarization to measure binding (Figure 16). The degree of polarization of fluorescently labeled ligand can be used to calculate binding constants based on the principle that large (protein) molecules tumble slower than small (ligand) molecules. Using polarized light as the source of excitation, only fluorophores having the dipole aligned with the excitation vector will be excited, and after a lifetime of a few nanoseconds, light is emitted. A ligand molecule that is not bound will tumble rapidly and emit light in a randomized orientation relative to the initial. A ligand-protein complex is much larger and will tumble slower, thus emitting light in directions closer to the orientation of the initial excitation polarization. Polarization is minimal in the absence of receptor and will increase with added receptor as the ligand bound, until it plateaus when all ligand is bound. A plot of [receptor] vs polarization yields a sigmoidal shaped curve and the binding can be found by using regression analysis.

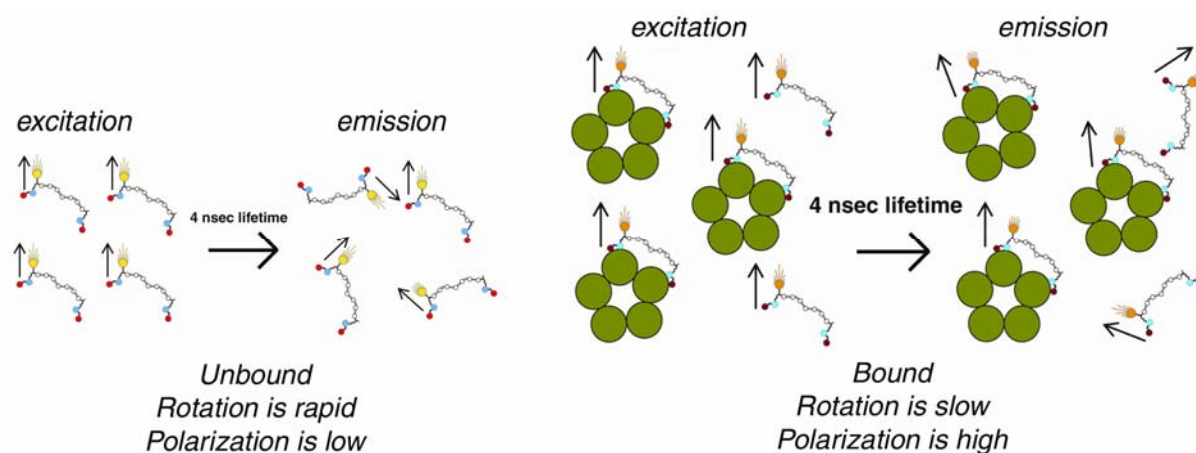


Figure 16 Cartoon depiction of how the size of a molecule and relates to the polarization.

The synthesis of the components of the ligand were similar to that outlined by Fan[36, 37] and Rickards.[38] Starting with commercially available 3,5-dinitrobenzoic acid, alternative solvent were explored to facilitate the S_NAr methoxide displacement reaction **7**. The original procedure calls for hexamethylphosphoramide (HMPA), however alternative polar aprotic solvents were tested as HMPA had a high boiling point (235°C) and would therefore be difficult to purify by distillation, not to mention the toxicity potential, especially in the large volumes (~500mL) needed for this reaction. Dimethylsulfoxide (DMSO), dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP) and dimethyl-tetrahydropyrimidinone (DMPU) were all tested and DMSO was found to be as effective a solvent as HMPA for this reaction (Figure 17). In the second step, phenol ether was hydrolyzed in refluxing aqueous HBr and acetic acid to the phenol. This step resulted in brown salts that were very problematic to remove. Efforts beyond extraction were required, which consisted of trituration with hot EtOAc: hexanes (1:3). When doing this, the product was soluble in the solvent, while the impurities oiled up as the bottom layer. Decanting and repeating the process resulted in a yellow-orange product **8** that served as the substrate for the glycosidic bond formation without further purification.

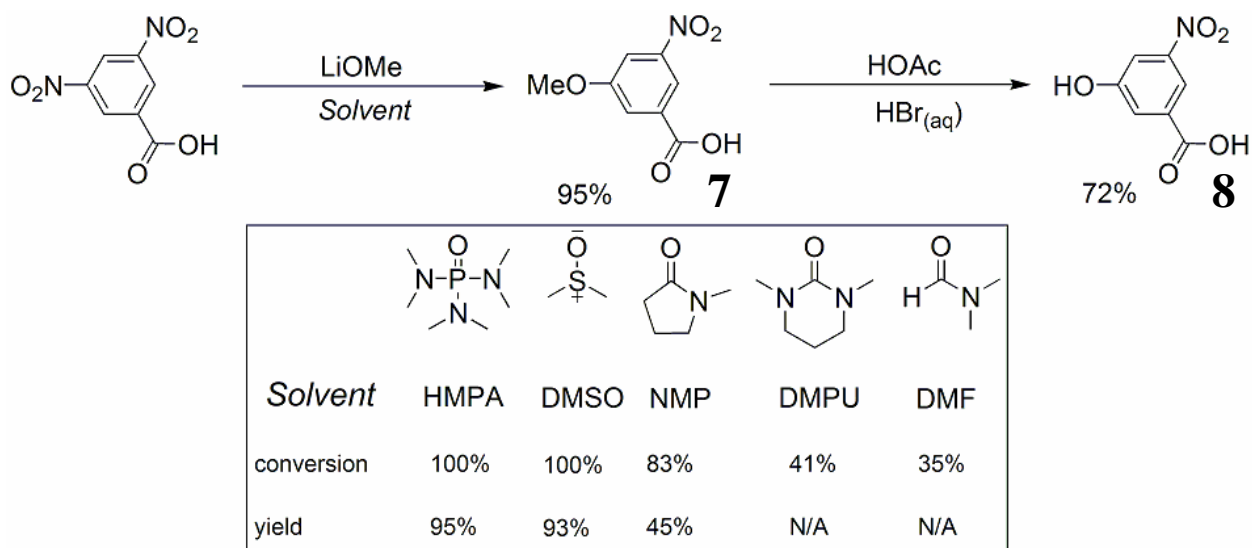


Figure 17 Synthesis of the substituted phenol.

Synthesis of the glycosidic linkage, as the desired α -anomer **9** (Figure 18), was the next step. Fan and coworkers used tin(IV) chloride in dichloromethane and claimed a selectivity of 40% for the α -anomer, in a 40% overall yield. However, using exactly the same procedure and conditions, the best that was achieved was an overall yield of 30% with an anomeric ratio of 1:4 (α : β). The net yield of this single reaction was 6% for the desired anomer. Obviously this was unacceptable, so alternate solvents and catalysts were explored.

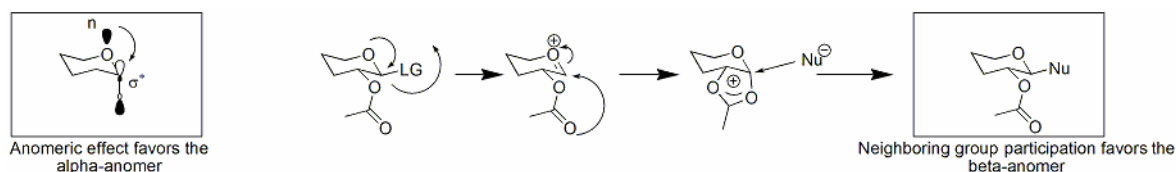


Figure 18 Conflicting preferences of the anomeric effect and the neighboring group effect.

The degree of anomeric selectivity was found to be dependant on several factors, including choice of the Lewis acid, choice of solvent, reaction time, and stoichiometry (Figure 19). Issues of solubility also arose, as it appeared that the phenol was not readily soluble in DCM. Changing the solvent to ACN with tin chloride resulted in greater solubility but identical selectivity and yield. Changing the Lewis acid to boron-trifluoride inverted the selectivity to the desired anomer. Increasing the equivalents of BF_3 added allowed the reaction time to be decreased by a factor of ten. The overall yield was still poor, with large of amounts of

unconverted starting material remaining. However this could be recovered and re-used. In the end, the objective of inverting the anomeric selectivity to the desired alpha anomer was achieved.

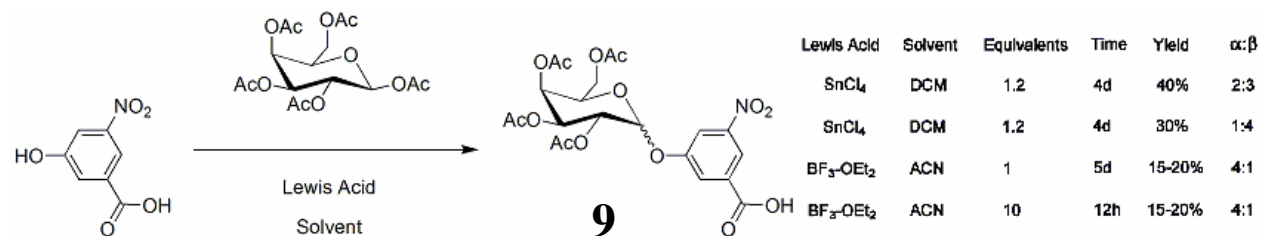


Figure 19 Table of formation of glycosidic bond. First entry is Fan published results.[37]

Simply being able to purify large amounts of material from the glycosylation reaction was a challenge, until a reversed phase C18 Isco column was used. Trying to separate a minor product from a mixture of other carboxylic acids is no easy task. However, reactions mixtures consisting of greater than 3g were routinely purified to ~90% purity using a 130g RP-C18 Isco column. The remaining steps in the synthesis (Figure 20) of the ligand were removal of the acetyl groups with NaOMe in methanol to give **10**, followed by selective enzymatic hydrolysis of the β -anomer using β -galactosidase, yielding **11**. This was required because it was not possible to resolve the anomers using chromatography, neither normal phase nor reversed-phase. At this point, the material was re-protected with acetic anhydride (**12**), purified, and used to synthesize bivalent ligands for cholera toxin.

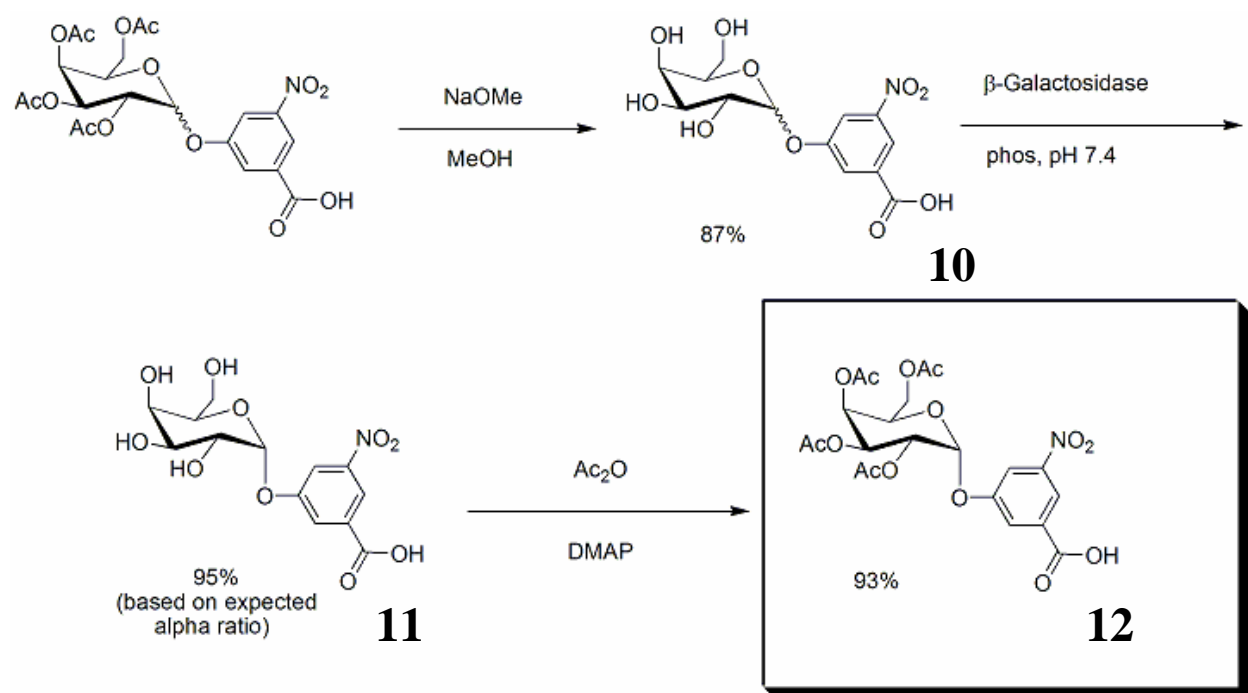


Figure 20 Remaining steps in the synthesis of the protected α -ligand

The first approach to make a bivalent ligand would involve the synthesis of a molecule varying in the number of building blocks separating the two ligands (Figure 22). Additional molecules would be synthesized varying in the number of β -Ala linking the ligand to the scaffold. These molecules were synthesized on the Rink Amide AM resin. The first residue was the diamino acid Fmoc-Ornithine (Orn) with an orthogonal 1% TFA cleavable methyltrityl (Mtt). The building blocks used in this part were all Boc-pro4(2S4S). An additional step in the synthesis of the building block was the conversion of the Cbz group to a Boc group in a one-pot reaction involving hydrogenolysis of the Cbz followed by spontaneous incorporation of a Boc group from Boc-anhydride, in tetrahydrofuran.[39] This procedure has been now been universally adopted in the synthesis of nearly all building blocks in our lab. The Boc-building block was a requirement for the synthesis of a bivalent ligand, as the best approaches to remove a Cbz groups (H_2 , Pd/C or triflic acid) were both incompatible with the nitroaromatic, glycosylated ligand.

The elongation of the sequence continued with additional building blocks until the last building block in the sequence. It was desired to couple another Orn followed by coupling of fluorescein and a ligand. Unfortunately, when an amino acid follows a building block, the

likelihood of being able to acylate the amine was slim, as the formation of the DKP was very rapid. To eliminate this reaction, a building block containing a primary amide instead of a methyl ester was synthesized (**13**). Reaction of the building block intermediate after Fmoc protection with HOBt/ HBTU and an ammonia/ dioxane solution resulted in desired product in good yield. The remaining steps of *tert*-butyl ester removal and Cbz-to-Boc were then performed to yield the building block-amide (Figure 21).

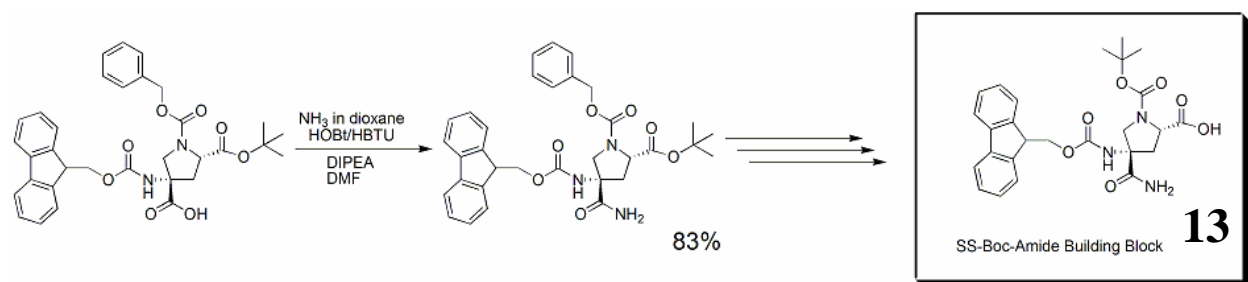


Figure 21 Synthesis of the building block-amide.

After incorporation of the building block-amide, the 2nd Orn was coupled, followed first by attachment of 5-carboxyfluorescein (5-FAM) using diisopropylcarbodiimide (DIC) and N-hydroxybenztriazole (HOBt). During the course of this overnight reaction, the beads changed to a yellow-orange color. This implied that the xanthate hydroxyls were confined in bonds made to other FAMs, creating a dendrimeric network. If this product was cleaved, the characteristic absorbance peaks for fluorescein were absent as was the predicted mass. Only after a few treatments of the resin with piperidine/DMF was the red color restored to the beads, and the typical absorbance spectrum of fluorescein returned and the desired mass appeared (**14**). After this, the resin was treated with 1% TFA to remove the 2 side chain protecting groups. Neutralization of the resin followed by coupling of the acetyl protected ligand using HOBt/HBTU produced the desired bivalent scaffolds (**15**). Use of HATU as the coupling reagent gave very little if any product.

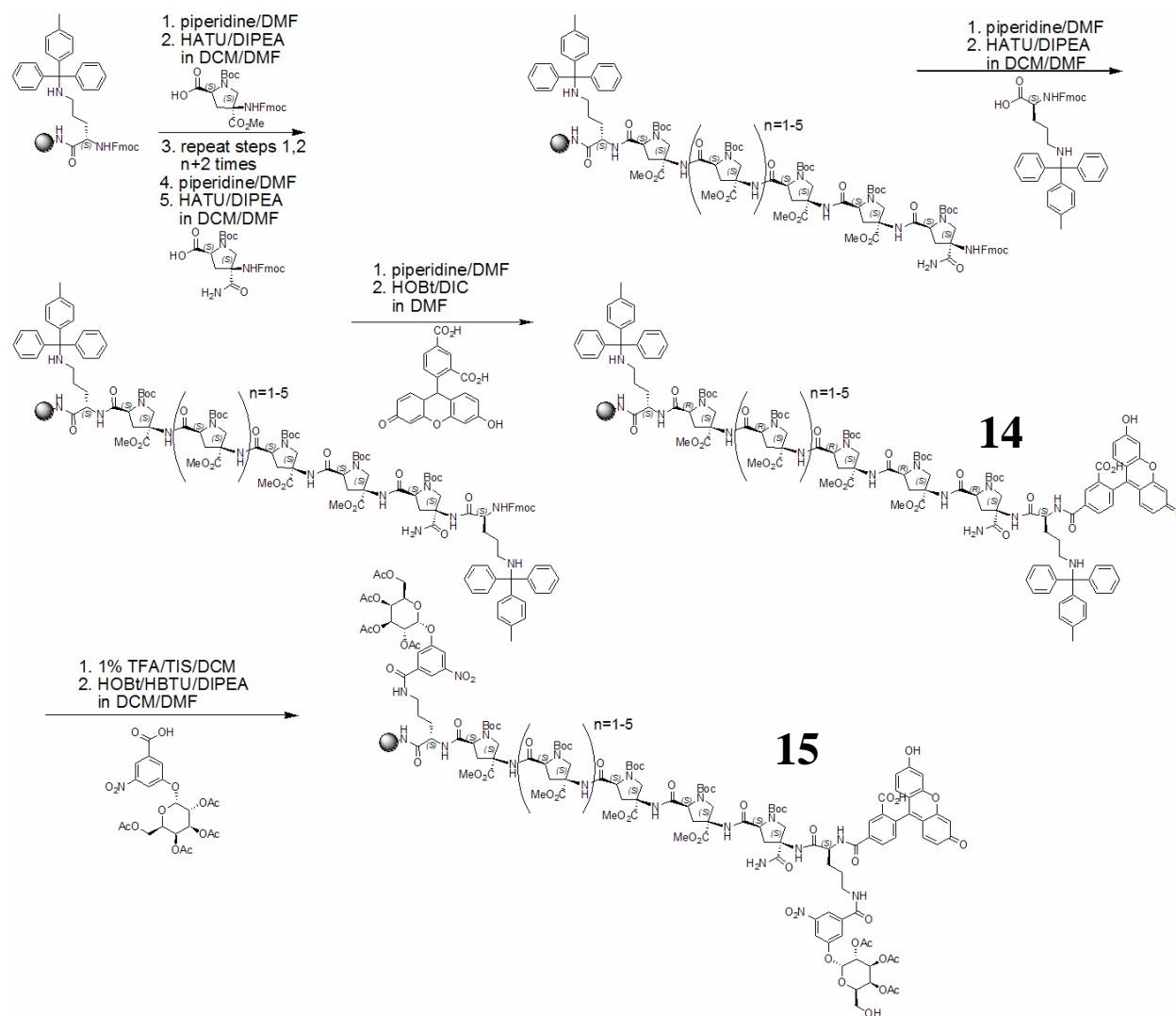


Figure 22 Solid phase synthesis of FAM-bivalent scaffolds.

Upon completion of the solid phase synthesis, the scaffold was cleaved (Figure 23) and the solvent removed (**16**, Figure 25). The residue was dissolved in MeOH and solution of NaOMe in methanol was added. The solution color changed from orange to bright red. This Zemplen deacetylation reaction needed only 10min for completion, after which it was quenched with HCl, changing the color back to orange-yellow (**17**, Figure 26). LCMS analysis at this point showed desired, deacetylated product, along with some amount of material with 1 or 2 diketopiperazines (DKPs) closed. The solvent was removed and the residue and salts were dissolved in 20% piperidine/NMP. Incubation at room temperature for 36hrs served to close the DKPs. The product was purified on an analytical HPLC system which afforded the fluorescently

labeled bivalent scaffolds (**18**, Figure 27) which were to be analyzed for binding using fluorescence polarization.

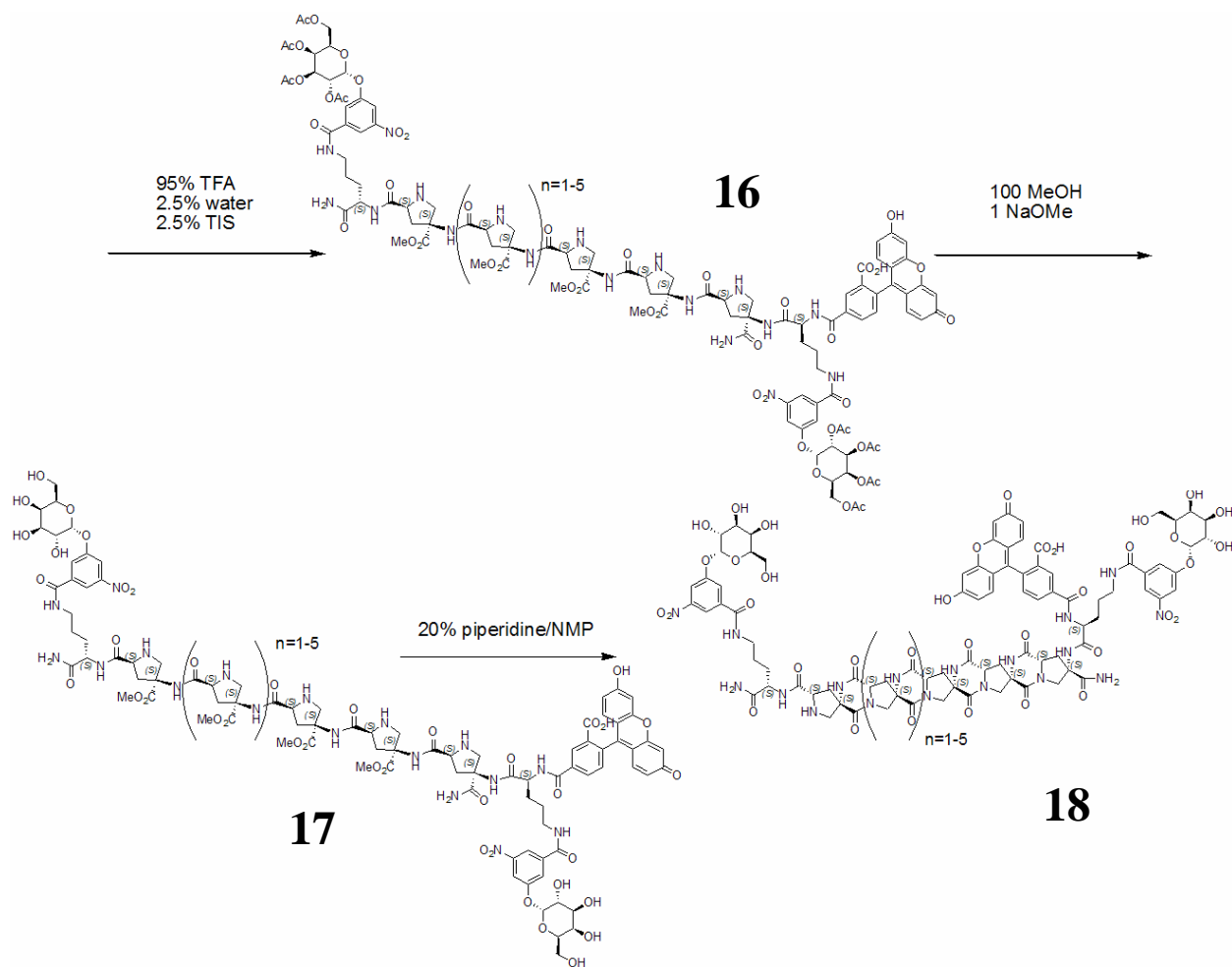


Figure 23 Cleavage, deacetylation, and DKP closure of FAM-bivalent scaffolds.

In addition to the five molecules above, of lengths 5-9 building blocks, additional molecules of this same length were also made in parallel (Figure 24). Two series, containing one (**19**) or two β -alanines (**20**) on both ends between the scaffold and ligand were made by coupling Fmoc- β -Ala to both ends, after the Mtt groups had been removed from the Orn. After the remaining steps were complete, 3 series of varying length having linkers of varying flexibility were successfully synthesized in quantities of $\sim 100\text{nmol}$, more than enough for FP measurements. In addition a monovalent positive control molecule was also made (**21**).

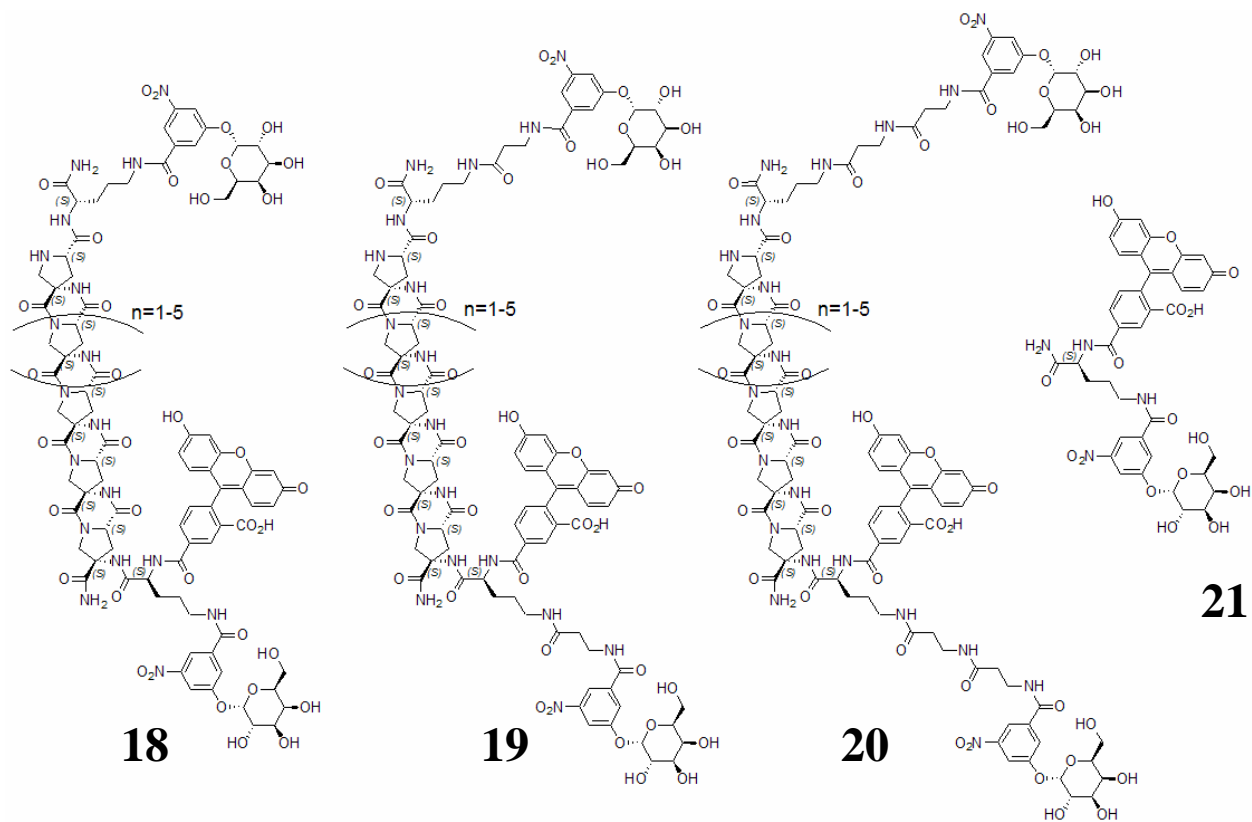


Figure 24 Bivalent scaffolds with linkers containing 0, 1, or 2 β -Ala and the monovalent control.

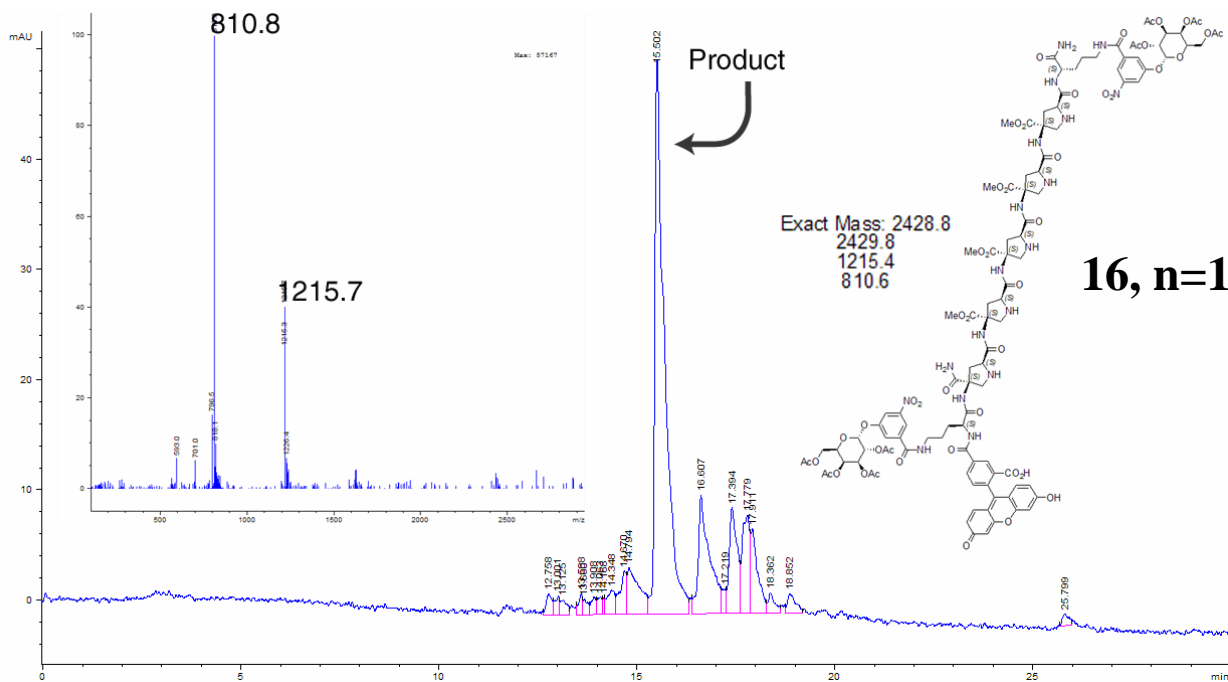


Figure 25 Representative cleavage of 5mer-FAM bivalent ligand.

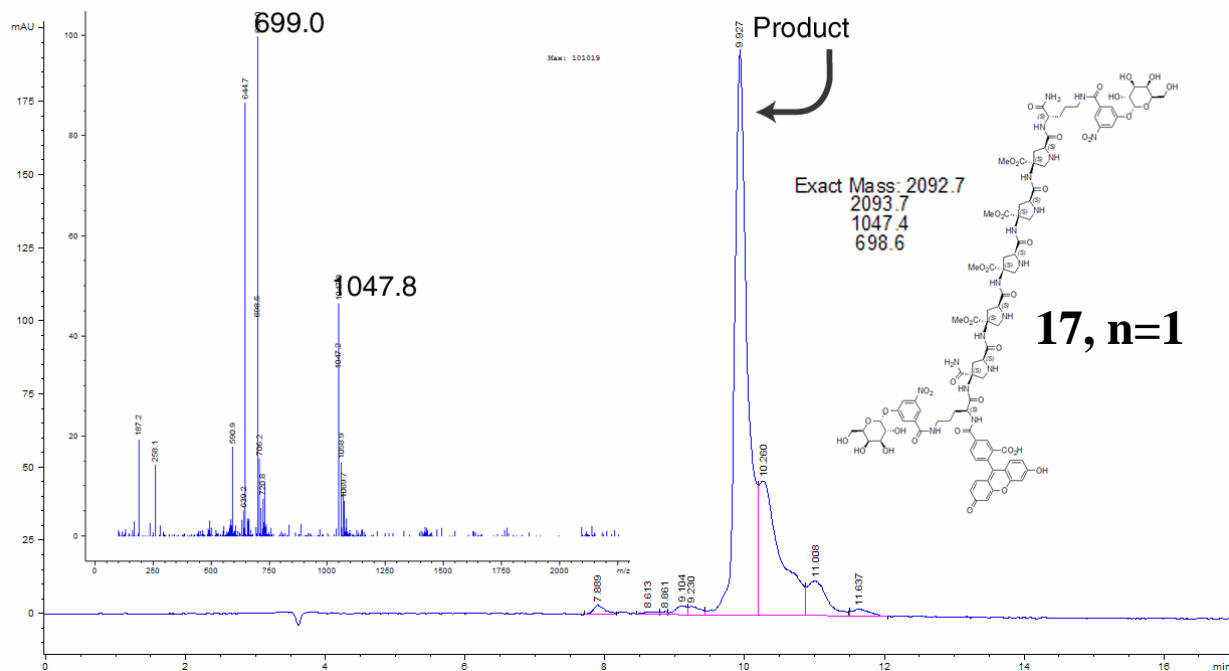


Figure 26 Representative deacetylation of the 5mer-FAM bivalent ligand.

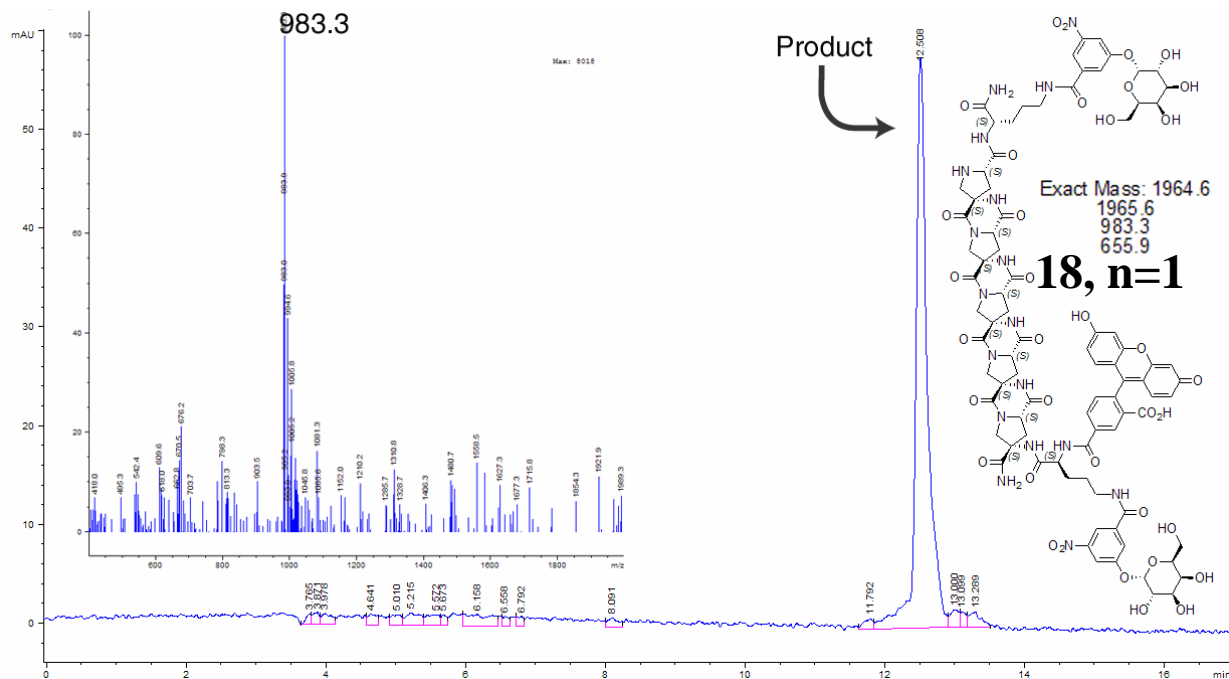


Figure 27 Representative final purified diketopiperazine closed product of the 5mer-FAM bivalent ligand.

The measurements of the fluorescence polarization were done on a LJI Analyst HT in the School of Public Health (Figure 28). The amount of fluorescent label in each 50 μ L well was evaluated using concentrations ranging from 100pM to 10nM. It was found that the intensity of

amounts above 500pM were linearly related to the concentration. However, the signal-to-noise ratio of concentrations below 1nM was borderline acceptable, so 3nM was chosen for all titrations. Inconsistent measurements of the polarization at low protein concentrations were likely due to adherence of the molecules to the plastic. When small amounts of bovine serum albumin or bovine gamma globulins were added to each well, the data at low concentrations of protein became linear and a binding curve was generated. Titrations involved the addition of recombinant Cholera Toxin β -subunit (CTB), ranging from 10nM up to 200 μ M.

Initial studies hoped to test the effects that distance and flexibility between the two ligands had on the binding affinity. Three series of five molecules were made containing 5-9 building blocks and linker composed of 0, 1, or 2 β -Ala. Unfortunately, the FP values for these molecules showed little difference from the monovalent control. Initial interpretations as to why no bivalent enhancement was obtained settled on the fact that perhaps the ideal distance and orientation of the two ligands was not achieved. Because these scaffold molecules have a helical pitch, changing the distance also changed the orientation, such that every building block added 90° rotation between the first ligand and the second. So perhaps one scaffold length held the two ligands at the ideal distance for bivalent binding, but the ligands happened to be pointing in non-ideal directions.

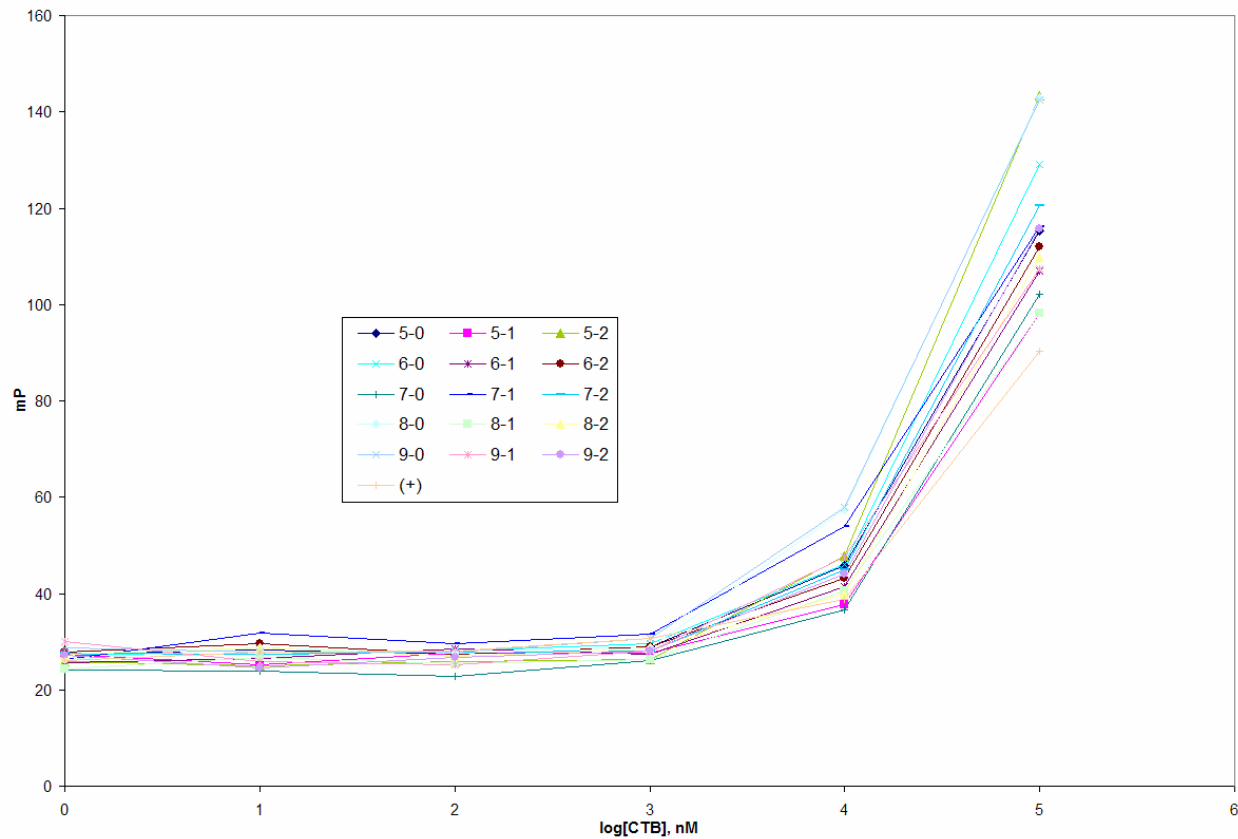


Figure 28 FP data on the 5-9mer series consisting of a linker with 0, 1, or 2 β -Ala.

The apparent solution to the problem of controlling distance and orientation between two ligands was to exploit the unique properties of molecular scaffolds. Scaffolds were made up of a series of fused rings and molecular modeling was used to search through all possible structures for sequences of building blocks that held the two ligands in an ideal binding orientation and distance. Using the coordinates from crystal structure of this ligand bound to the CTB pentamer, sequences were “scored” on their ability to hold two ligands in a “perfect” binding orientation. The ends of the bivalent ligand had a few rotatable bonds, which were limited to rotomers adopting a low energy conformation. The energy of the two ends was also factored into the score, so that the ideal score would have the two flexible ends in a low energy conformation and a given scaffold sequence would position the ligands precisely into the binding pocket. The variables for the *in silico* screening were 8mers or 9mers using all four stereoisomers of the pro4 building block (SS, RR, SR, RS) with a L-Dpr or D-Dpr at each end, which generated 1.3 million scaffolds. Six scaffolds (Figure 30; seq3 disappeared upon DKP closure), having the highest

score and the greatest likelihood of being able to hold two ligands in an ideal bivalent binding orientation, were synthesized (Figure 29, 31) and tested using FP. Additionally, two new positive control compounds were synthesized to investigate whether the fluorescein had any effect on the binding of the ligand by varying the distance between the ligand and the fluorescein (Figure 30, compounds **23**, **24**).

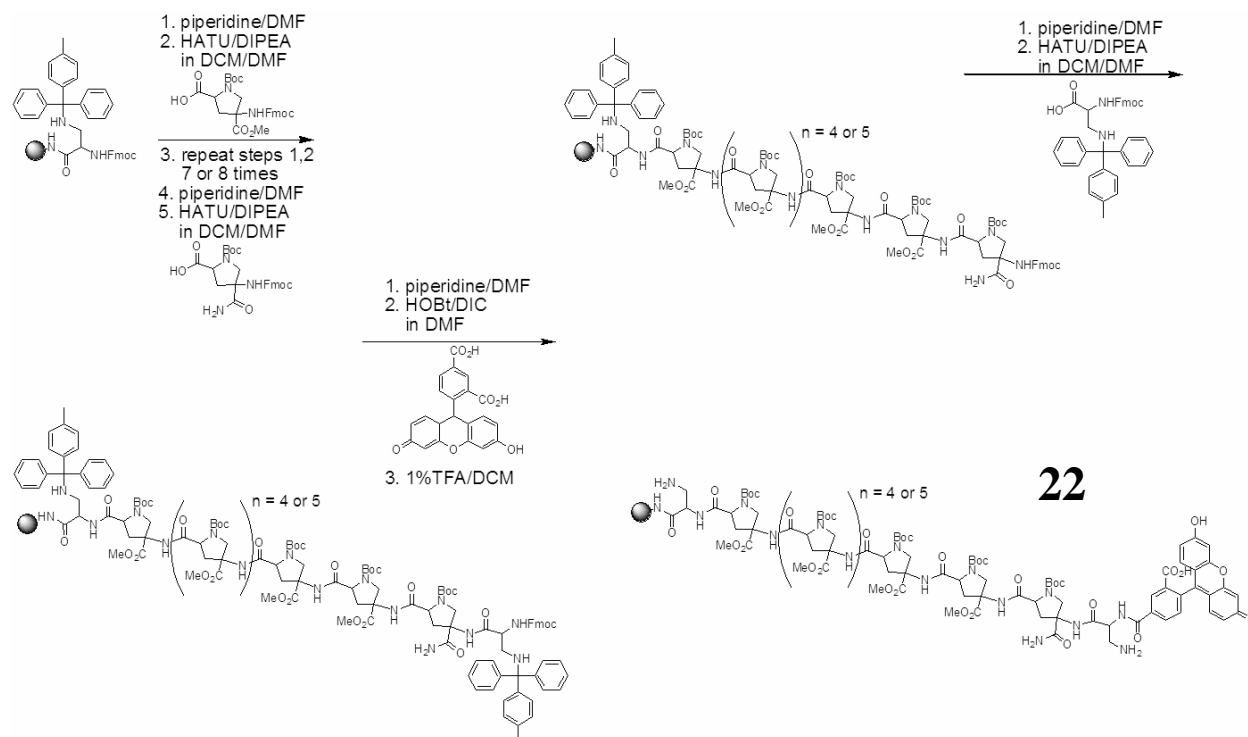


Figure 29 Synthesis of the resin bound predicted bivalent intermediate

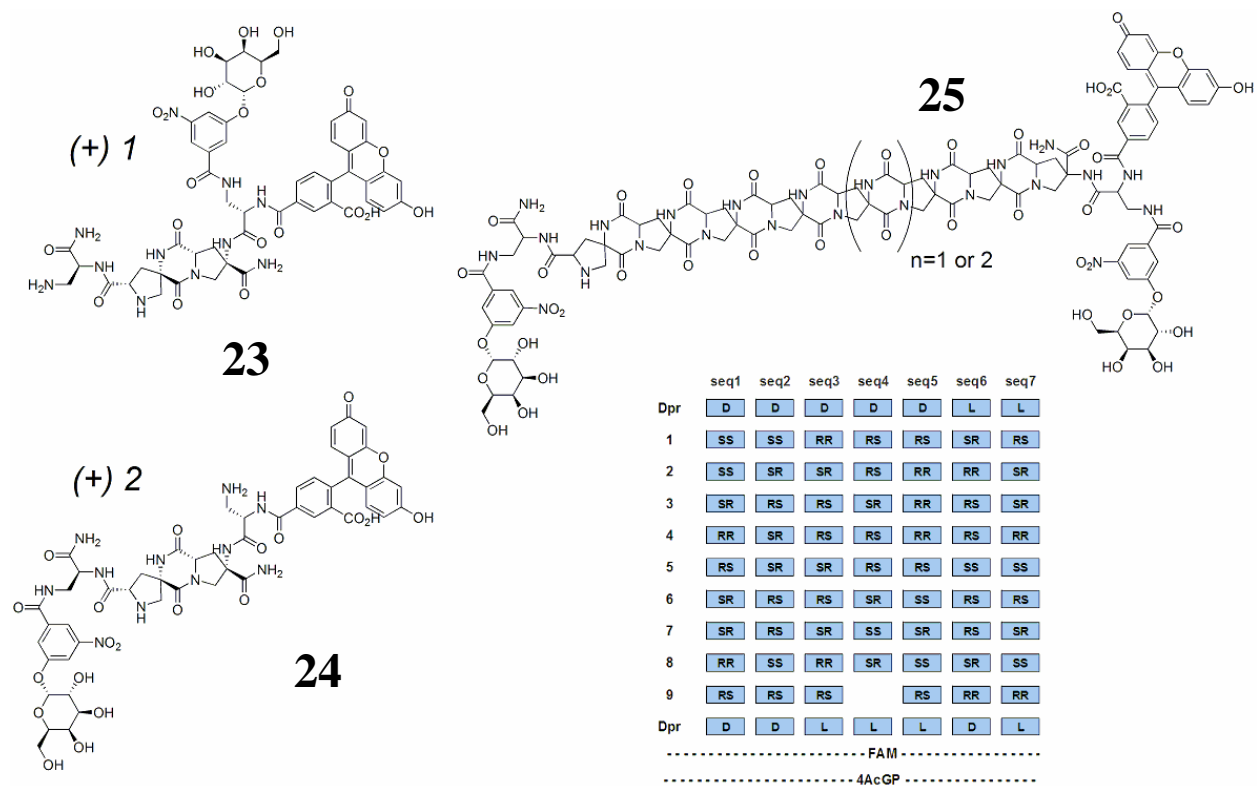


Figure 30 "Predicted" bivalent ligands (25) and new monovalent controls.

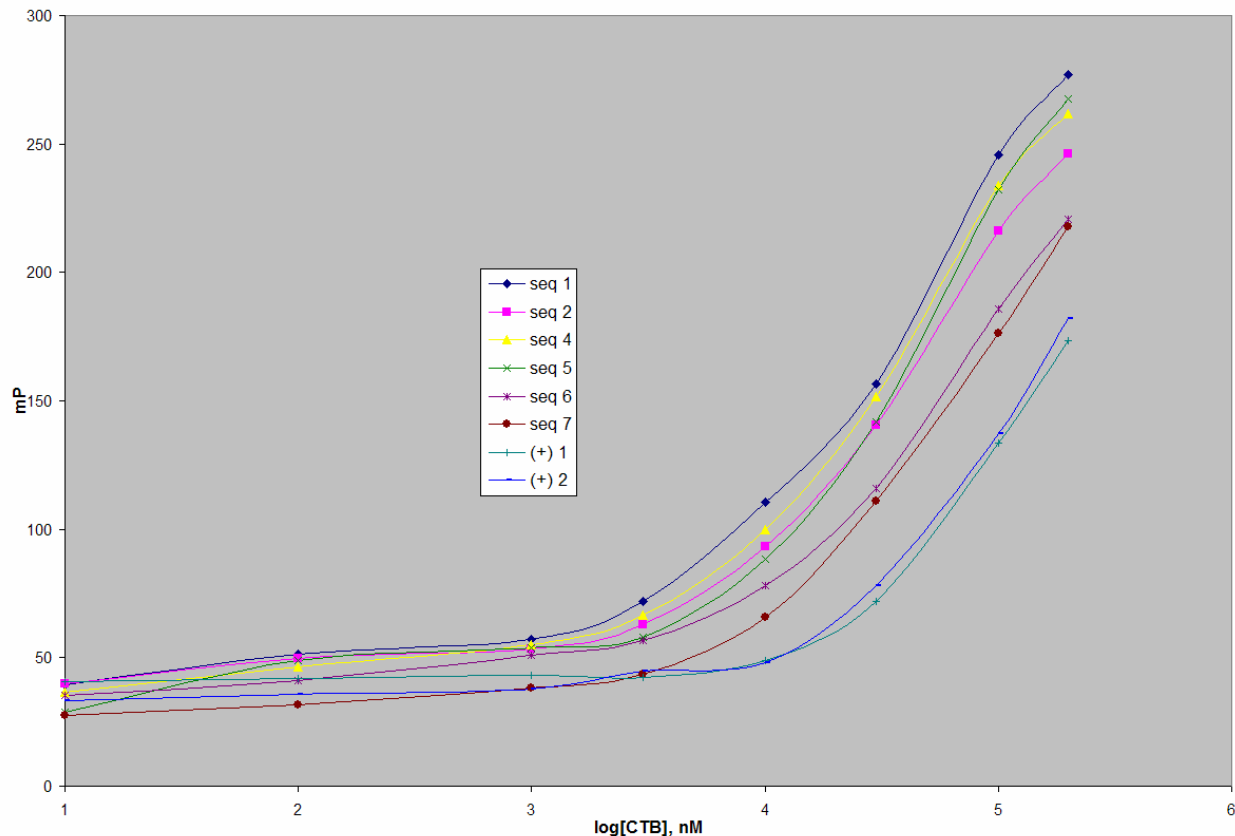


Figure 32 FP binding titrations of (+) controls and predicted sequences.

The results (Figure 32) for the bivalent predicted sequences (**25**) implied that these ligands were not binding bivalently, as the enhancement seen was no more than a factor of five. Further study of the structure of these molecules was required. 2D-Electron Paramagnetic Resonance (EPR) techniques are capable of measuring distances between two unpaired electrons with angstrom precision. Attachment of two nitroxyl spin labels to seq5, 6, and 7, (**26**) instead of a ligand, would lend insight into whether the two ligands were spaced the proper distance apart, allowing experimental validation of the molecular modeling predictions. The distances that were measured (Figure 33) were between 30Å-33Å, indicating that these bivalent scaffolds were entirely capable of holding two ligands the desired distance apart (30Å -35Å).

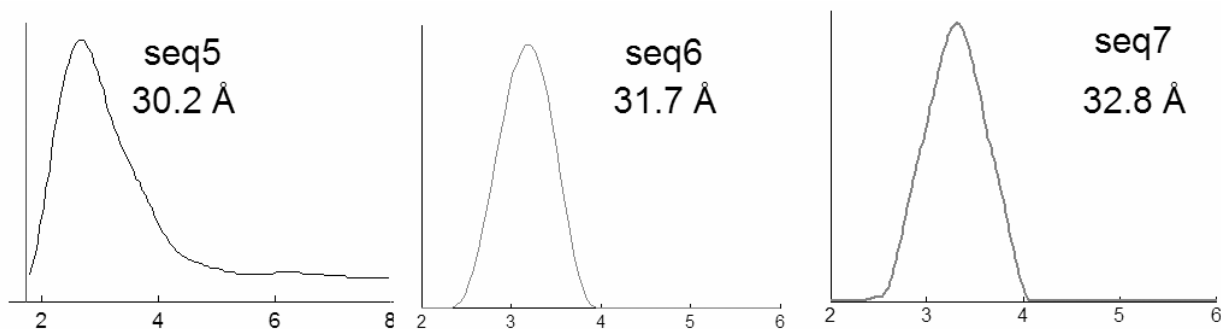


Figure 33 2-D EPR measurements of the distance between two nitroxide spin labels for seq5, seq6, and seq7.

Two final molecules were synthesized as controls. As mentioned in the introduction, many groups have seen impressive bivalent enhancements with a simple PEG linker. A PEG linker was attached to seq5 (**28**) between the ligand and scaffold (Figures 31, 34). Also, since it was known that the sugar portion of this ligand was primarily responsible for the specific binding interactions in the pocket, replacement of the sugar portion with a methyl group (**7**) should eliminate any binding in the bivalent **27** (Figures 31, 34).

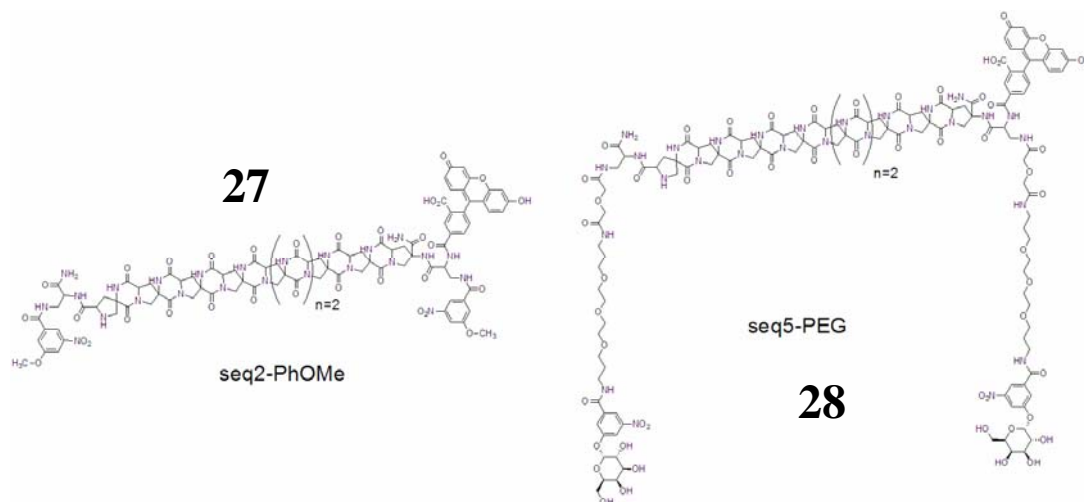


Figure 34 Structures of the negative control and PEG-bivalent ligand.

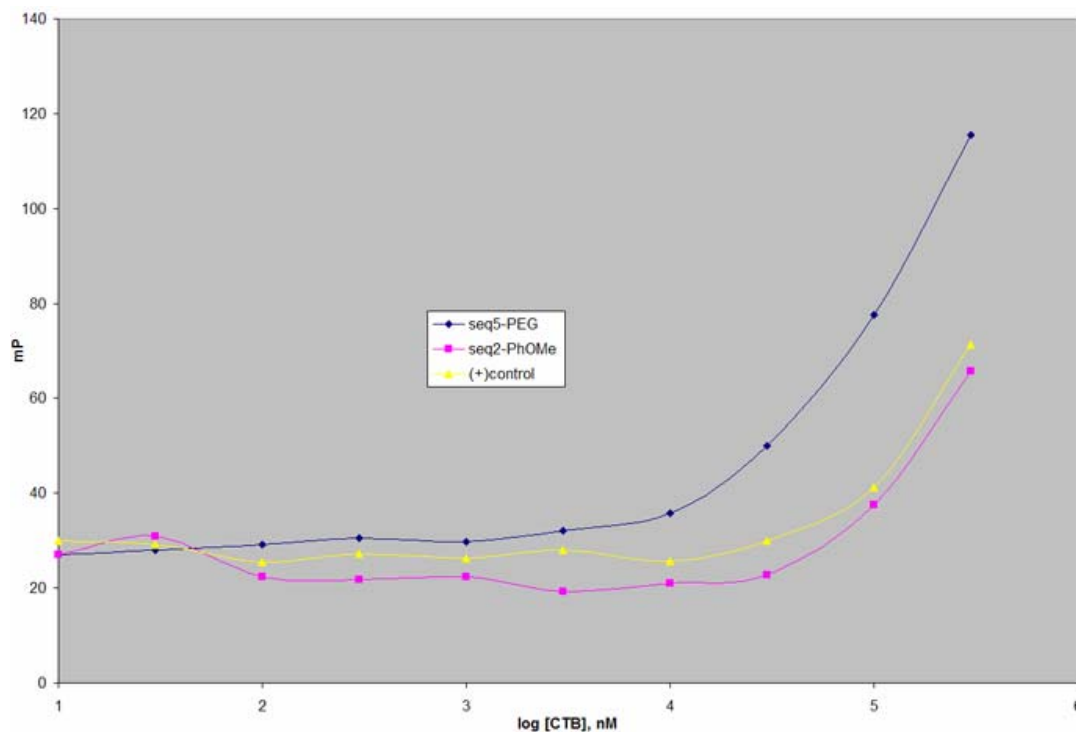


Figure 35 FP titration of PEG-bivalent ligand (-) control and (+) control.

The results (Figure 35) were that the PEG-bivalent ligand **28** bound only a factor of two better than the positive control **21** and that the *des*-galactose bivalent ligand **27** bound with similar affinity to the monovalent control. The interpretation of this result was damaging, because, not only did it appear that there was no potential to create a bivalent ligand because the PEG-seq5 failed to reveal an enhancement, it was reasoned that the ligand itself was not interacting in the binding site. A bivalent scaffold, stripped of the galactose portion, was binding with similar affinity to the monovalent ligand control. Our conclusion at this point was we had been led astray by at least one of three possible root causes: the protein was bad, the ligand was a non-specific binder, or the assay conditions were flawed.

2.2.2 Lactose Ligand

The next ligand to be synthesized, attached to scaffolds, and evaluated for bivalent enhancements was a lactose-thiazoline-benzoic acid derivative that had some appealing features.[40, 41] First, the synthesis was fairly quick and straightforward, devoid of multiple purifications. Each step

was reasonably high-yielding. But most appealing was the fact the glycosidic linkage was the β -anomer, which was much easier to control, as the selectivity for it was near unity. As with the synthesis of the previous ligand, this published procedure was modified and optimized.

Starting with Boc-protected propargyl amine **29** (Figure 36), a Sonogashira reaction between the alkyne and *meta*-iodomethylbenzoate produced the desired product **30**, which was purified using normal phase chromatography. TFA deprotection yielded a brown sludge which was initially used in the next step without further purification, as Vrasidas suggested. Purification of a free amine **31** would normally be challenging, but the RP-C18-Isco column proved highly capable. Five grams of crude amine salt was purified using a water/ACN gradient. Removal of the solvent afforded a tan solid in >75% recovery, and use of this material resulted in higher yields and purity in subsequent reactions.

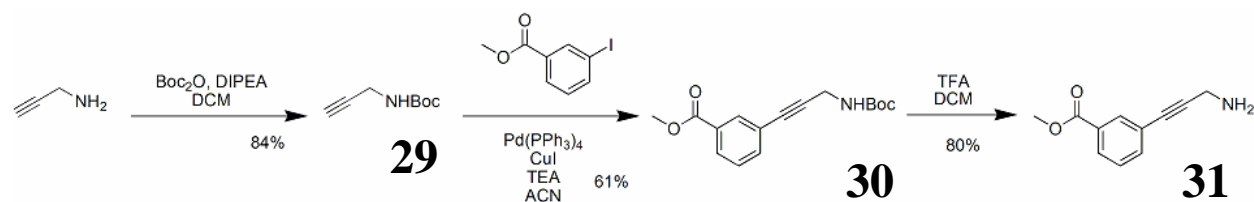


Figure 36 Synthesis of the aromatic-propargyl-amine fragment.

The synthesis of the lactose amine (Figure 37) involved two standard sugar-chemistry reactions: first peracetylation of lactose with acetic anhydride and DMAP to form a mixture of anomeric acetates **32**, followed by HBr in AcOH treatment, which resulted in α -peracetyl-lactose bromide **33**. At this point, the authors recommend a neat melt of the lactose bromide in the presence of KSCN for large scale preparation the β -lactose isothiocyanate **36**. This reaction produced multiple products and was deemed unworkable in our hands. Otherwise, the authors recommended a three-step procedure involving initial phase-transfer catalyzed (PTC) formation of the lactose azide **34**, followed by reduction with H₂, Pd/C (**35**), and immediate reaction with thiophosgene to form **36**. This sequence proved to much more useful, as it yielded the desired product as ~10:1 mixture of anomers, favoring the desired β -isomer. The anomeric impurity was traced back to the reduction step, which was logical because having the electron withdrawing amine group in the β -position was destabilizing. In the presence of trace water or acid, the pyranose ring could open and reform, resulting in the amine (**35**) adopting the more stable α -position. This α -impurity could be removed in subsequent steps. However, a literature search

revealed yet another way to make sugar-isothiocyanates, which utilized the same PTC along with KSCN to make the desired β -product **36**, in 1 step vs. 3 steps, but more importantly, the anomeric ratio was $>50:1$. This reaction was done on a 5g scale to afford large amounts of material for subsequent steps.

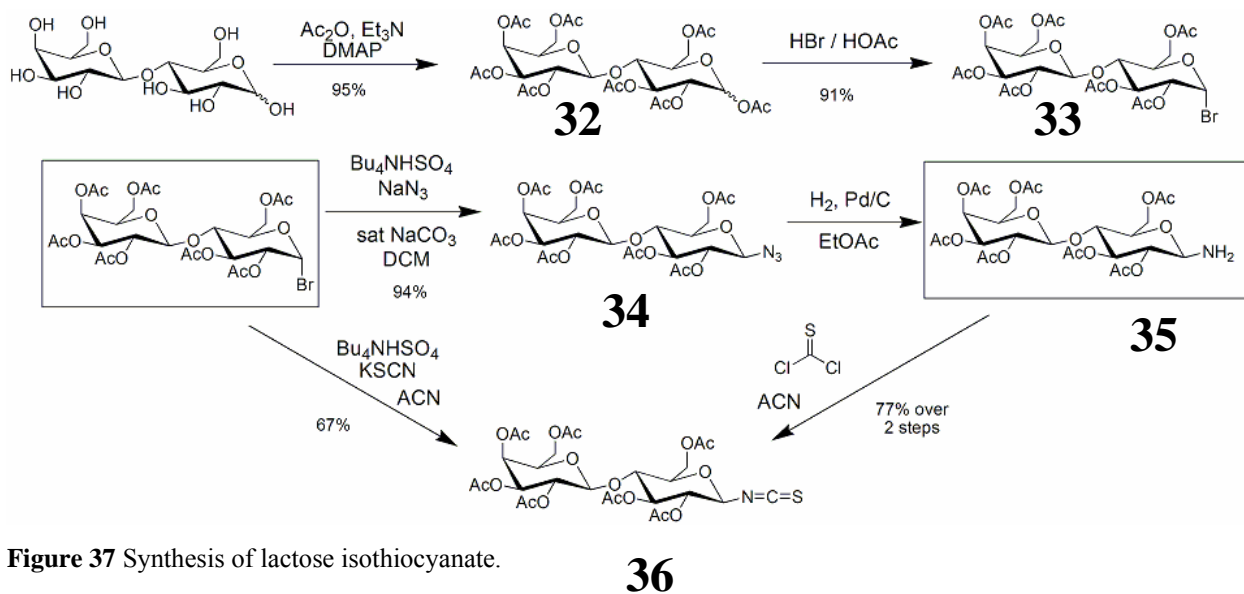


Figure 37 Synthesis of lactose isothiocyanate.

Reaction between the isothiocyanate and amine (Figure 38), to initially form the thiourea (**37**), proceeded cleanly with the purified amine, using a modified procedure. When amine was the limiting reagent or excess base was used, which was a requirement when using the crude oil from the TFA deprotection, an extra peak on the LCMS was seen, corresponding to a mass of 2-NCS, 1-amine. The reaction proceeded with $\sim 100\%$ conversion when the purified amine was the excess reagent and 1eq base was used. The reaction was found to be complete in less than 1hr, as opposed to the 12hr reaction time used by Vrasidas. After the initial reaction time, 10eq of acetic acid were added to form the thiazoline cyclized product (**38**). This also was a modification, as the authors worked up the reaction before adding the acid. The thiazoline was purified on the RP-C18-Isco column, as a single injection of the crude product dissolved in acetic acid. As Vrasidas desired to keep the methyl ester (**39**, $\text{R}=\text{Me}$) while removing acetyl groups from the lactose, we diverged from them in the next step. Saponification of all esters was accomplished in a solution of methanol and lithium hydroxide, and by extending the reaction time from 1hr to 12hrs (**39**, $\text{R}=\text{H}$). Initial inspection of the crude product by LCMS showed three peaks, all with the same mass. Addition of HCl until the pH was neutral caused the three LCMS peaks to coalesce into a single peak. Care was needed in removal of some of the

methanol in order for the highly polar product to be retained by the RP-C18-Isco column because it was found that the elevated temperatures used to expedite the removal of solvent *in vacuo* would scramble the product distribution from a single peak to multiple peaks, again having the same mass. Removal of the methanol without heating resulted in little, if any, rearranged product.

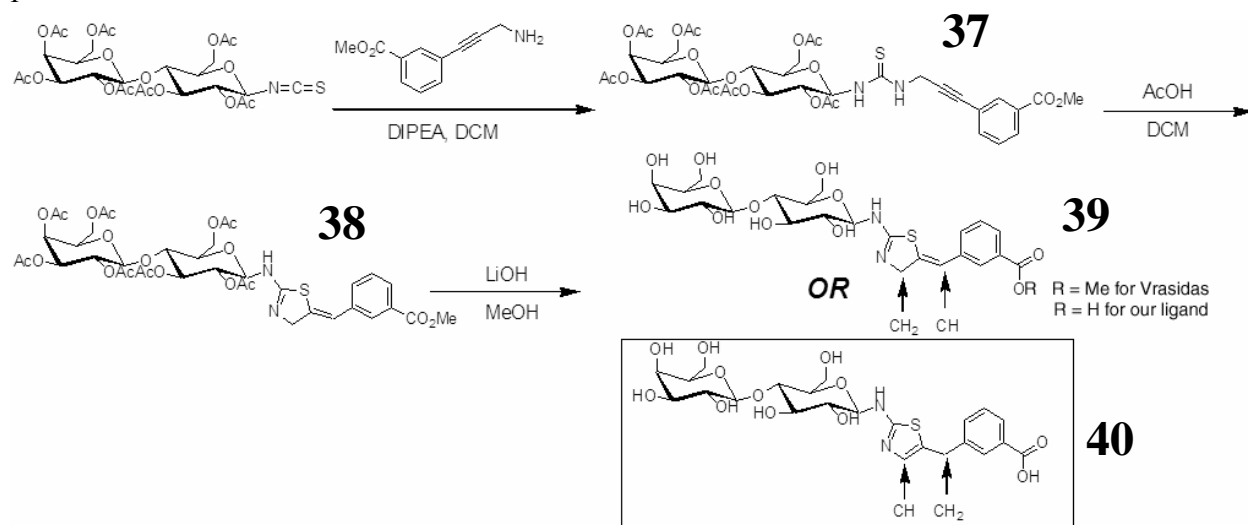


Figure 38 Remaining steps of the synthesis of the lactose containing ligand, with the two possible tautomers highlighted.

Comparison of the $^1\text{H-NMR}$ spectra for this product with the published spectra of the methyl ester showed differences that appeared to be not solely due to the differences between a carboxylic acid and a methyl ester. The experimental $^1\text{H-NMR}$ and the published $^1\text{H-NMR}$ was mainly congruent, however one discrepancy was apparent, besides the difference between methyl ester and free acid. The $-\text{CH}_2-$ proton peak had shifted from about δ 5ppm to δ 4ppm in the carboxylic acid ligand product. Furthermore, when the free acid was converted to the methyl ester using TMSCHN_2 , the two $^1\text{H-NMR}$ spectra should have been identical, but they were not, with the $-\text{CH}_2-$ peak again being farther upfield. 2-D NMR spectra were acquired of the ligand carboxylic acid consisting of an HSQC and an HMBC (Figure 39) to elucidate whether the structure was more consistent with **39** or **40**. The HSQC produced cross peaks for J^{HC} 1-bond coupling, which identified the protons attached directly to carbons. The HMBC showed cross peaks for J^{HC} 3-4 bond coupling, which identified the protons and carbons that were nearest to a given signal. From the HMBC spectrum, we determined that the $-\text{CH}_2-$ was closer to the aromatic ring than the $-\text{CH}-$, which was consistent with a benzylic $-\text{CH}_2-$ (**40**).

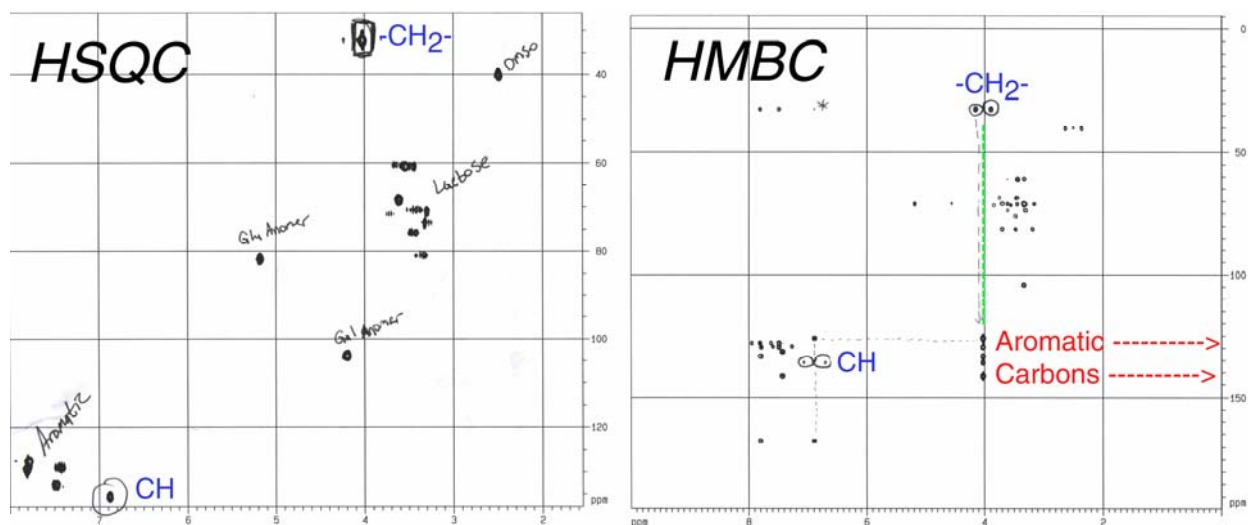


Figure 39 2-D NMR spectra (HSQC and HMBC) of the final ligand used in tautomer identification (**39** or **40**).

Thus far, the CTB that had been used was expressed in *E. Coli* and purified on a galactose affinity column, followed by diafiltration to remove the excess galactose (plasmid was a gift from Minke at UWash). This recombinant CTB (rCTB) formed a complex with anti-CTB monoclonal antibody which had a molecular mass around 10kDa. LCMS analysis of this protein gave a mass of ~11803 Da. Sequence analysis predicts a mass of ~11606 Da. Additionally, comparison of the fluorescence spectrum (Figure 40) of the rCTB with what was seen in the literature or with CTB purchased from Sigma proved brought more suspicion upon the rCTB. From this point on, we exclusively used CTB purchased from Sigma.

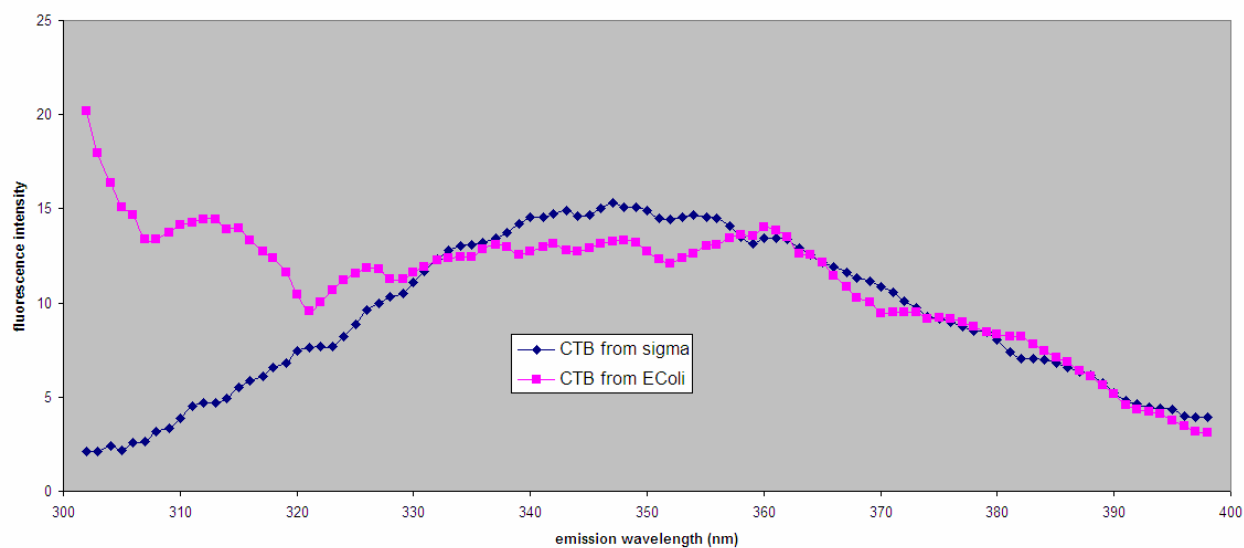


Figure 40 FI spectra comparing two sources of CTB.

The ligand (**39** or **40**) was evaluated for binding using a tryptophan fluorescence assay (Figure 41).[40] CTB contains 1 Trp residue and the fluorescence intensity (FI) was plotted against increasing concentration of ligand, while the protein concentration was maintained at 100nM, 250nM or 500nM. Trp FI is very sensitive to its environment, and since this Trp is found in the binding pocket, the FI is directly correlated with the extent of binding. As more ligand binds, fluorescence of the Trp decreases, until saturation occurs, then no more change in FI is seen. Plotting of Δ FI vs. ligand concentration generated a sigmoidal curve, and regression analysis yielded a binding constant. The synthetic ligand-carboxylic acid (**39** or **40**) was tested for binding to CTB, at a CTB (monomer) concentration of 500nM. The ligand was varied from 0 to 150 μ M. These are the exact conditions used by Vrasidas, and he obtained a binding constant of \sim 23 μ M. Our carboxylic acid ligand had a binding constant of \sim 42 μ M, which was derived from a Scatchard plot of (fraction bound/free ligand) vs. (fraction bound). The slope of the line is equal to $-K_a$. Additionally, the x-intercept of the Scatchard line = V_{max} , and was 595 Δ FI units. It was gratifying to finally see an irrefutable sigmoidal binding curve and derive a binding constant that made sense.

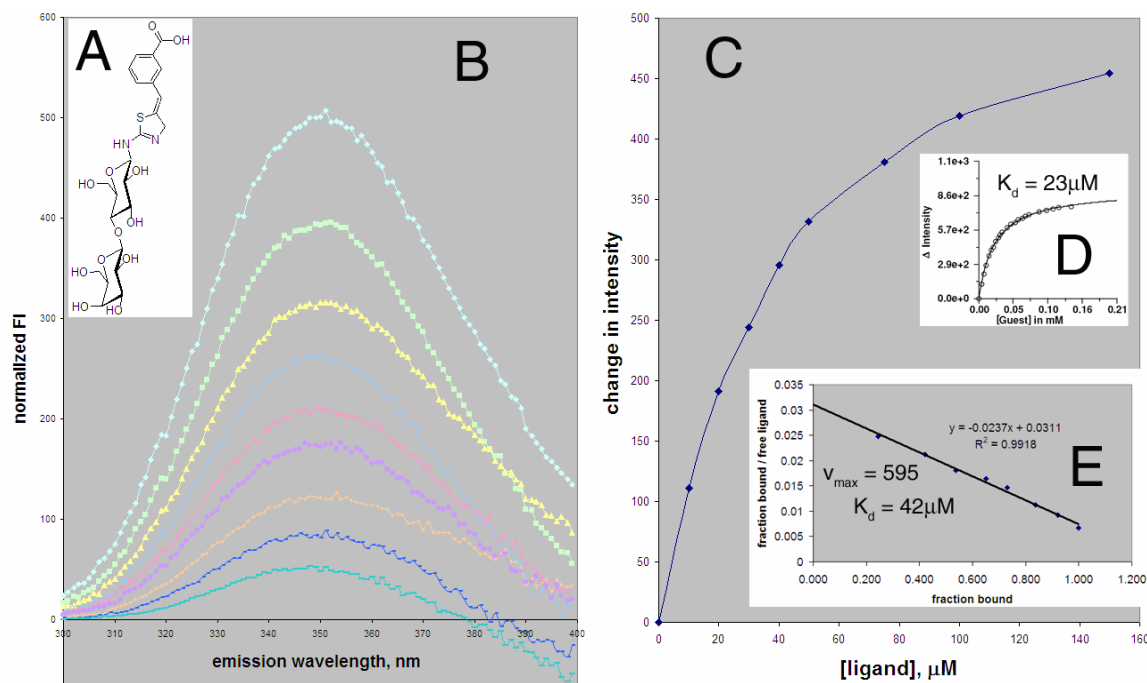


Figure 41 (A) structure of carboxylic acid ligand (**39** or **40**) used for FI assay (B) fluorescence intensity curves with increasing amounts of ligand (C) binding curve of Δ FI vs. [ligand] (D) Vrasidas binding curve from publication (E) Scatchard plot of (C)

The initial test of the ligand (**39** or **40**), shown above, validated that the synthetic ligand bound to CTB with similar affinity to that of Vrasidas. However, precise structural characterization of the ligand by 2-D NMR methods was not done until months later, by this time this initial batch of ligand was long gone. The 2-D NMR spectra in Figure 39 shows that the ring structure in our molecule is different than Vrasidas'. Comparison of the FI binding curves (Figure 42) of the "First titration" with that of the structurally assigned ligand, called "Last titration", showed that the two bound with different affinities. One conclusion of this is that the "First" ligand may have been structurally different than the "Last" ligand, which only could have been the result of differential reaction conditions when the esters were saponified in the final step with LiOH. The tautomer of the "First" may have been similar to Vrasidas, that of the conjugated exocyclic double bond (**39**), while the "Last" was confirmed to be the tautomer containing a benzyl -CH₂- between two aromatic rings (**40**). If this conclusion is accurate, this tautomer, having a K_d of ~3μM, represents the strongest binding monomeric CTB ligand discovered to date. A medicinal chemist would have been thrilled with this 10-fold improvement in binding affinity, however the consequences for this project were negative, because now the dynamic range for the FI assay to measure potential bivalent enhancement had also been reduced by a factor of 10.

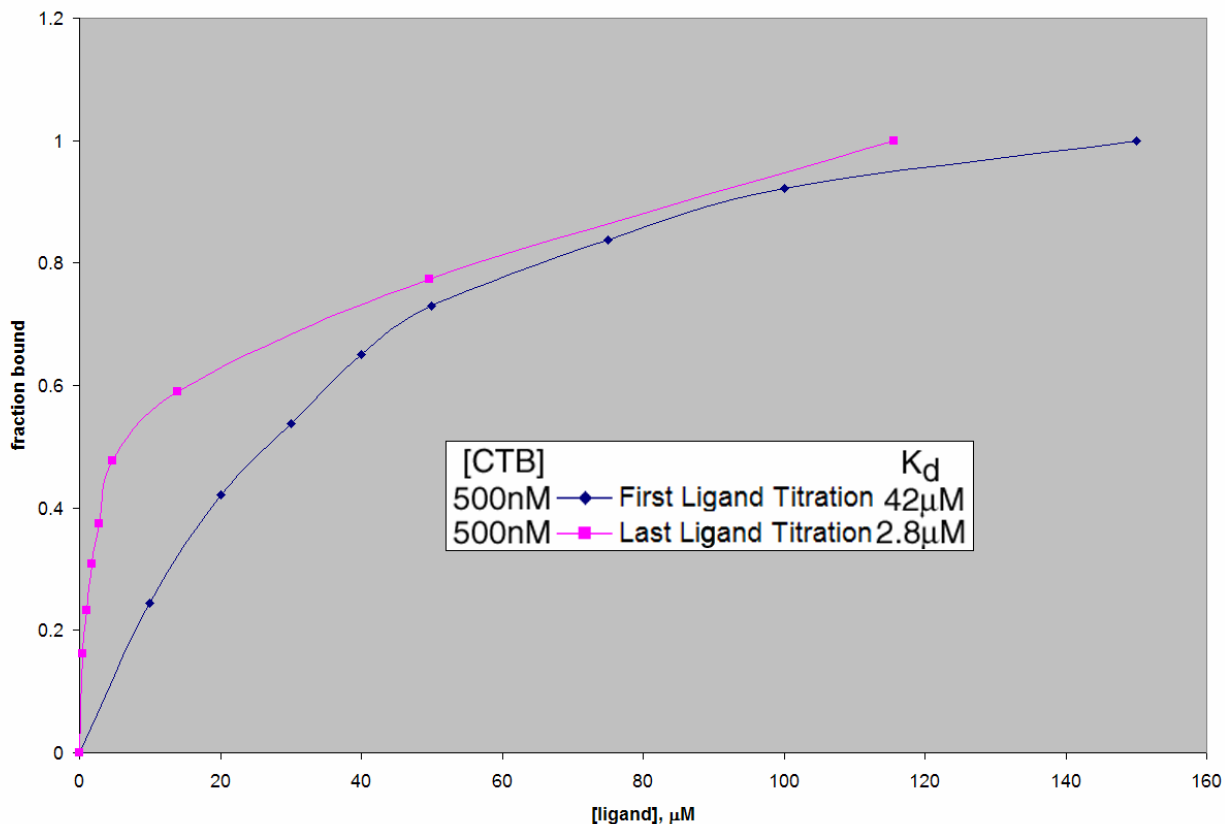


Figure 42 FI binding curves comparing the ligand binding of the very first titration with that of a later batch of ligand.

Incorporation of this ligand into the scaffold was initially fraught with difficulty. The final ligand (**39/40**) was protected with acetyl groups, as this was thought to aid in making the coupling reaction more specific. Using similar techniques as in the previous synthesis of bivalent galactose-based ligands, involving coupling of the acetyl-protected ligand, followed by cleavage, de-acetylation, and finally DKP formation were found to be incompatible with this ligand. For reasons not understood, incubation of the ligand, with or without acetyl groups, in the 20% piperidine/ NMP solution needed to close the DKPs produced a large, broad LCMS peak not seen before. A possible solution to this problem was to couple the ligand in solution after the scaffold DKPs had been closed. This failed because of the difficulty involved in separating bivalent product from monovalent and starting material. Clean bivalent product was an absolute requirement for the evaluation of any bivalent enhancements.

An alternative solution to this problem was to close the DKP first on the resin, then couple the ligand on the resin. This had routinely been done in the lab by others, however they

employed a resin that was stable to the TFA Boc-deprotection conditions, with cleavage being done with triflic acid. Triflic acid, and even TFA, are known to readily hydrolyze glycosidic bonds. Fortunately, the glycosidic bond in this ligand showed negligible hydrolysis when exposed to TFA for less than two hours, but complete hydrolysis in triflic acid occurred in less than 5 minutes. This meant that the Rink Amide resin had to be used, but non-TFA Boc-deprotection conditions would be needed.

Other on-resin-Boc-deprotection conditions were tried, both thermally (190°C in acetic acid for 2hr) and chemically (cerium ammonium nitrate), but both were ineffective. The third set of conditions attempted utilized the Lewis acid TMSOTf (trimethylsilyl triflate), which would complex with the carbonyl oxygens, and 2,6-lutidine would then attack the carbonyl.[42] The difference in reactivity of carbamates vs. amides allowed these conditions to selectively remove the Boc group. Although the Rink linker was not cleaved under these conditions, other parts of the linker were reactive when using the Rink Amide AM resin. Switching to the plain Rink Amide resin resulted in clean removal of the Boc group, in three treatments of 3min each, while keeping the scaffold attached to the solid support (Figure 43). This was a major breakthrough, especially when it was found that the DKPs could be quantitatively closed on the resin with NMP/piperidine in only 10hrs, simply by raising the temperature to 37°C (**41**).

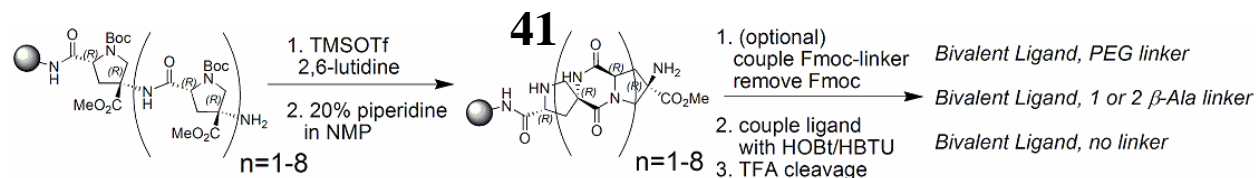


Figure 43 On-resin Boc-deprotection and DKP closure, followed by acylation to form bivalent ligands with varying linkers.

With conditions in hand to close DKPs on-resin, the next step was to attach the ligand. It was found that the ligand, without the hydroxyls protected, could be coupled cleanly and efficiently using HOBt/HBTU activation, 2 x 2eq (per 2 amines) in DMSO:DMF for 2 x 1hr with stirring. The cleavage conditions were found to be critical. If the cleavage in 95%TFA was allowed to proceed for more than 1hr, significant amounts of hydrolysis of the glycosidic bond became apparent. This was particularly problematic because both the desired product and the *des*-galactose products had nearly identical retention times by RP-HPLC, as the sugar portion seemed to be the dictator of polarity. On several occasions, material had to be discarded as

inseparable, but limiting the cleavage time to one hour and using the Speedvac to quickly remove the TFA after cleavage proved to remedy this problem. The final bivalent products were easily purified on a preparative HPLC to homogeneity. An HPLC calibration curve was used to estimate the concentration, and the HPLC fraction was aliquoted into fractions consisting of enough material for one FI titration, and then lyophilized. This was required because it was found that large amounts of bivalent ligand were presumably adhering to the plastic tube when the sample was allowed to stand in buffer at 4°C. When the time for titration came, the pellet was resuspended and the absorbance spectrum was measured. Using the extinction coefficient ($\epsilon = 8855$) derived from a Beers law plot of the glycine methyl ester acylated with the ligand carboxylic acid, the concentration of the bivalent ligand was determined.

A series of bivalent ligands were synthesized containing various linker lengths. These series included bivalent ligands with PEG linkers, bivalent ligands with 1 or 2 β -Ala ligands, and finally, bivalent ligands with no linker, as seen in Figure 44.

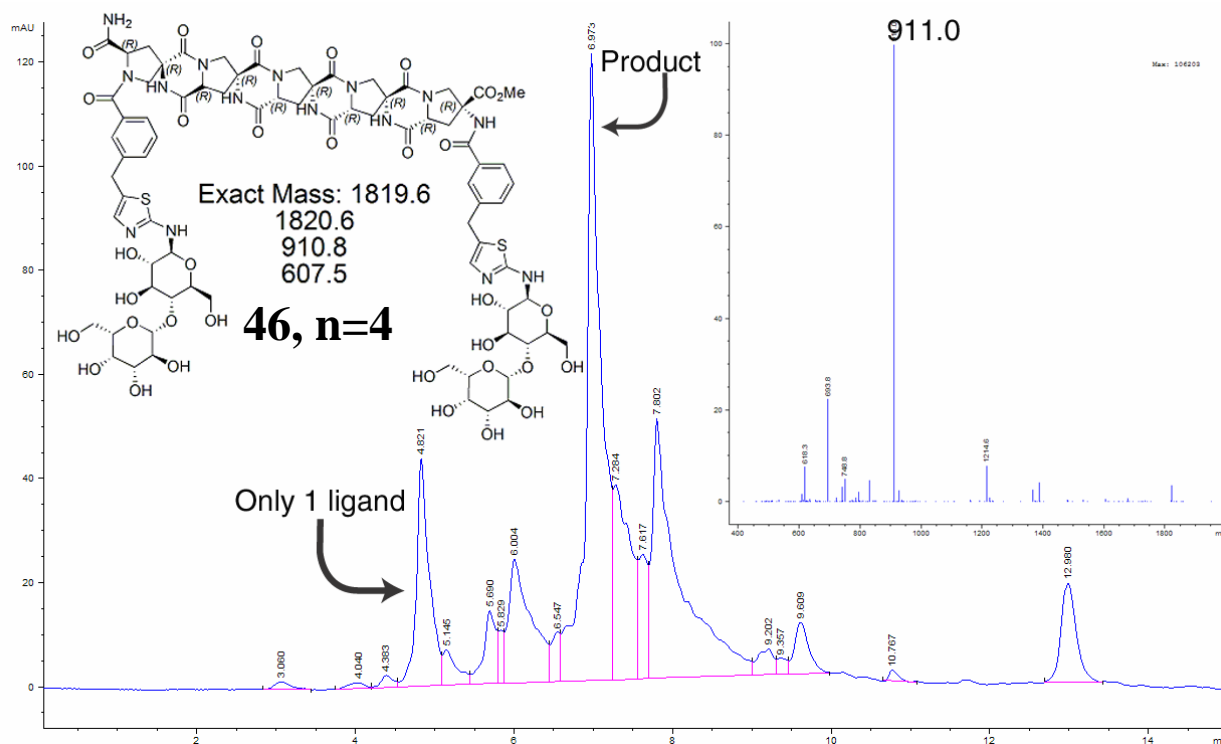


Figure 44 Representative LCMS chromatogram illustrating the effectiveness of the scaffold synthesis, Boc-deprotection, on-resin DKP closure, and coupling of the unprotected lactose ligand. 5mer-bivalent ligand described in Figure 50.

Scaffolds containing 5-9 building blocks with a PEG linker (Figure 45) connecting the ligand to the scaffold were synthesized (**42**) and purified. Of these, the 5mer and 6mer were chosen for binding measurements, this time using 100nM protein. The apparent binding constant of these samples were less than 100nM (Figure 46). As the K_d is defined as the concentration of free ligand where half of the protein is bound, the lowest K_d that could be conceivably measured was 50nM. However, when deriving binding constants at less than 100nM, our fitting software became temperamental and was prone to producing nonsensical answers. To test the effect of the PEG linker on binding, an additional bivalent ligand consisting of only diaminopropionic acid (Dpr) with two PEG tethered ligands (**43**). The binding constant of this molecule was also less than 100nM (Figure 47). If the goal of these bivalent experiments was to make a bivalent molecule that binds with enhanced affinity, this would have been achieved. However, the goal was to observe bivalent selectivity, where one length of a scaffold binds strongly and other lengths, both shorter and longer, bind with less affinity. In order to observe this, shorter linkers were necessary, consisting of too short, too long, and just right (Goldilocks effect).

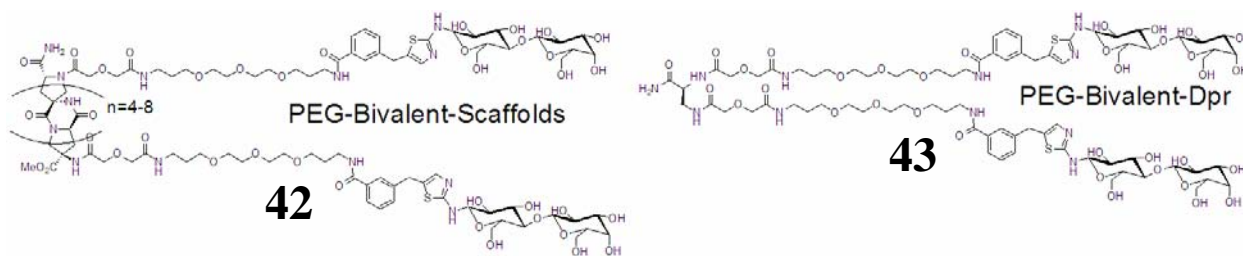


Figure 45 PEG-bivalent ligands and Dpr-PEG bivalent ligand.

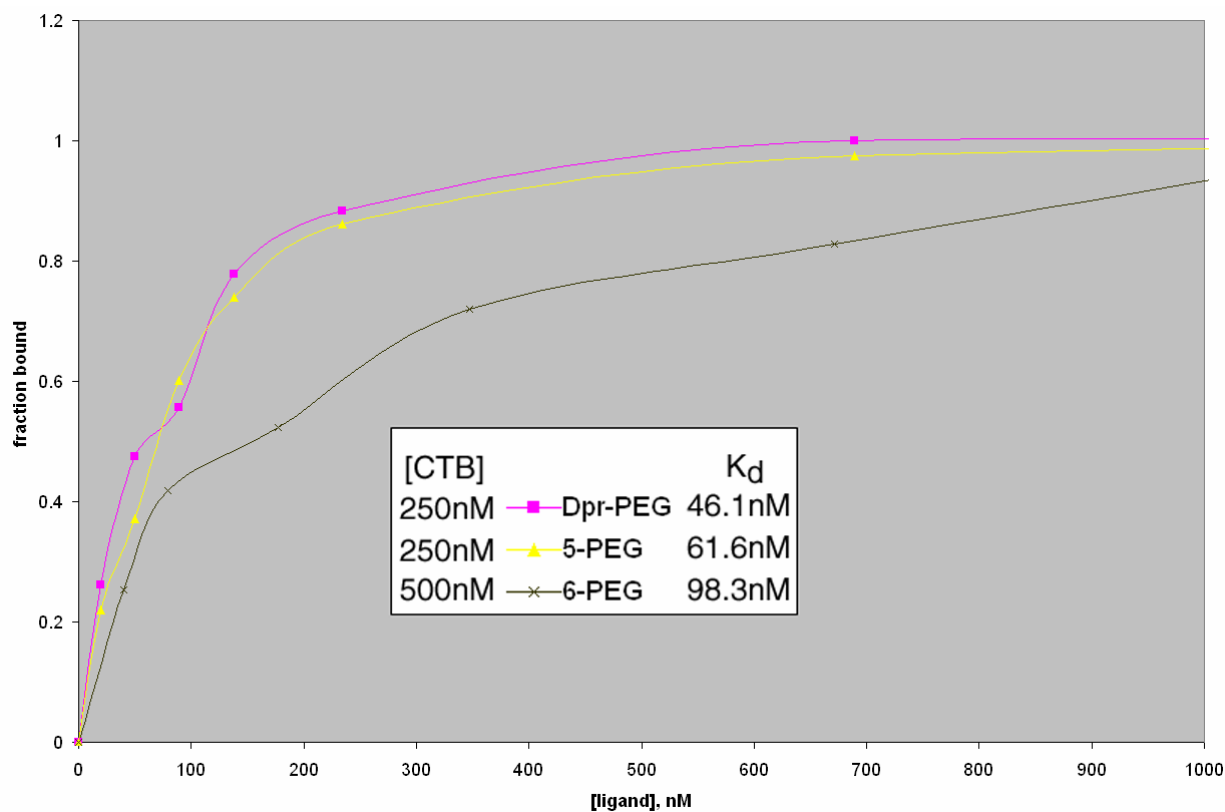


Figure 46 PEG-linker-Bivalent FI-Titration Curves

The next set of molecules varied in length from 5-9 building blocks and had either one or two β Ala between the ligand and the scaffold (Figure 47). All ten molecules were successfully synthesized and purified as described previously. The shortest scaffold was a 5mer (**44**) with a one- β Ala linker. The binding of this to CTB was measured and found to be under 100nM (Figure 48). A 7mer (**45**) with a 2- β Ala linker was also tested and found to have a K_d also less than 100nM. It was at this point that it was realized that the linker would have to be removed in order to have any chance of bivalent selectivity.

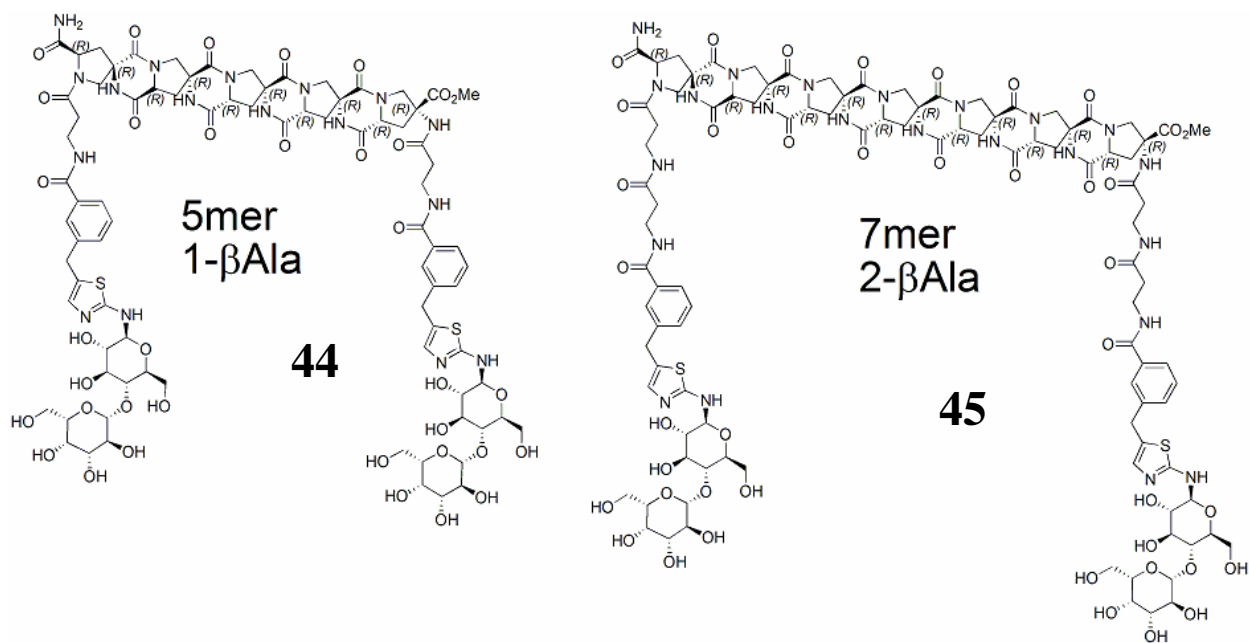


Figure 47 β-Ala derivatives tested for binding by FI.

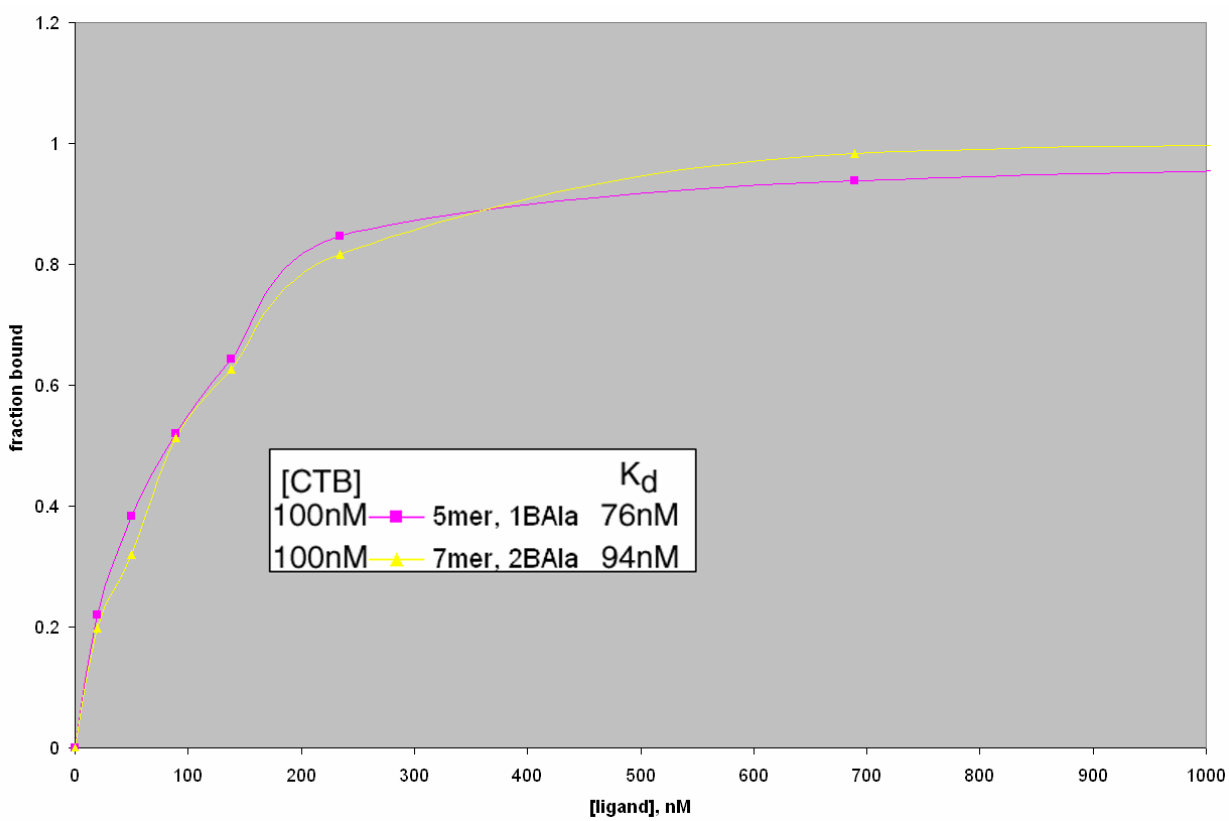


Figure 48 βAla-linker-Bivalent FI-titration curve

Accordingly, two sets of molecules were synthesized (Figure 50) consisting of a series of 2-7 building blocks with a ligand at the trailing end and, either a glycine DKP (**47**) or a second ligand at the leading end (**46**). These bivalent ligands position the two ligands between 10Å and 35Å apart. From this, it was anticipated that the shortest ligands would be unable to span the 35Å between adjacent binding sites and bivalent selectivity would be achieved. In essence, we were looking for a bivalent ligand that bound monovalently, and we believed that the shortest bivalent ligands would achieve this. This series of bivalent ligands, containing scaffold of lengths 2-7 building blocks, were tested using the FI assay (Figure 49). GM1 ganglioside, the natural ligand for CTB was also tested. In this assay, GM1 had a K_d of 63.8 nM, which is similar to what Bernardi measured for the GM1 oligosaccharide (without the hydrophobic tail) employing an identical assay (219nM).[33] When the binding constants were derived for the bivalent ligands and GM1, it was clear that all bound stronger than the GM1. Although differences can be seen, due to the imprecision of this measurement, closer inspection is not warranted. The only statement that can be made with confidence is that the binding constants were all less than 100nM. Of particular note is that the 2mer-2TZ and the 3mer-2TZ were both known to be too short to span the distance required to bind to two binding sites at once. One explanation for this would be that the ligand was binding between two protein pentamers and not within a single pentamer.[34] This would be considered preliminary evidence of an aggregational mechanism as opposed to chelation, but more experiments are necessary and ongoing, especially the development of assays capable of accurately measuring binding constants into the picomolar range.

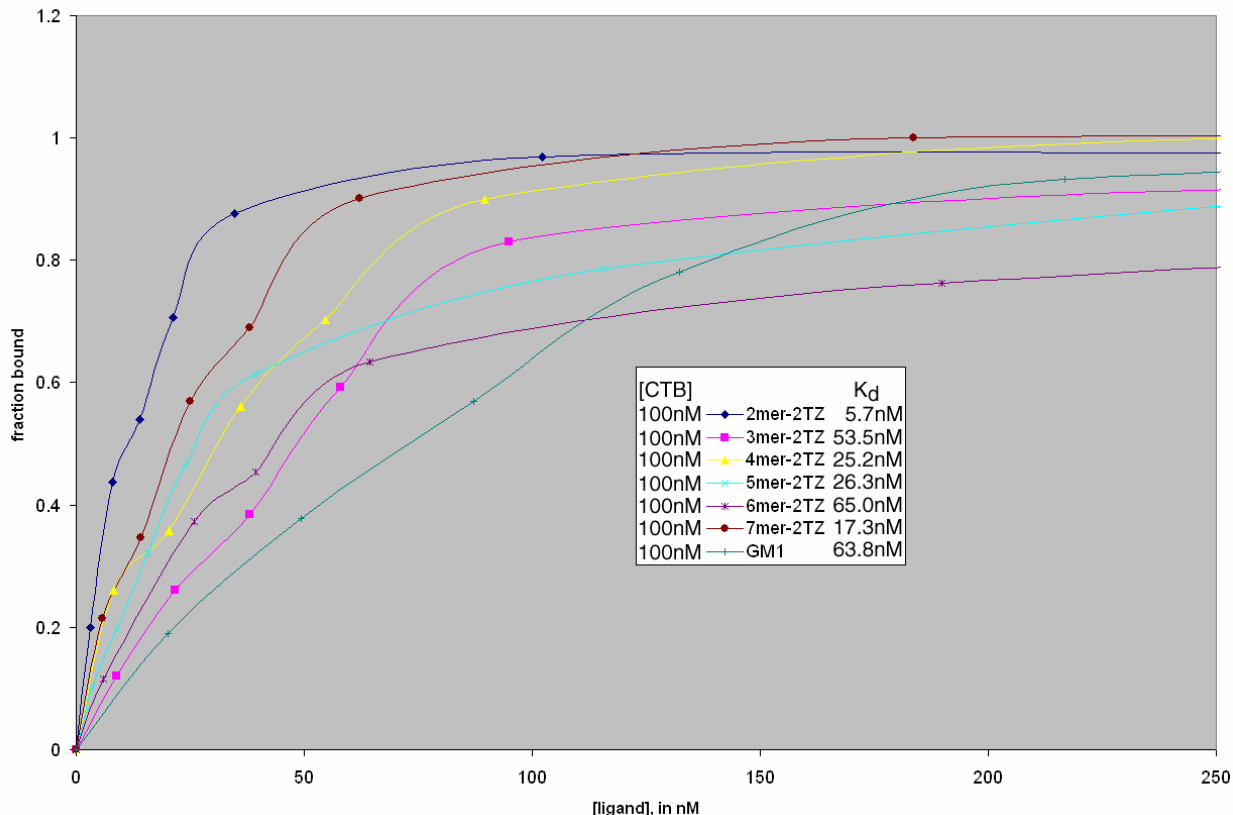


Figure 49 Bivalent scaffold FI-titration curve.

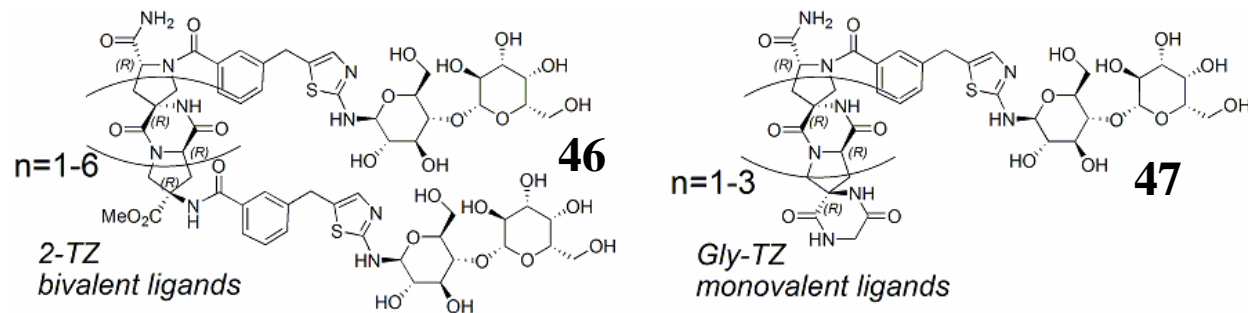


Figure 50 Bivalent ligands directly attached to the scaffold and Glycine-monovalent scaffold ligands.

Additionally, the monovalent scaffold (**47**) series was synthesized in order to evaluate Fan's explanation for an enhancement of a bivalent ligand incapable of spanning the required distance between two binding sites.[31] Fan and coworkers put forth steric blocking as an explanation for the observance of a bivalent enhancement for non-spanning bivalent ligands. In our case, this monovalent series was designed to assess whether the scaffold had any contribution to the binding affinity. Additionally, a ligand-amide monovalent control was synthesized as the basis for comparing the binding affinity of this monovalent-scaffold series.

From the binding constants, two things are apparent (Figure 51). First, all samples have similar binding constants of $\sim 200\text{nM}$. No difference was seen between the TZ-GlyOMe (Figure 52, compound **48**) and the monovalent scaffold series. Therefore, it didn't appear that the scaffold made any contribution to the binding affinity. The second thing that was apparent was that the TZ-GlyOMe (**48**) bound about 10 times more tightly than the free acid, but more investigation of the observation is needed. If this stands, though, it would definitely be the highest affinity monovalent ligand for CTB ever made.

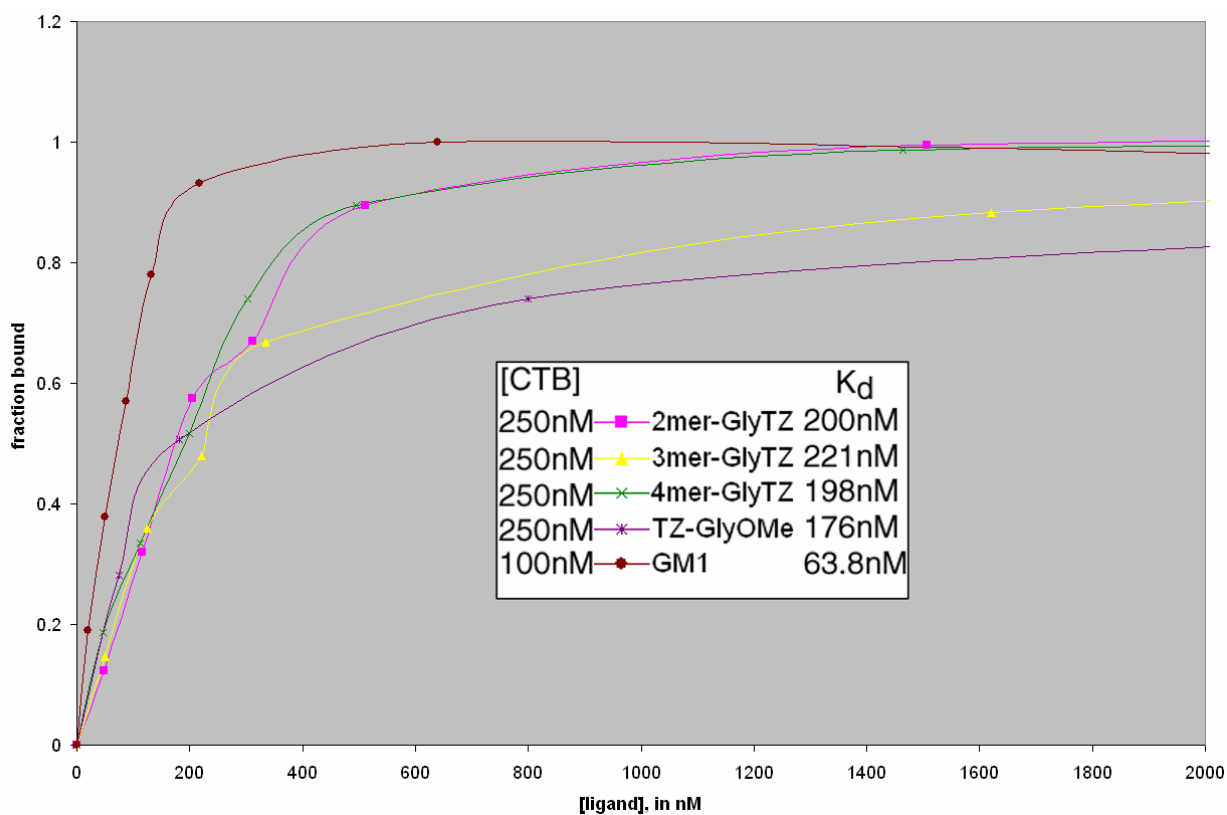


Figure 51 Monovalent Glycine/Ligand FI-titration curve

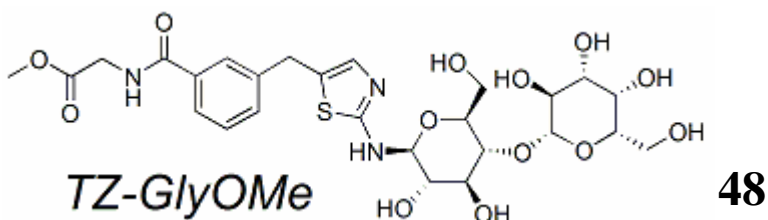


Figure 52 Ligand-glycine methyl ester control used for the monovalent-amide binding control, as well as for the Beer's Law plot.

Fluorescein labeled bivalent ligands were also synthesized (Figure 53) with the intention that FP would be used to accurately measure higher affinity binding. Incorporation of an Fmoc-Dab(ivDde)-OH as the first residue, followed by n pro4(2R4R) building blocks ($n=2-7$) yielded the series of scaffold lengths. Removal of the Boc groups on-resin, followed by on-resin DKP closure gave a resin bound product with two free amines (**49**). Acylation with the free ligand (**50**), followed by removal of the ivDde proved to be compatible. Reaction between the Dab amine and fluorescein was slightly challenging. Using methods developed for the galactose-based bivalent ligands failed to selectively form the desired product. Using fluorescein-N-hydroxy succinyl ester was promising. Desired product was seen when using sub-stoichiometric amounts of DIPEA, but numerous impurities were also present as well. Base-free conditions, over the period of 2-4 days yielded desired product (**51**) cleanly and efficiently.

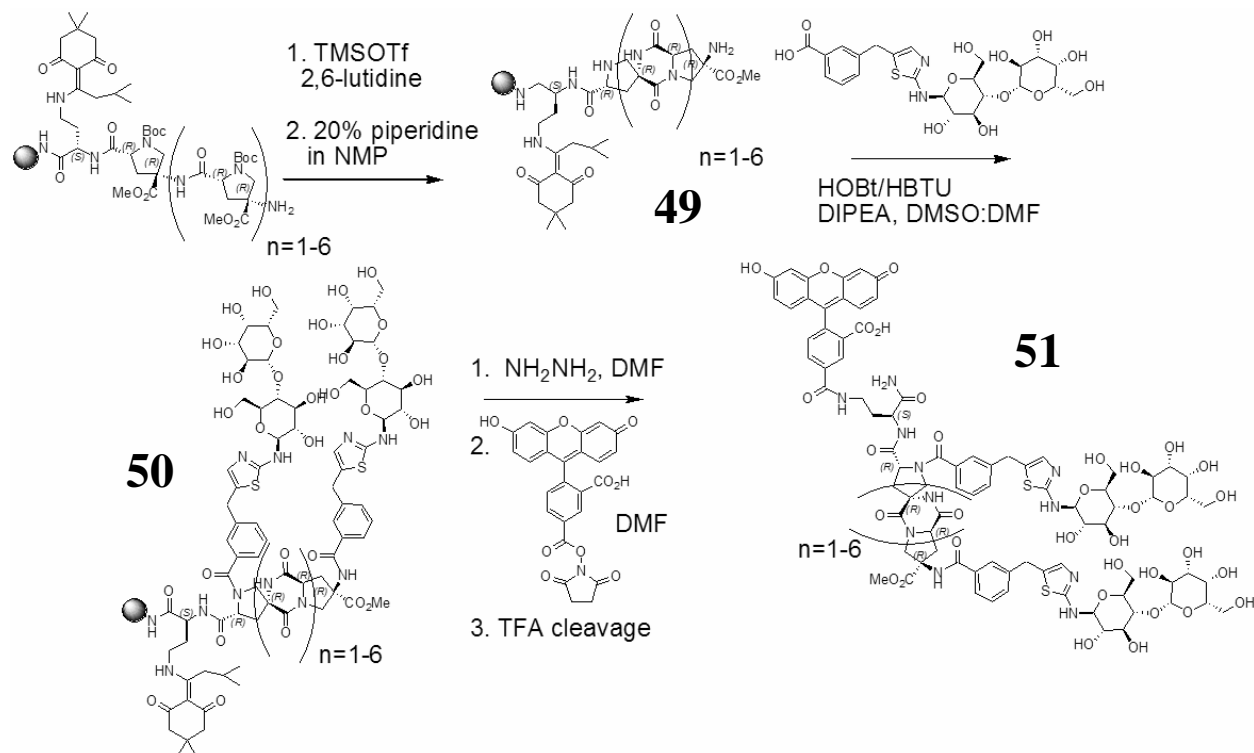


Figure 53 Synthesis of FAM-labeled bivalent ligands.

The FAM-labeled bivalent 6mer was tested for bivalent binding using FP. Concentrations of CTB (Sigma) ranged from 10nM to 3 μ M were used, however no change in polarization was seen until the last data point. Therefore FP may prove to be useful for

measuring bivalent enhancements into the low nM-pM range, but further study and optimization are needed.

2.3 CONCLUSIONS

Two different types of ligands were successfully synthesized and incorporated into scaffolds. It was found that conditions leading to the carboxylic acid-thiazoline based ligand also produced a tautomer which is one of the tightest binding ligands known for CTB. Bivalent ligands, varying in number of building blocks, linker type, and linker length were synthesized. Using fluorescence polarization to measure binding constants requires further optimization. Tryptophan fluorescence intensity shifts were employed to measure equilibrium binding constants of a number of series of molecules, including PEG-linked bivalent scaffolds, 1 or 2 β Ala-linked bivalent scaffolds, bivalent scaffolds directly attached to the ligands, and monovalent controls, consisting of the full scaffold with only 1 ligand attached. A bivalent enhancement was seen for all bivalent ligands, although the lower limit of the dynamic range of this assay was quickly surpassed. Further work is ongoing to implement assays having the ability to measure low nanomolar-to-picomolar binders, capable of yielding impressive bivalent enhancements, and allowing the further study of factors affecting the strength and specificity of bivalent interactions, as well as the mechanism. In summary, bivalent display of two ligands using an oligomeric *bis*-amino acid scaffold has been validated as a potential “killer app” for the *bis*-amino acid technology being developed in this lab and should be pursued with vigor on multiple fronts.

2.4 EXPERIMENTAL

General: Dichloromethane was distilled from CaH_2 . All other reagents were used as received, unless stated otherwise. Column chromatography was performed using ICN Silitech 32-63 D (60 Å) grade silica gel and TLC analysis was performed on EM Science Silica Gel 60 F₂₅₄ plates

(250 μ m thickness). NMR spectroscopy was performed using a Bruker 300 MHz instrument. Chemical shifts were reported in parts per million downfield relative to trimethylsilane and categorized as br = broad, s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, q = quartet, and m = multiplet. Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. N,N-diisopropylethylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and N-Hydroxybenzotriazole hydrate (HOBt) were obtained from Novabiochem. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and an inert atmosphere over the reaction. HPLC-MS analysis was performed on a Hewlett-Packard Series 1050 instrument equipped with a Waters Xterra MS C₁₈ column (3.5 μ m packing, 4.6 mm x 150 mm) and a diode-array detector, while the MSD-ESI was Series 1100. Preparative purification was done using a Varian Prostar 500 equipped with a Waters Xterra Prep MS-C₁₈ column (5 μ m packing, 10 mm x 100 mm), equipped with a dual wavelength detector.

3-methoxy-5-nitrobenzoic acid (7).

To a flame dried 1L, 3-neck round bottom flask equipped with a water-cooled condenser, N₂ line, and rubber septa, 65mL (1.5mol) of ultra-dry methanol was added through the rubber septum. To this, ~1.4g (189mmol) of lithium ribbon was added with vigorous stirring, which was prepared by washing away the preservative mineral oil in a 100mL beaker of hexanes. Care was taken to minimize air exposure of the washed lithium, which needed to quickly be weighed and returned to the hexanes until added to the reaction in a series of about 5 additions. Vigorous reaction occurred between the methanol and lithium, producing heat and hydrogen gas (**CAUTION**). A rubber septum was removed from the flask to minimize the dangerous pressure. Once the reaction subsided, the septum was replaced and the reaction allowed to stir for 30min. The solvent was then removed *in vacuo* and allowed to dry *in vacuo* for 30min. The white solid was dissolved in 250mL of freshly distilled dimethylsulfoxide. To this purple solution, 10.0g

(47.1mmol) of 3,5-dinitrobenzoic acid was added, and the reaction was stirred at $\sim 50^{\circ}\text{C}$ under nitrogen for 18hrs. The entire reaction contents were slowly poured into 750g ice and 75mL of concentrated H_2SO_4 (**CAUTION**), with care to avoid splattering. An orange precipitate which formed, which was mainly product, was filtered and put aside. The remaining liquid was extracted 3 x 250mL EtOAc. The organic layers were pooled to redissolve the orange precipitate. The organic layers were washed 3 x 100mL water and 2 x 100mL brine, dried over sodium sulfate, and filtered. The solvent was removed *in vacuo* to yield 8.5g (91%) product which was used without further purification. ^1H NMR (300MHz, 25°C , d6-acetone) δ 8.36 (m, 1H), 7.96 (t, 1H), 7.90 (m, 1H), 4.02 (s, 3H).

3-hydroxy-5-nitrobenzoic acid (8).

To a 500mL round bottom flask equipped with a water cooled condenser, 150mL of aqueous hydrobromic acid (**CAUTION**) was added. In a 250mL Erlenmeyer flask, 100mL of acetic acid was heated on a hot plate to $\sim 50^{\circ}\text{C}$ and 6.8g (34.5mmol) of 3-methoxy-5-nitrobenzoic acid was added, and the mixture was allowed to stir until all product dissolved, with additional heating if necessary. This solution was added to the HBr in the round bottom and the mixture was heated to 145°C under nitrogen for 12hrs. The solvent was removed with heating *in vacuo*, until a brown oil resulted. After cooling, 250mL of EtOAc:hexanes (1:3) was added and stirred for 5min. The yellow top layer was carefully decanted away from the brown bottom layer. This process was repeated at least three more times. In most cases, additional brown oil would settle to the bottom. Again, the yellow solution was carefully decanted away, and the solvent was removed *in vacuo* to yield 6.3g of product (86% yield). The product could be recrystallized from 25% aqueous HCl if needed, but in most cases the product was sufficiently pure by NMR and LCMS. ^1H NMR (300MHz, 25°C , d6-acetone) δ 8.27 (m, 1H), 7.90 (m, 2H).

3-O-(2,3,4,6-tetraacetyl-D-galactopyranose)-5-nitrobenzoic acid (9).

To a 250ml flame dried round bottom flask, 1.6g (8.7mmol) of 3-hydroxy-5-nitrobenzoic acid was added along with 2.9g (7.4mmol) peracetyl galactose, which was dried at 50°C *in vacuo* for 2days. Then 90mL of extra-dry acetonitrile was added, along with a septum and nitrogen needle. The mixture was stirred under nitrogen for 10min, then 11.0mL (87.4mmol) boron trifluoride-diethyletherate was added, and the reaction was stirred for 12hrs. The solvent was removed *in*

vacuo. The crude product reaction was dissolved in 15mL of methanol and loaded as a single injection onto a 120g-RP-C18-Isco column, equilibrated in 5% ACN (0.05% formic acid) / 95% water (0.1% formic acid). A gradient from 5-95% ACN separated the mixture. Product fractions were identified by reinjection onto an HPLC or LCMS and pooled, and the solvent was removed by lyophilization to yield about 600mg of product, usually in ~15% yield. ¹H NMR (300MHz, 25°C, d₆-acetone) δ 8.68 (m, 1H), 8.46 (t, 1H), 8.37 (m, 1H), 6.32 (d, 1H, J = 3.6Hz), 5.94 (d, 0.2H, J = 7.8), 5.76 (m, 2H), 5.59 (dd, 1H, J = 3.6Hz, 10.7Hz), 4.75 (t, 1H, J = 6.3Hz), 4.39 (d, 2H, J = 6.4Hz), 2.38 (s, 3H), 2.27 (s, 3H), 2.18 (s, 3H), 2.04 (s, 3H).

3-O-(D-galactopyranose)-5-nitrobenzoic acid (10).

To a 250mL round bottom flask, 780mg (1.52mmol) of 3-O-(2,3,4,6-tetraacetyl-D-galactopyranose)-5-nitrobenzoic acid was added into 50mL of methanol. To this, 1.1mL of 25%NaOMe/MeOH was added and allowed to stir for 15min (color change to yellow when methoxide added). The reaction was quenched by addition of 1mL TFA in 20mL of methanol, which changed the color back to clear. The solvent was removed *in vacuo* to yield a crude product that was used without further purification or characterization besides LCMS.

3-O-(α-D-galactopyranose)-5-nitrobenzoic acid (11).

The crude product from the previous step was dissolved in autoclaved buffer consisting of 50mM Tris-HCl (pH 7.4), 250mM NaCl, and 1mM MgCl₂, at an estimated concentration of 10mM. To this, a few flakes (~10-100μg) of β-galactosidase were added, and the enzyme was allowed to react in a 50mL conical for 12hrs. The reaction was deemed to be complete (no more beta anomer) when the starting material peak and the hydrolyzed phenol peak ceased to change, as seen by HPLC. The reaction was lyophilized, redissolved an 5mL of 10% ACN/water, and injected onto the 40g-RP-C18-Isco and separated using a 5-45% ACN gradient using ACN (0.05% formic acid) and 95% water (0.1% formic acid). Fractions containing pure product were identified by LCMS or HPLC and lyophilized to give desired product, typically 100-300mg depending on initial scale and anomeric purity. ¹H NMR (300MHz, 25°C, d₄-methanol) δ 8.37 (m, 1H), 8.20 (m, 1H), 8.10 (m, 1H), 5.71 (d, 1H, J = 3.0Hz), 4.02 (m, 3H), 3.93 (t, 1H, J = 6.0Hz), 3.73 (d, 2H, J = 6.0Hz).

3-O-(2,3,4,6-tetraacetyl- α -D-galactopyranose)-5-nitrobenzoic acid (12).

The purified alpha product (200mg, 0.58mmol) was transferred to a 25mL round bottom flask and 3mL of acetic anhydride was added along with 10mg dimethylaminopyridine. The reaction proceeded as a suspension initially, but after ~30min all product was in solution. At this point, the unsymmetrical carboxy-anhydride as well as the excess acetic anhydride were both hydrolyzed by the addition of 10mL ACN:water (1:1). Two layers initially formed, but after 10min a single layer remained. The reaction could be accelerated by addition of ~0.1mL TFA, in which case, a single layer formed immediately. The reaction was directly injected onto the 40g-RP-C18-Isco column, and purified using a 5-95% ACN gradient using ACN (0.05% formic acid) and 95% water (0.1% formic acid). Fractions containing pure product were identified by LCMS or HPLC and lyophilized to give desired product (280mg) in a ~90% yield. ¹H NMR (300MHz, 25°C, d₆-acetone) δ 8.68 (m, 1H), 8.46 (t, 1H), 8.37 (m, 1H), 6.32 (d, 1H, J = 3.6Hz), 5.76 (m, 2H), 5.59 (dd, 1H, J = 3.6Hz, 10.7Hz), 4.75 (t, 1H, J = 6.3Hz), 4.39 (d, 2H, J = 6.4Hz), 2.38 (s, 3H), 2.27 (s, 3H), 2.18 (s, 3H), 2.04 (s, 3H).

4-Carbamoyl-4-Fmoc-1-Cbz-pyrrolidine-2-carboxylic acid-*tert*-butyl ester (2S4S in this case).

To a 25mL round bottom flask, 917mg of 4-Carboxy-4-Fmoc-1-Cbz-pyrrolidine-2-carboxylic acid-*tert*-butyl ester[17] (1.56mmol) was added along with 8.0mL of DMF. To this, 715mg of HATU (1.88mmol) was added, followed by 0.8mL of DIPEA (4.0mmol). After stirring for 5min, a yellow color was observed, to which 3.6mL of 0.5M solution of ammonia in dioxane (1.8mmol) was added, and allowed to stir under nitrogen for 3hr. The crude reaction was injected onto a 40g Isco column, equilibrated in chloroform. A gradient of chloroform to 10% methanol/chloroform served to purify the product. The product fractions were pooled and the solvent removed *in vacuo* to yield 760mg of product (83%).

4-Carbamoyl-4-Fmoc-1-Cbz-pyrrolidine-2-carboxylic acid (2S4S in this case).

To a 50mL round bottom flask, 500mg of 4-Carbamoyl-4-Fmoc-1-Cbz-pyrrolidine-2-carboxylic acid-*tert*-butyl ester (0.85mmol) was added, followed by 0.5mL of DCM, then 17mL of TFA. The reaction was stirred for 2hrs. The solvent was removed *in vacuo*, and methanol or DCM

were alternatively added and then removed *in vacuo* to azeotrope the TFA. The material was used for the next step without further purification.

4-Carbamoyl-4-Fmoc-1-Boc-pyrrolidine-2-carboxylic acid (2S4S in this case) (13).

To a 100mL round bottom flask, ~450mg of crude 4-Carbamoyl-4-Fmoc-1-Cbz-pyrrolidine-2-carboxylic acid (0.85mmol) was added, followed by 25mL of tetrahydrofuran. To this stirred solution, 0.78mL of Boc-anhydride (3.4mmol) and 100mg of Pd/C were added. A double-thick balloon filled with hydrogen was added to a three-way valve attached to the greased round bottom. An aspirator was added and at least 5 cycles of aspiration followed by H₂ backfill served to degas the solvent and charge the palladium with hydrogen. The reaction was stirred for between 24-96hr, until the Cbz had entirely been exchanged for a Boc, as judged by LCMS. The reaction was filtered, and the Pd/C was washed several times with DCM. The solvent was removed *in vacuo*. The crude reaction was dissolved in 5mL of chloroform and injected onto a 40g Isco column, equilibrated in chloroform. A gradient of chloroform to 10% methanol/chloroform served to purify the product. The product fractions were pooled and the solvent removed *in vacuo* to yield 370mg of product (87% over two steps). ¹H NMR (300MHz, 25°C, CDCl₃) δ 7.75 (d, 2H, J=7.4Hz), δ 7.54 (d, 2H, J=7.2Hz), δ 7.39 (m, 4H), δ 6.32 (br 1H), δ 6.13 (br, 1H), δ 5.85 (br, 1H), δ 4.52 (m, 2H), δ 4.19 (m, 1H), δ 4.01 (m, 1H), δ 3.85 (m, 1H), δ 2.56 (m, 2H), δ 1.45 (s, 9H),

Synthesis of the fluorescein labeled Orn (+) control (21).

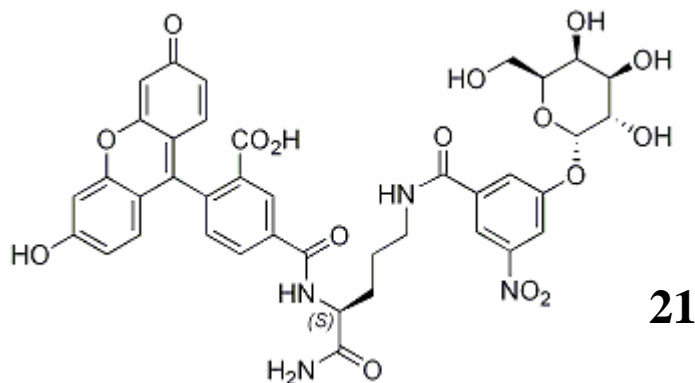


Figure 54 Fluorescein labeled Orn-(+) control

To a 1.25mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide Resin (Novabiochem) (5mg, 3.2 μ mol loading). The resin was swelled for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 9.6mg (15.8 μ mol) of Fmoc-L-Orn(Mtt)-OH (Novabiochem) and 6.0mg of HATU (15.8 μ mol) in 80 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 5.5 μ L (31.5 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

(ALL steps forward done in the dark) In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 11.8mg (31.5 μ mol) of 5-carboxyfluorescein (Probes) and 4.8mg of HOBt (31.5 μ mol) in 165 μ L of DMF. This solution was mixed using a micropipettor, after which 4.9 μ L (31.5 μ mol) of diisopropylcarbodiimide was added to make the active ester. After 10min activation time, the solution was aliquoted to the deprotected resin, and allowed to react by argon mixing for 12hr. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, and DMF for 2min each.

The resin was swelled in DCM with slight bubbling for 30min. The Orn side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 8.1mg (15.8 μ mol) of the acetyl-protected ligand (**12**) and 6.0mg of HATU (15.8 μ mol) in 80 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 5.5 μ L (31.5 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF,

the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The product was cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solution was drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The acetylated ligand was dissolved in 200 μ L of methanol and mixed with a pipettor. Then, 2 μ L of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400 μ L of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75 μ L of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The sample was stored in the dark at 4°C until needed.

Synthesis of the fluorescein labeled resin-bound intermediate (14).

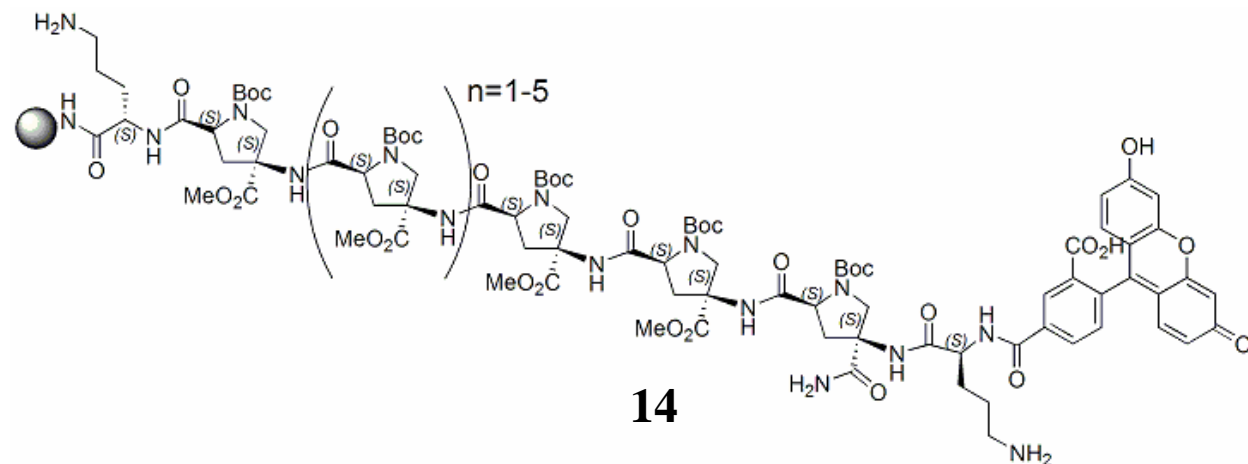


Figure 55 Resin-bound scaffold used in the synthesis of fluorescein labeled bivalent ligands, varying in β Ala.

To a 15mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide Resin (Novabiochem) (25mg, 16 μ mol loading). The resin was swelled for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the

number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 45.8mg (75 μmol) of Fmoc-L-Orn(Mtt)-OH (Novabiochem) and 28.5mg of HATU (75 μmol) in 375 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 26.1 μL (150 μmol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 15.3mg (30 μmol) of Boc-protected pro4(2S4S)[17] building block and 11.4mg of HATU (30 μmol) in 150 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 10.5 μL (60 μmol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

This process of coupling/deprotection was repeated three additional times. After the fourth Fmoc deprotection, the resin was washed with DCM several times, and allowed to dry for five minutes via aspiration. The beads were now easily removed from the reactor and then weighed. An appropriate weight fraction (i.e. 1/5th removed after 4th building block was coupled, then 1/4th after 5th building block, etc.) was then removed, placed in a fresh 1.0 mL reactor and stored in a desiccator. The remaining resin was then swelled in DMF for 10 min. The process of coupling/deprotection/aliquot removed was repeated four additional times, making 5 portions of equimolar amounts of 5 different lengths, 4-8 building blocks, with the final amine deblocked, yielding the sequence, resin-Orn(Mtt)-SS₃-(SS)_n-free amine ($n=1-5$).

The final building block coupled to all of the sequences was the SS-Amide (**13**). In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 14.9mg (30 μmol) of Boc-protected pro4(2S4S)-amide building block and 11.4mg of HATU (30 μmol) in 150 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 10.5 μL (60 μmol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 45.8mg (75 μmol) of Fmoc-L-Orn(Mtt)-OH (Novabiochem) and 28.5mg of HATU (75 μmol) in 375 μL of

20% DCM/DMF. This solution was mixed using a micropipettor, after which 26.1 μ L (150 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

(ALL steps forward done in the dark) In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 56.4mg (150 μ mol) of 5-carboxyfluorescein (Probes) and 23.0 of HOBt (150 μ mol) in 750 μ L of DMF. This solution was mixed using a micropipettor, after which 23.2 μ L (150 μ mol) of diisopropylcarbodiimide was added to make the active ester. After 10min activation time, the solution was aliquoted to the deprotected resin, and allowed to react by argon mixing for 12hr. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, and DMF for 2min each (**14**)

The resin was swelled in DCM with slight bubbling for 30min. The Orn side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% trisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*. Each length sample were aliquoted into 3 equal portions for incorporation of n β Ala ($n = 0, 1, \text{ or } 2$).

Synthesis of fluorescein labeled bivalent ligands (**18**).

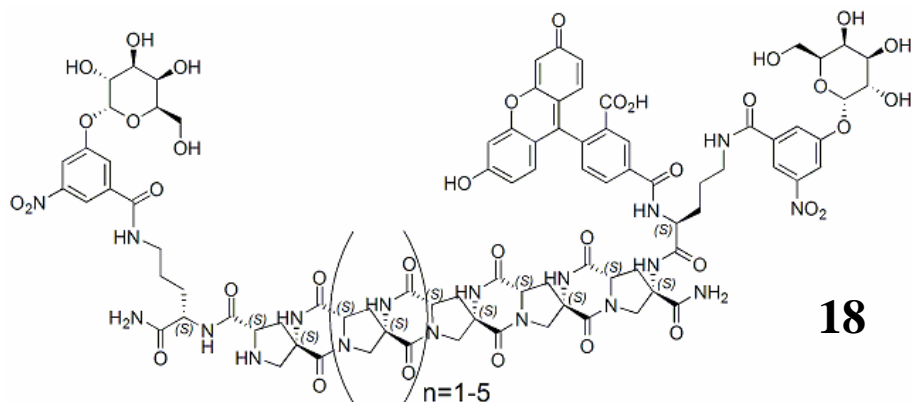


Figure 56 Fluorescein labeled bivalent ligands, with lengths of 5-9 building blocks.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 33.6mg (60 μ mol) of the acetyl-protected ligand (**12**) and 22.8mg of HATU (60 μ mol) in 600 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20.9 μ L (120 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The bivalent-acetylated ligands were dissolved in 200 μ L of methanol and mixed with a pipettor. Then, 2 μ L of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400 μ L of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75 μ L of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4°C until needed.

Synthesis of fluorescein labeled bivalent ligands containing 1 β Ala (19**).**

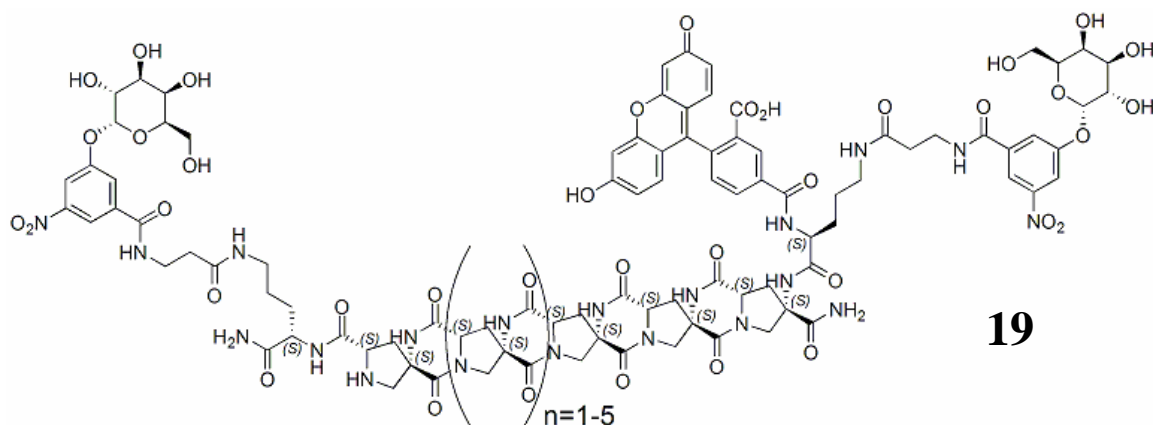


Figure 57 Fluorescein labeled bivalent ligands containing 1 β Ala, with lengths of 5-9 building blocks.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 22.3mg (75 μ mol) of Fmoc- β Ala-OH (Novabiochem) and 28.5mg of HATU (75 μ mol) in 375 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 26.1 μ L (150 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 33.6mg (60 μ mol) of the acetyl-protected ligand (**12**) and 22.8mg of HATU (60 μ mol) in 600 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20.9 μ L (120 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The bivalent-acetylated ligands were dissolved in 200 μ L of methanol and mixed with a pipettor. Then, 2 μ L of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400 μ L of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75 μ L of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4 $^{\circ}$ C until needed.

Synthesis of fluorescein labeled bivalent ligands containing 2 β Ala (20).

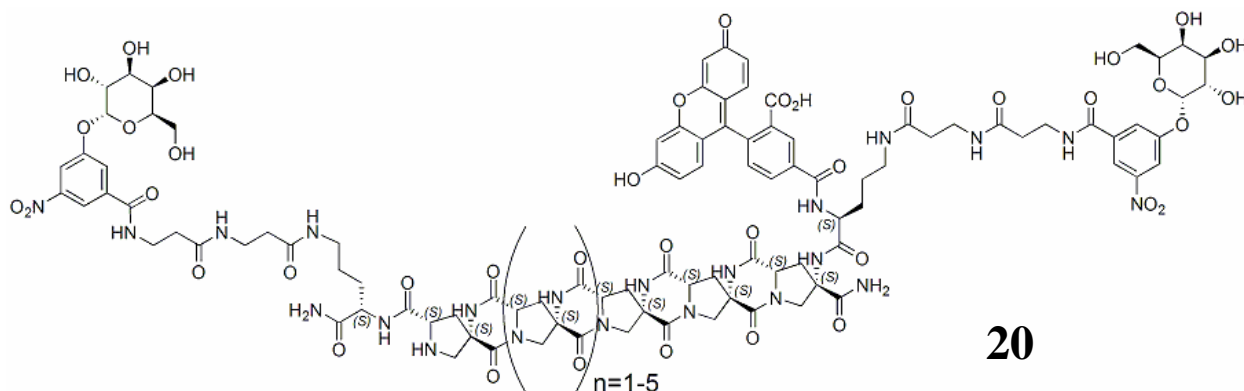


Figure 58 Fluorescein labeled bivalent ligands containing 2 β Ala, with lengths of 5-9 building blocks.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 22.3mg (75 μ mol) of Fmoc- β Ala-OH (Novabiochem) and 28.5mg of HATU (75 μ mol) in 375 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 26.1 μ L (150 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 22.3mg (75 μ mol) of Fmoc- β Ala-OH (Novabiochem) and 28.5mg of HATU (75 μ mol) in 375 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 26.1 μ L (150 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 33.6mg (60 μ mol) of the acetyl-protected ligand (**12**) and 22.8mg of HATU (60 μ mol) in 600 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20.9 μ L (120 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF,

the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The bivalent-acetylated ligands were dissolved in 200 μ L of methanol and mixed with a pipettor. Then, 2 μ L of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400 μ L of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75 μ L of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4°C until needed.

Synthesis of the fluorescein labeled Dpr (+) controls (23, 24).

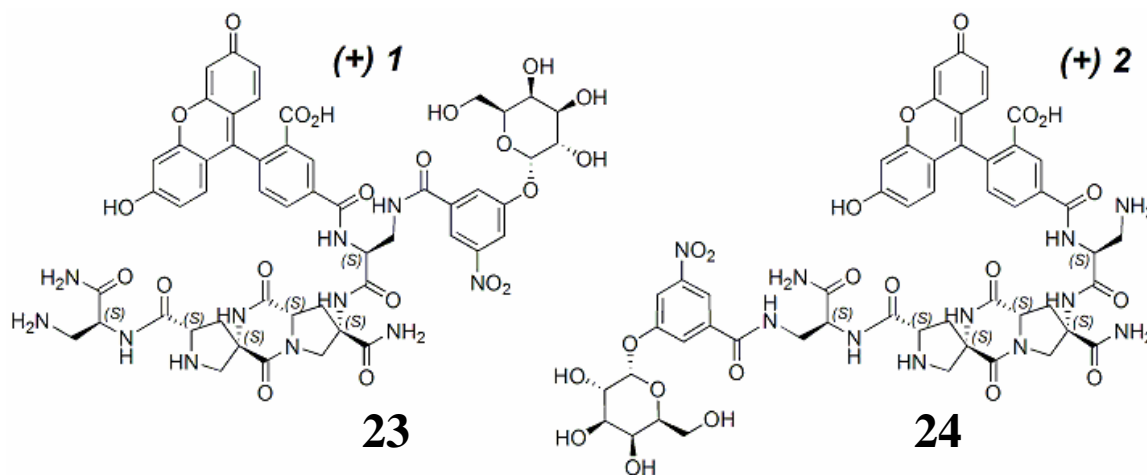


Figure 59 Fluorescein labeled Dpr-(+) controls

To 2 x 1.25mL polypropylene solid phase peptide synthesis (SPPS) reaction vessels were added Rink Amide Resin (Novabiochem) (5mg, 3.2 μ mol loading). The resins were swelled for

1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In 2 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 9.2mg (15.8 μmol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) *OR* 6.7mg (15.8 μmol) of Fmoc-L-Dpr(Boc)-OH (Novabiochem) and 6.0mg of HATU (15.8 μmol) in 80 μL of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 5.5 μL (31.5 μmol) of DIPEA was added to make the active ester. After 10min activation time, the solutions were added to the deprotected resin, and allowed to react by argon mixing for 30min. The resins were then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In 2 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 3.2mg (6.3 μmol) of Boc-protected pro4(2S4S) building block [17] and 2.4mg of HATU (6.3 μmol) in 40 μL of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 2.2 μL (12.6 μmol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

The final building block coupled to all of the sequences was the Boc-protected Amide-pro4(2S4S) (**13**). In 7 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 3.2mg (6.3 μmol) of Boc-protected pro4-amide building block and 2.4mg of HATU (6.3 μmol) in 40 μL of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 2.2 μL (12.6 μmol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

In 2 x 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 6.7mg (15.8 μmol) of Fmoc-L-Dpr(Boc)-OH (Novabiochem) *OR* 9.2mg (15.8 μmol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 6.0mg of HATU (15.8 μmol) in 80 μL of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 5.5 μL (31.5 μmol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

(ALL steps forward done in the dark) In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 11.8mg (31.5 μ mol) of 5-carboxyfluorescein (Probes) and 4.8mg of HOBt (31.5 μ mol) in 165 μ L of DMF. This solution was mixed using a micropipettor, after which 4.9 μ L (31.5 μ mol) of diisopropylcarbodiimide was added to make the active ester. After 10min activation time, the solution was aliquoted to the deprotected resin, and allowed to react by argon mixing for 12hr. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, and DMF for 2min each.

The resin was swelled in DCM with slight bubbling for 30min. The Orn side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 8.1mg (15.8 μ mol) of the acetyl-protected ligand (**12**) and 6.0mg of HATU (15.8 μ mol) in 80 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 5.5 μ L (31.5 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The product was cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solution was drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The acetylated ligand was dissolved in 200 μ L of methanol and mixed with a pipettor. Then, 2 μ L of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400 μ L of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75 μ L of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the

desired product were identified by reinjection on the LCMS and lyophilized. The sample was stored in the dark at 4°C until needed.

Synthesis of resin bound predicted fluorescein labeled scaffolds (22).

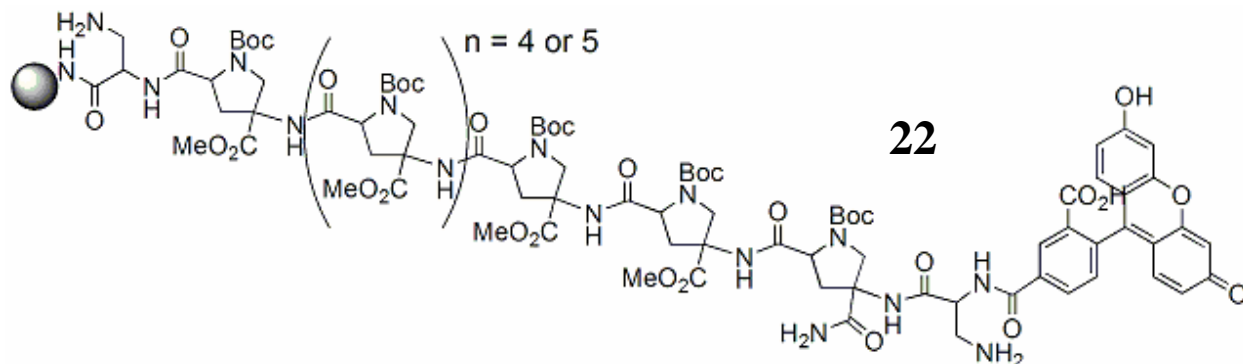


Figure 60 Generic resin bound scaffold structure used in the synthesis of predicted fluorescein labeled bivalent ligands.

To 7 x 1.25mL polypropylene solid phase peptide synthesis (SPPS) reaction vessels were added Rink Amide Resin (Novabiochem) (5mg, 3.2 μ mol loading). The resins were swelled for 1hr in DMF. The terminal Fmoc-protected amines were deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resins were washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

	seq1	seq2	seq3	seq4	seq5	seq6	seq7
1st Dpr	D-Mtt	D-Mtt	D-Mtt	D-Mtt	D-Mtt	L-Mtt	L-Mtt
Building block 1	SS	SS	RR	RS	RS	SR	RS
Building block 2	SS	SR	SR	RS	RR	RR	SR
Building block 3	SR	RS	RS	SR	RR	RS	SR
Building block 4	RR	SR	RS	RS	RR	RS	RR
Building block 5	RS	SR	SR	RS	RS	SS	SS
Building block 6	SR	RS	RS	SR	SS	RS	RS
Building block 7	SR	RS	SR	SS	SR	RS	SR
Building block 8	RR	SS	RR	SR	SS	SR	SS
Building block 9	RS	RS	RS		RS	RR	RR
2nd Dpr	D-Mtt	D-Mtt	L-Mtt	L-Mtt	L-Mtt	D-Mtt	L-Mtt
FAM	10eq	10eq	10eq	10eq	10eq	10eq	10eq
4AcGP	10eq	10eq	10eq	10eq	10eq	10eq	10eq

Figure 61 Predicted sequences 1-7, detailing the building block and Dpr isomers used in each.

In 7 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 9.2mg (15.8 μ mol) of Fmoc-Dpr(Mtt)-OH (Novabiochem) and 6.0mg of HATU (15.8 μ mol) in 80 μ L of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 5.5 μ L (31.5 μ mol) of DIPEA were added to each to make the active ester. After 10min activation time, the solutions were added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin were then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In 7 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 3.2mg (6.3 μ mol) of Boc-protected pro4 building block [17] and 2.4mg of HATU (6.3 μ mol) in 40 μ L of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 2.2 μ L (12.6 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

This process of coupling/deprotection was repeated 6 or 7 additional times. The final building block coupled to all of the sequences was the SS-Amide (**13**). In 7 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 3.2mg (6.3 μ mol) of Boc-protected pro4-amide building block and 2.4mg of HATU (6.3 μ mol) in 40 μ L of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 2.2 μ L (12.6 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling, washing, and Fmoc release.

In 7 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 9.2mg (15.8 μ mol) of Fmoc-Dpr(Mtt)-OH (Novabiochem) and 6.0mg of HATU (15.8 μ mol) in 80 μ L of 20% DCM/DMF. This solutions were mixed using a micropipettor, after which 5.5 μ L (31.5 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

(ALL steps forward done in the dark) In 7 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 11.8mg (31.5 μ mol) of 5-carboxyfluorescein (Probes) and 4.8 of HOBt (31.5 μ mol) in 160 μ L of DMF. These solutions were mixed using a micropipettor, after which 4.9 μ L (31.5 μ mol) of diisopropylcarbodiimide was added to make the active ester. After 10min activation time, the solutions were aliquoted to the deprotected resin, and allowed to react by argon mixing for 12hr The resins were then washed 3 x 2min with DMF,

the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, and DMF for 2min each.

The resins were swelled in DCM with slight bubbling for 30min. The Orn side chain protecting groups, methytrityl, were removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*. Each of the 7 sequences were split into ~4 portions, to be used for bivalent ligands, bivalent PEG ligands, bivalent spin labels or bivalent (-) controls.

Synthesis of predicted fluorescein labeled bivalent scaffolds (25).

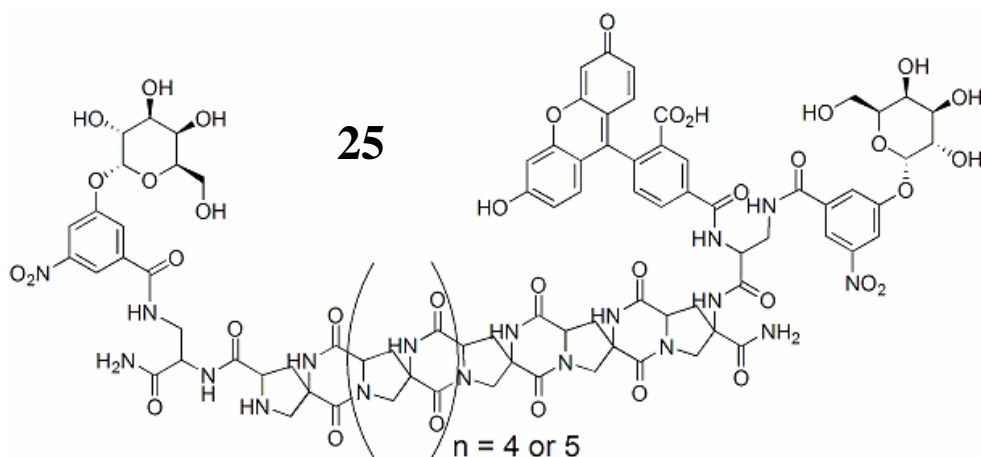


Figure 62 Fluorescein labeled predicted bivalent ligands (seq1-7).

In 7 x 1.5mL microcentrifuge tubes, the coupling solutions were made by dissolving 7.1mg (12.6 μ mol) of the acetyl-protected ligand (**12**) and 4.8mg of HATU (12.6 μ mol) in 65 μ L of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 4.4 μ L (25.2 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resins were then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The bivalent-acetylated ligands were dissolved in 200 μ L of methanol and mixed with a pipettor. Then, 2 μ L of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400 μ L of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75 μ L of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4 $^{\circ}$ C until needed.

Synthesis of fluorescein labeled bivalent spin probes (26).

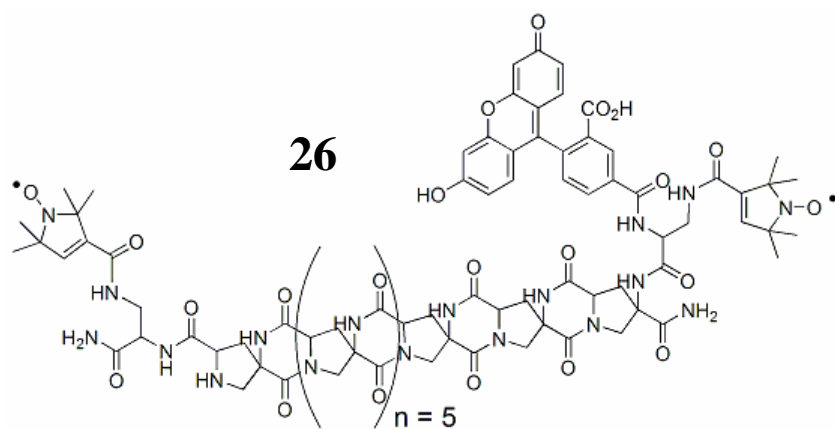


Figure 63 Fluorescein labeled predicted bivalent spin probes (seq5-7).

In 3 x 1.5mL microcentrifuge tubes, the coupling solutions were made by dissolving 5.8mg (31.5 μ mol) of the 2,2,5,5-Tetramethyl-3-pyrroline-1-oxyl-3-carboxylic Acid (Acros) and 11.9mg of HATU (31.5 μ mol) in 165 μ L of 20% DCM/DMF. These solutions were mixed using a micropipettor, after which 11.0 μ L (63.0 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted to the resin seq5-7 as above, followed by washing, and a second coupling. The resins were then washed 3 x 2min with DMF, the 3 x 1min ~5% piperidine/DMF,

repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The crude product was dissolved in 75μL of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4°C until needed.

Synthesis of fluorescein labeled bivalent (-) control scaffold (27).

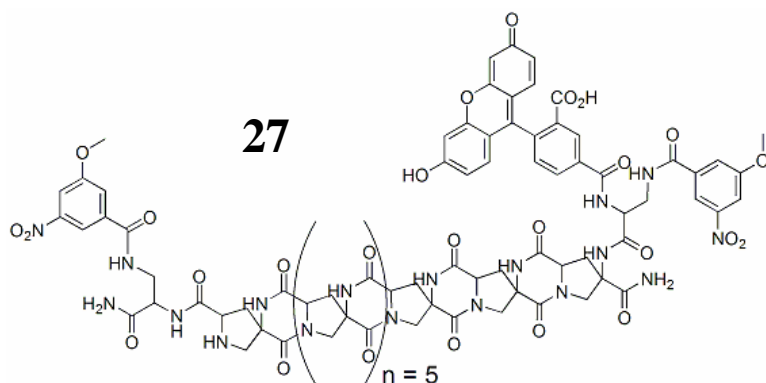


Figure 64 Fluorescein labeled predicted bivalent (-) control (seq2).

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 6.2mg (31.5μmol) of the 3-methoxy-5-nitrobenzoic acid (7) and 11.9mg of HATU (31.5μmol) in 165μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0μL (63.0μmol) of DIPEA was added to make the active ester and then after 10min, added to resin seq2 as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solution was drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac. The sample was redissolved in 0.5mL of 12.5% ACN in water with 0.5% formic acid and analyzed by LCMS.

The crude product was dissolved in 75μL of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4°C until needed.

Synthesis of predicted PEG-linker fluorescein labeled bivalent scaffold (28).

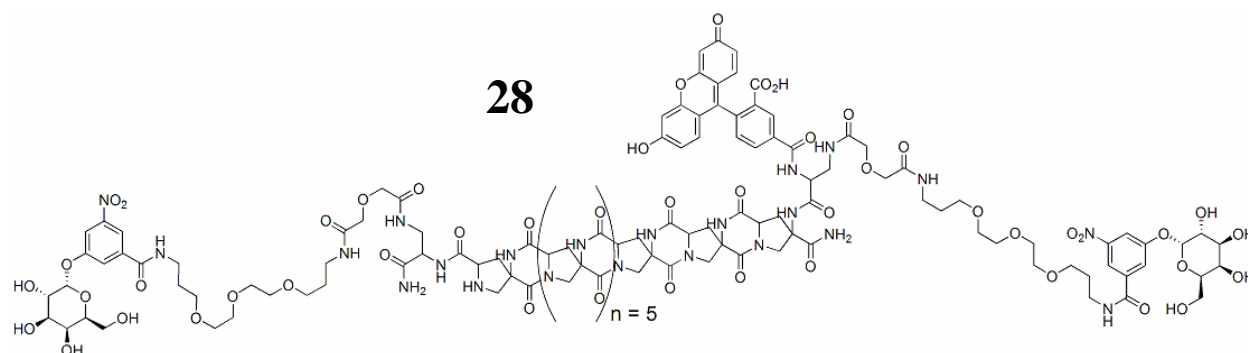


Figure 65 Fluorescein labeled PEG-linked predicted bivalent ligand (seq5).

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 7.0mg (12.6μmol) of Fmoc-PEG-OH (Novabiochem) and 4.8mg of HATU (12.6μmol) in 75μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 4.4μL (25μmol) of DIPEA was added to make the active ester. After 10min activation time, the solution was aliquoted to each resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above, followed by washing with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 7.1mg (12.6μmol) of the acetyl-protected ligand (**12**) and 4.8mg of HATU (12.6μmol) in 65μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 4.4μL (25.2μmol) of

DIPEA was added to make the active ester and then after 10min, aliquoted to the resins as above, followed by washing, and a second coupling. The resin was then washed 3 x 2min with DMF, the 3 x 1min ~5%piperidine/DMF, repeating these two washes 3x more. Then the resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hour. The solution was drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac.

The bivalent-acetylated ligand was dissolved in 200μL of methanol and mixed with a pipettor. Then, 2μL of 25% NaOMe/MeOH was added to each sample, mixed via pipettor, and allowed to react for 15min. The reaction was quenched with 400μL of 0.1M HCl, frozen, and lyophilized.

The crude product, along with NaCl salts, was dissolved in 75μL of 20% piperidine/NMP and allowed to react at room temperature for 36hrs. The crude reaction was filtered, and injected as a single injection onto the analytical HPLC column for purification. Fractions containing the desired product were identified by reinjection on the LCMS and lyophilized. The samples were stored in the dark at 4°C until needed.

Fluorescent Polarization (FP) Assay

The FP assay was performed in a Corning Costar 384-well black square bottom microtiter plate (Fisher, 07-200-653). The samples in each well were arranged in columns, consisting of 2 columns of buffer, 2 columns of just FAM-ligand, and then 16 columns of 8 increasing protein concentration, ½ log difference between each (i.e. 6, 30, 60, 300, 600...). The buffer was 50mM Tris-HCl (pH7.4), 200mM, 3 mM NaN₃, 1 mM EDTA and 0.2mg/mL bovine gamma globulins and was used for all dilutions of protein or FAM-ligand. Depending on the number of samples being analyzed at once, between 8-32 points duplicate points for protein concentration were done. The protein dilutions, or just buffer were aliquoted using a multichannel pipettor, 25μL per well, with concentrations ranging from ~10nM to 300μM CTB depending on the sample. Then the lights were turned out and the FAM-ligand was diluted to ~2-10nM, but usually 6nM. Then,

25 μ L of FAM-ligand or just buffer was aliquoted into each well, and mixed by pipette at least five times to give final FAM concentration of 3nM and CTB concentration of 3, 10, 30, 100, 300, etc. The microplate was covered with foil and allowed to sit at room temperature overnight. The plate was read on a LJL Analyst HT plate reader using the following settings: microplate format = Corning 384 Square Opaque PS, shake time = 10s, excitation filter = fluorescein 485nm, emission filter = fluorescein 530 nm, beamsplitter = FL 505, detector counting = counts, one reading per well, integration time = 100000 μ s, attenuator mode off, Z height = 1.5mm, excitation polarizer filter = initial parallel, but dynamic, and emission polarizer filter = static.

Boc-propargylamine (29).

To an ice cooled 500mL round bottom containing 250mL distilled dichloromethane, 4.8mL of propargylamine (75.1mmol) was added and allowed to stir for 10min. To this, 17.2g of Boc-anhydride (78.8mmol) was added along with 21.1mL of triethylamine (150.1mmol). The reaction was stirred for 12hrs under nitrogen. The solvent was removed *in vacuo*. The residue was dissolved in 300mL EtOAc, and washed 2 x 150mL 0.1M HCl, 2 x 100mL brine, and dried with sodium sulfate and filtered. The solvent was removed *in vacuo* to yield the crude product, which was dissolved in 200mL hot hexanes. The insolubles were filtered, and the product was allowed to crystallize at 4°C for 36hrs. The crystals were filtered and dried *in vacuo* to yield 9.8g of product (84%). ¹H NMR (300MHz, 25°C, CDCl₃) δ 4.68 (b, 1H), δ 3.91 (d, 2H, J = 3.2Hz), δ 2.20 (t, 1H, J = 2.5Hz), δ 1.43 (s, 9H).

***meta*-(Boc-amino-prop-1-ynyl)-benzoic acid methyl ester (30).**

To a flame-dried 250mL round bottom flask, 50mL of extra-dry acetonitrile was added, along with 2.2g Boc-propargylamine (14.2mmol), 5.0g *meta*-methyl-iodobenzoate (19.2mmol), 2.4mL triethylamine (17.7mmol), 45mg copper iodide (0.24mmol), and 140mg tetrakis(triphenylphosphine)palladium (0.12mmol). Freeze-pump-thaw degassing cycles were conducted by freezing in liquid N₂, pumped under *vacuo*, and warmed in a gloved hand. This was repeated at least 5 times, and the last the frozen sample was transferred to a stir plate, allowed to thaw, and stirred under nitrogen overnight. 15g of celite was then added

and the solvent removed *in vacuo*. The celite was transferred to a loading cartridge. The product was purified on a ~300g steel Isco column equilibrated in hexanes. Separation was achieved with a gradient of hexanes to EtOAc:hexanes (3:2). Fractions were pooled and the solvent removed *in vacuo* to yield 2.4g product (59%). ¹H NMR (300MHz, 25°C, CDCl₃) δ 8.05 (m, 1H), δ 7.95 (m, 1H), δ 7.55 (m, 1H), δ 7.36 (m, 1H), δ 4.80 (b, 1H), δ 4.13 (m, 2H), δ 3.88 (s, 3H), δ 1.44 (s, 9H).

***meta*-(amino-prop-1-ynyl)-benzoic acid methyl ester (31).**

To a 250mL round bottom flask, 2.4g of *meta*-methylbenzoate-Boc-propargylamine was added along with 150mL of TFA:DCM (2:5). The reaction was stirred for 2hrs, and the solvent was removed *in vacuo*. The crude product was dissolved in 10mL methanol and injected onto the 120g RP-C18-Isco column and separated using a 5-45% ACN gradient using ACN (0.05% formic acid) and 95% water (0.1% formic acid). Fractions containing pure product were identified by LCMS or HPLC and lyophilized to give 1.3g desired product (81%).

Peracetyl-D-lactose (32).

To a 500mL round bottom flask, 55mL of acetic anhydride (585mmol) was added, along with 10g D-lactose (29.2mmol), 30mL of triethylamine (215mmol), and finally, 180mg dimethylaminopyridine (1.5mmol). The reaction, initially a cloudy-white suspension, was stirred for 1hr, after which the solution was yellow, with all material now dissolved. The reaction was cooled on ice, and 50mL of water was added to hydrolyze excess acetic anhydride. 300mL of water and 300mL of EtOAc were added and the mixture transferred to a separatory funnel. The organic layer was set aside, and the aqueous layer extracted 2 x 150mL EtOAc. The organic layers were pooled, and carefully washed with 6 x 100mL saturated sodium bicarbonate, which produced vigorous bubbling. More washes may be needed until no further bubbling occurred. The organic layer wash then washed with 2 x 100mL water and 2 x 100mL brine, dried with sodium sulfate, and filtered. The solvent was removed *in vacuo* to produce 19.1g product (97%). ¹H NMR (300MHz, 25°C, CDCl₃) δ 6.19 (d, 0.6H), δ 5.83 (d, 0.4H, J = 9.4Hz), δ 5.45 (t, 1H, J = 8.8Hz), δ 5.34 (m, 1H), δ 5.07 (m, 2H), δ 4.94 (m, 1H), δ 4.83 (m, 1H), δ 4.45 (m, 1H), δ 4.24 (m, 6H), 2.20-1.88 (21H).

Heptaacetyl- α -bromo-D-lactose (33).

The peracetylactose (19.1g, 28.2mmol) was dissolved in 50mL distilled dichloromethane in a 250mL round bottom flask, and 50mL of hydrobromic acid in acetic acid (**CAUTION**) was carefully added over a few minutes. The reaction was stirred for 3hr at room temperature. The reaction was cooled on ice and 400mL of ice-water was slowly added over 10min. Then 30g sodium bicarbonate, in ~2g scoops, with waiting until bubbling subsides. 300mL of EtOAc were added and the mixture transferred to a separatory funnel. The organic was drained, and the aqueous layer was extracted 2 x 150mL of EtOAc. The organic layers were pooled, washed with 100mL of saturated sodium bicarbonate, then washed with 2 x 100mL water and 2 x 100mL brine, dried with sodium sulfate, and filtered. The solvent was removed *in vacuo* to produce 18.2g product (93%). ^1H NMR (300MHz, 25°C, CDCl_3) δ 6.51 (d, 1H, $J = 4.0\text{Hz}$), δ 5.56 (t, 1H, $J = 9.6\text{Hz}$), δ 5.34 (d, 1H, $J = 3.3\text{Hz}$), δ 5.13 (dd, 1H, $J = 7.9\text{Hz}, 10.3\text{Hz}$) δ 4.96 (dd, 1H, $J = 3.4\text{Hz}, 10.4\text{Hz}$) δ 4.76 (dd, 1H, $J = 4.1\text{Hz}, 10.0\text{Hz}$), δ 4.50 (m, 2H), δ 4.21 (m, 4H), δ 3.89 (m, 2H), 2.14-1.94 (21H).

Heptaacetyl- β -isothiocyanate-D-lactose (36).

Extra-dry acetonitrile (300mL) was added to a flame-dried 500mL round bottom flask. ~40g of activated molecular sieves (in microwave until red) were added with stirring for ~1hr. Then, 2.5g of tetrabutylammonium hydrogen sulfate (7.15mmol) followed by 2.1g of potassium thiocyanate (21.5mmol) were added, producing a red color change. A condenser was attached and the suspension was refluxed under nitrogen for 2-3hr. The suspension was allowed to cool to room temperature, and the lactose bromide was added and the reaction was stirred for 14hrs. The sieves were filtered using a Buchner funnel, and the flow-through collected into a 2L round bottom using an adaptor. The sieves were washed extensively (5 x 150mL) with dichloromethane. The solvent was removed *in vacuo*. The crude product was adsorbed onto 20g celite. The product was purified on a ~300g steel Isco column equilibrated in hexanes. Separation was achieved with a gradient of hexanes to EtOAc:hexanes (3:2). Fractions were pooled and the solvent removed *in vacuo* to yield 3.0g product (66%). ^1H NMR (300MHz, 25°C, CDCl_3) δ 5.33 (d, 1H), δ 5.20 (m, 4H), δ 4.47 (m, 2H), δ 4.13 (m, 4H), δ 3.85 (m, 2H), δ 3.65 (m, 1H), δ 2.13-1.92 (21H).

***Meta*-[[heptaacetyl- β -D-lactosamino]-4*H*-thiazol-(5*Z*)-ylidenemethyl]-benzoic acid methyl ester (38).**

To a 100mL round bottom flask containing 30mL distilled dichloromethane, 1.0g of the lactose-isothiocyanate (1.48mmol) was added. Then, 525mg of *meta*-methylbenzoate-propargylamine (2.22mmol) was added, followed by 1mL of diisopropylethylamine (2.96mmol). The reaction was stirred for no more than 2hrs. Acetic acid (6mL) was then added, and allowed to stir for ~4hrs, after which the initial thiourea product was converted to the thiazoline. The dichloromethane was removed *in vacuo*, and the crude product, in acetic acid, was loaded onto a 120g-RP-C18-Isco column, equilibrated in 5% ACN (0.05% formic acid) / 95% water (0.1% formic acid). A gradient from 5-95% ACN separated the mixture. Product fractions were identified by reinjection onto an HPLC or LCMS and pooled, and the solvent was removed by lyophilization to yield about 900mg of product, usually in ~75% yield. ¹H NMR (300MHz, 25°C, CDCl₃) δ 7.86 (s, 1H), δ 7.82 (m, 1H), δ 7.38 (d, 2H), δ 6.53 (d, 1H), δ 5.30-4.82 (m, 8H), δ 4.44 (m, 2H), δ 4.12 (m, 2H), δ 3.88 (s, 3H), δ 3.81 (m, 3H), δ 2.10-1.91 (21H).

***Meta*-[2[β -D-lactosamino]-thiazol-5-ylmethyl]-benzoic acid (39/40).**

To the 900mg *meta*-[[heptaacetyl- β -D-lactosamino]-4*H*-thiazol-(5*Z*)-ylidenemethyl]-benzoic acid methyl ester (1.11mmol) in a 100mL round bottom flask, 50mL of methanol:0.5M aqueous LiOH (1:1) was added and the reaction was allowed to stir overnight for 12hrs. The reaction was neutralized by addition of 1M HCl until ~pH7. The methanol was carefully removed at ambient temperature *in vacuo* and injected onto the 40g-RP-C18-Isco and separated using a 5-30% ACN gradient using ACN (0.05% formic acid) and 95% water (0.1% formic acid). Fractions containing pure product were identified by LCMS or HPLC and lyophilized to give ~300mg desired product (50%). ¹H NMR (300MHz, 25°C, d₆-dimethylsulfoxide) **ADD NMR PEAKS HERE.**

***Meta*-[2[β -D-lactosamino]-thiazol-5-ylmethyl]-benzyl-glycine methyl ester**

To 10mg of *meta*-[2[β -D-lactosamino]-thiazol-5-ylmethyl]-benzoic acid (17.9 μ mol) in a 1.5mL microcentrifuge tube, 200 μ L of DMF was added along with a stir bar. To this, 5.5mg HOBt (35.8 μ mol) and 13.6mg HBTU (35.8 μ mol) were added, followed by 25 μ L of DIPEA (143.1 μ mol) and stirring for 5min. Then 5.1mg of glycine methyl ester hydrochloride hydrate

(35.8 μ mol) was added and stirred for 2hrs. The reaction was quenched by addition of 1mL 12.5% ACN in water, 0.5% formic acid. The crude reaction was purified on the preparative HPLC system, in four injections. Fractions containing pure product were identified by LCMS reinjection. Lyophilization gave the ~6mg of desired product (53%), which was confirmed by MS analysis

Synthesis of the resin bound bivalent intermediate (41).

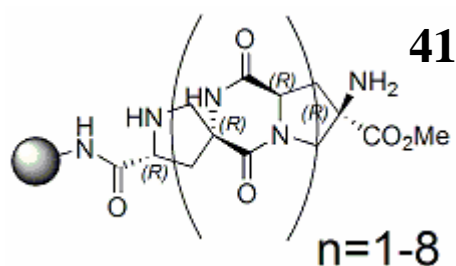


Figure 66 Resin-bound scaffold used in the synthesis of 4 types of bivalent ligands.

To a 50mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide (**NOT AM!**) Resin (Novabiochem) (100mg, 60 μ mol loading). The resin was swelled for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 5mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 61.2mg (120.0 μ mol) of Boc-protected pro4(2R4R)[17] building block and 45.6mg of HATU (120.0 μ mol) in 600 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 41.8 μ L (41.8 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

This process of coupling/deprotection was repeated n ($n=1-8$) additional times. After each Fmoc deprotection, the resin was washed with DCM several times, and allowed to dry for

five minutes via aspiration. The beads were now easily removed from the reactor and then weighed. An appropriate weight fraction (i.e. $1/8^{\text{th}}$ removed after 2^{nd} building block was coupled, then $1/7^{\text{th}}$ after third building block, etc.) was then removed, placed in a fresh 1.0mL reactor and stored in a desiccator. The remaining resin was then swelled in DMF for 10 min. The process of coupling/deprotection/aliquot removed was repeated seven additional times, making 8 portions of equimolar amounts of 8 different lengths, 2-9 building blocks, with the final amine deblocked, yielding the sequence, resin-RR-(RR) $_n$ -free amine ($n=1-8$).

Because the solid support was the ordinary Rink Amide resin, the Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 20mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf-from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min to each of the 8 resin lengths, with slight bubbling, the Boc groups were removed while still attached to the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on each resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 12hrs at 35°C - 40°C .

The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each, then with DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*. Each of the lengths $n=1-8$ was split into 4 equal portions to be used in the synthesis of the 4 types of bivalent ligands described below. The resins were stored in the desiccator until needed.

Synthesis of the PEG-bivalent scaffolds (42).

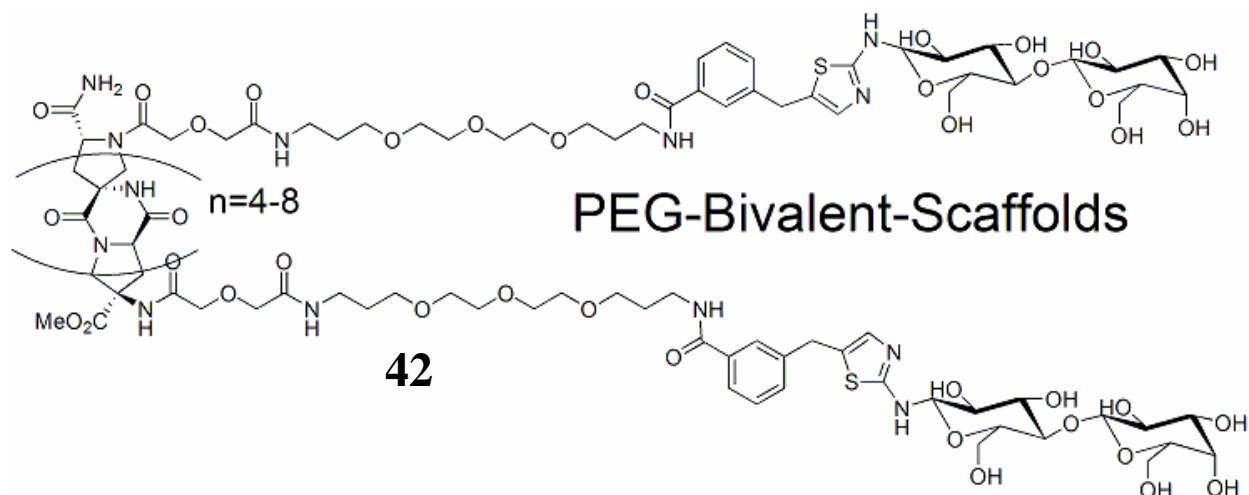


Figure 67 PEG-bivalent scaffolds, of length 5-9 building blocks.

The resin length portions $n=4-8$ were each swelled in DMF for 30min. The estimated amount of material in each reactor was $60\mu\text{mol} / 8 / 4 = \sim 2\mu\text{mol}$, with $\sim 10\mu\text{mol}$ in total. In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 27.9mg ($50\mu\text{mol}$) of Fmoc-PEG-OH (Novabiochem) and 19.0mg of HATU ($50\mu\text{mol}$) in 250 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 8.7 μL ($100\mu\text{mol}$) of DIPEA was added to make the active ester. After 10min activation time, the solution was aliquoted to each resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above, followed by washing with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 22.4mg ($40\mu\text{mol}$) of the synthetic ligand (**39/40**), 6.1mg of HOBt ($40\mu\text{mol}$) and 15.2mg of HBTU ($40\mu\text{mol}$) in 400 μL of 50% DMSO/DMF. This solution was mixed using a micropipettor, after which 10.5 μL ($120\mu\text{mol}$) of DIPEA was added to make the active ester and then after 10min, aliquoted as above, followed by washing, and a second coupling. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 1hour (NO LONGER). The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the

cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac. Each sample was redissolved in 0.5mL of 12.5% ACN in water with 0.5% formic acid and analyzed by LCMS.

The final bivalent ligands of varying lengths ($n=4-8$) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. Using an HPLC calibration curve derived from the glycine methyl ester ligand (**48**), the total nmols of material in the pooled purified product were estimated, and aliquoted into $\sim 10-30$ nmol portions, depending on the amount of material and the desired final concentration. The microcentrifuge tubes were lyophilized and stored at 4°C . When the time came for binding measurements, a single tube was resuspended in 0.8mL buffer and the concentration was measured based on the extinction constant derived from the glycine methyl ester ligand (**48**).

Synthesis of the β Ala (1 or 2)-bivalent scaffolds (**44/45**).

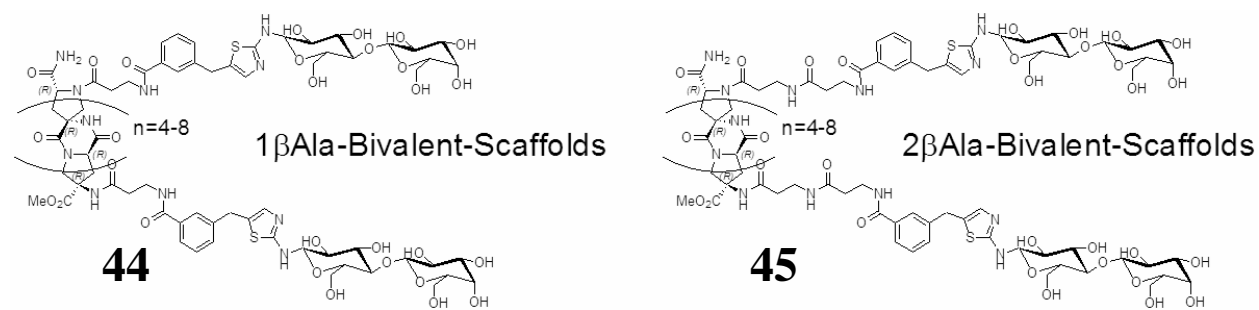


Figure 68 β Ala (1 or 2)-bivalent scaffolds, of length 5-9 building blocks.

Two sets of resin length portions $n=4-8$ were each swelled in DMF for 30min. The estimated amount of material in each reactor was $60\mu\text{mol} / 8 / 4 = \sim 2\mu\text{mol}$, with $\sim 10\mu\text{mol}$ in each of the two length sets. In 2 x 1.5mL microcentrifuge tube, the coupling solutions were made by dissolving 31.1mg ($100\mu\text{mol}$) of Fmoc- β Ala-OH (Novabiochem) and 38.0mg of HATU ($100\mu\text{mol}$) in $500\mu\text{L}$ of 20% DCM/DMF. These solution was mixed using a micropipettor, after which $34.8\mu\text{L}$ ($200\mu\text{mol}$) of DIPEA was added to each to make the active ester. After 10min activation time, the solution was aliquoted to each resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for

quantitative acylation, as judged by subsequent Fmoc release in the same manner described above, followed by washing with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

One set of 5 different lengths were ready for ligand coupling, but the other set needed another β Ala. By repeating the above steps of β Ala double coupling followed by Fmoc removal, this sample was made ready for ligand incorporation.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 44.8mg (80 μ mol) of the synthetic ligand (**39/40**), 12.2mg of HOBt (80 μ mol) and 30.4mg of HBTU (80 μ mol) in 800 μ L of 50% DMSO/DMF. This solution was mixed using a micropipettor, after which 21.0 μ L (240 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted into the 10 reactors, followed by washing, and a second coupling. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 1hour (NO LONGER). The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac. Each sample was redissolved in 0.5mL of 12.5% ACN in water with 0.5% formic acid and analyzed by LCMS.

The final bivalent ligands of varying lengths (n=4-8) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. Using an HPLC calibration curve derived from the glycine methyl ester ligand (**48**), the total nmols of material in the pooled purified product were estimated, and aliquoted into ~10-30nmol portions, depending on the amount of material and the desired final concentration. The microcentrifuge tubes were lyophilized and stored at 4°C. When the time came for binding measurements, a single tube was resuspended in 0.8mL buffer and the concentration was measured based on the extinction constant derived from the glycine methyl ester ligand (**48**).

Synthesis of bivalent scaffolds (46).

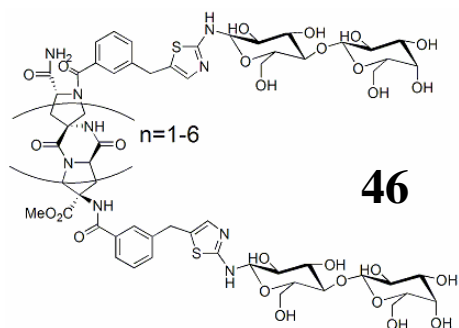


Figure 69 Bivalent scaffolds, of length 2-7 building blocks.

The resin length portions $n=1-6$ were each swelled in DMF for 30min. The estimated amount of material in each reactor was $60\mu\text{mol} / 8 / 4 = \sim 2\mu\text{mol}$, with $\sim 12\mu\text{mol}$ in total. In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 22.4mg ($40\mu\text{mol}$) of the synthetic ligand (**39/40**), 6.1mg of HOBt ($40\mu\text{mol}$) and 15.2mg of HBTU ($40\mu\text{mol}$) in 400mL of 50% DMSO/DMF. This solution was mixed using a micropipettor, after which $10.5\mu\text{L}$ ($120\mu\text{mol}$) of DIPEA was added to make the active. After 10min activation time, the solution was aliquoted to each resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF, followed by a second coupling. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 1hour (NO LONGER). The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac. Each sample was redissolved in 0.5mL of 12.5% ACN in water with 0.5% formic acid and analyzed by LCMS.

The final bivalent ligands of varying lengths ($n=1-6$) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. Using an HPLC calibration curve derived from the glycine methyl ester ligand (**48**), the total nmols of material in the pooled purified product were estimated, and aliquoted into $\sim 10-30\text{nmol}$ portions, depending on the amount of material and the desired final concentration. The microcentrifuge tubes were lyophilized and stored at 4°C . When the time came for binding measurements, a single tube was

resuspended in 0.8mL buffer and the concentration was measured based on the extinction constant derived from the glycine methyl ester ligand (**48**).

Synthesis of the monovalent scaffolds (**47**).

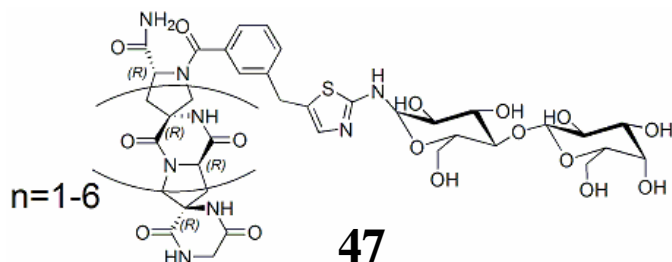


Figure 70 Monovalent scaffolds, of length 2-7 building blocks.

To a 15mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide (***NOT AM!***) Resin (Novabiochem) (20mg, 12 μ mol loading). The resin was swelled for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 12.2mg (24 μ mol) of Boc-protected pro4(2R4R)[17] building block and 9.1mg of HATU (24.0 μ mol) in 120 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 8.4 μ L (48.0 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

This process of coupling/deprotection was repeated n ($n=1-6$) additional times. After each Fmoc deprotection, the resin was washed with DCM several times, and allowed to dry for five minutes via aspiration. The beads were now easily removed from the reactor and then weighed. An appropriate weight fraction (i.e. 1/6th removed after 2nd building block was coupled, then 1/5th after third building block, etc.) was then removed, placed in a fresh 1.0 mL reactor and stored in a desiccator. The remaining resin was then swelled in DMF for 10 min. The process of coupling/deprotection/aliquot removed was repeated six additional times, making

6 portions of equimolar amounts of 6 different lengths, 2-7 building blocks, with the final amine deblocked, yielding the sequence, resin-RR-(RR)_n-free amine (*n*=1-6).

The resin length portions *n*=1-6 were each swelled in DMF for 30min. In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 17.8mg (60.0μmol) of Fmoc-Gly-OH (Novabiochem) and 22.8mg of HATU (60.0μmol) in 300μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 21.0μL (120.0μmol) of DIPEA was added to make the active ester. After 10min activation time, the solution was aliquoted to each resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF and the coupling/washing was repeated.

Because the solid support was the ordinary Rink Amide resin, the Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 20mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf-from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min to each of the 8 resin lengths, with slight bubbling, the Boc groups were removed while still attached to the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on each resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 12hrs at 35°C -40°C. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each, then swelled in DMF again.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 13.5mg (24.0μmol) of the synthetic ligand (**39/40**), 3.7mg of HOBt (24.0μmol) and 9.1mg of HBTU (24.0μmol) in 240 μL of 50% DMSO/DMF. This solution was mixed using a micropipettor, after which 4.2μL (48.0μmol) of DIPEA was added to make the active ester and then after 10min, aliquoted as above, followed by washing, and a second coupling. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 1hour (NO LONGER). The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac. Each

sample was redissolved in 0.5mL of 12.5% ACN in water with 0.5% formic acid and analyzed by LCMS.

The final monovalent ligands of varying lengths ($n=2-6$) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. Using an HPLC calibration curve derived from the glycine methyl ester ligand (**48**), the total nmols of material in the pooled purified product were estimated, and aliquoted into $\sim 10-30$ nmol portions, depending on the amount of material and the desired final concentration. The microcentrifuge tubes were lyophilized and stored at 4°C . When the time came for binding measurements, a single tube was resuspended in 0.8mL buffer and the concentration was measured based on the extinction constant derived from the glycine methyl ester ligand (**48**).

Synthesis of fluorescein containing bivalent scaffolds (**51**).

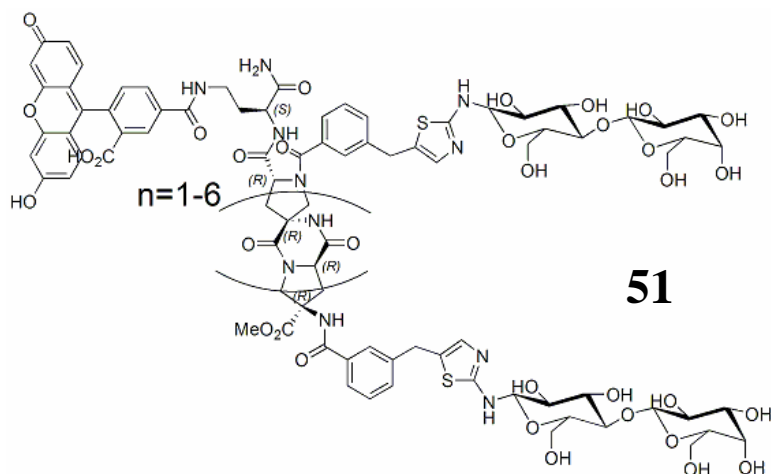


Figure 71 Bivalent fluorescein labeled scaffolds, of length 2-7 building blocks.

To a 15mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide (**NOT AM!**) Resin (Novabiochem) (20mg, $12\mu\text{mol}$ loading). The resin was swelled for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 32.8mg ($60\mu\text{mol}$) of Fmoc-L-Dab(ivDde)-OH (Novabiochem) and 22.8mg of HATU ($60\mu\text{mol}$) in $300\mu\text{L}$

of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20.9 μ L (120 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 12.2mg (24 μ mol) of Boc-protected pro4(2R4R)[17] building block and 9.1mg of HATU (24.0 μ mol) in 120 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 8.4 μ L (48.0 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

This process of coupling/deprotection was repeated n ($n=1-6$) additional times. After each Fmoc deprotection, the resin was washed with DCM several times, and allowed to dry for five minutes via aspiration. The beads were now easily removed from the reactor and then weighed. An appropriate weight fraction (i.e. 1/6th removed after 2nd building block was coupled, then 1/5th after third building block, etc.) was then removed, placed in a fresh 1.0 mL reactor and stored in a desiccator. The remaining resin was then swelled in DMF for 10 min. The process of coupling/deprotection/aliquot removed was repeated six additional times, making 6 portions of equimolar amounts of 6 different lengths, 2-7 building blocks, with the final amine deblocked, yielding the sequence, resin-RR-(RR) $_n$ -free amine ($n=1-6$).

Because the solid support was the ordinary Rink Amide resin, the Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 20mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf-from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min to each of the 8 resin lengths, with slight bubbling, the Boc groups were removed while still attached to the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on each resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 12hrs at 35°C -40°C. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each, then swelled in DMF again.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 26.9mg (48.0 μ mol) of the synthetic ligand (**39/40**), 7.3mg of HOBT (48.0 μ mol) and 18.2mg of HBTU

(48.0 μ mol) in 240 μ L of 50% DMSO/DMF. This solution was mixed using a micropipettor, after which 8.4 μ L (96.0 μ mol) of DIPEA was added to make the active ester and then after 10min, aliquoted as above, followed by washing, and a second coupling. Then the resins were extensively washed with DMF, IPA, DMF, IPA, and DMF.

The Dab side chain protecting groups, ivDde, were removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the side chain. Then the resins were extensively washed with DMF, IPA, DMF, IPA, and DMF.

The resins were acylated with fluorescein-N-hydroxysuccinyl ester (56.8mg, 120.0 μ mol) in 600 μ L DMF with stirring in the dark for 72hrs. Then the resins were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds were cleaved in 0.75mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 1hour (NO LONGER). The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The washes were pooled with the cleavage solutions in a 13mm glass tube and the solvent was removed in a SpeedVac. Each sample was redissolved in 0.5mL of 12.5% ACN in water with 0.5% formic acid and analyzed by LCMS.

The final fluorescently labeled ligands of varying lengths (n=2-6) were purified on an analytical HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. The tubes were lyophilized and stored at 4°C in the dark.

Fluorescence Intensity (FI) Assay

Two cuvettes were used for each titration (Semi-micro fluorometer cell, NSG Precision Cells, Type 517). In the first, 500 μ L of FI buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.02% NaN₃) was added, and in the second, 500 μ L of buffer along with CTB concentration = 100nM, 250nM, or 500nM, depending on the ligand being tested. The FI was measured using a Varian Cary Eclipse Fluorescence spectrophotometer with excitation at 282nm and the emission monitored from 300-400nm, with a scan rate of 100nm/min and 5 scans per point. The voltage of the detector was adjusted to give ~200-400 of 1000, for the initial protein-only point. Eight

incremental additions of a ligand stock solution were added using Hamilton syringes. The background FI was averaged for each addition and then subtracted from each of the five scans, which were then averaged. The 5-largest FI intensities between 330nm-360nm were then averaged to give the FI for each point. The change in FI was calculated and plotted against the ligand concentration to get a sigmoidal curve. Binding constants were derived either from a Scatchard plot or from regression analysis using Mathematica.

3. PHAGE DISPLAY ON AN ARTIFICIAL RECEPTOR

3.1 INTRODUCTION

The power of phage display lies in the expansive number of discrete peptide ligands which can be screened simultaneously. Typically, in excess of 1 billion different peptide ligands can be tested for high-affinity interactions with a target. The ligands which bind most tightly to the receptor are selectively retained, while the non-binders are washed away. The strong binders are eluted, amplified, and again screened against the target receptor, this time with washing conditions designed to disrupt weaker interactions. After repeated cycles of screening / amplification, it is possible to get enrichment on the order of several million, in going initially from 10^9 to much less than 10^2 , but often near-complete enrichment to a few ligands is possible.

Filamentous M13 phage are a member of the family of viruses which infect bacteria. Being a virus, phage are simply protein-coated DNA. The DNA is found as a single circular strand consisting of a few thousand nucleotides encoding a few proteins. Phage are long cylindrical particles with a diameter of about 50\AA , while the length is ten to a hundred times longer. The coat proteins of the phage are mainly pVIII, which, at copy levels of several thousand, makes up the entire outer barrel of the phage, while other proteins, like pIII, make up the tip of the phage, at copy numbers of five.[43]

These two proteins are the main surrogates for insertion of small peptide libraries, although all coat proteins have been tried, but by far the most success has been achieved with pVIII and pIII. The question of the desired copy number and size of the peptide are important when deciding which coat protein to chose. Commercially available phage libraries mainly use pIII, although pVIII has much promise and will likely be commercialized in the future. Several features of pIII make it useful, such as its tolerance of large inserts, beyond the length scale of a

simple peptide, as well as the likelihood for monovalent interactions between ligand and receptors.

The selection process, between a phage-peptide library and the target receptor, is conceptually very simple: the phage library is incubated with an immobilized target, then non-binders are washed away, binders are eluted and amplified, and the process repeated, normally two more times, or until sufficient enrichment is achieved. The conditions used for elution are diverse, ranging from low pH (~2),[44] to denaturants (6M urea),[45] to displacement with specific ligands,[46] or, alternatively, direct infection without elution.[47]

The incredible power of selection is the driving force behind the utility of phage display, but there is also a dark side to the selective pressure. It has been said before that “one gets what one is selecting for.” In the absence of the desired strong, productive interaction between peptide and receptor, selection rears its other side by selecting for plastic binder or streptavidin binders. Alternatively, “freaks” can be selected for, such that internal duplication results in the display of additive weakly interacting peptides which amount to a stronger binder. Additionally, unnecessary rounds of selection and amplification result in the selection for a growth advantage, or in the case of trace contamination, wild-type phage not burdened by the additional peptide insert.

Since phage display was first described over 20 twenty years ago,[44] there have been tens of thousands of published examples exploiting the powers of affinity selection and amplification. A 12 residue peptide was found that modulates the fluorescent properties of the dye, Texas-Red.[48] A taxol binding peptide was found to be of a sequence similar to anti-apoptotic human protein Bcl-2, demonstrating that peptides displayed on the surface of bacteriophage particles can mimic the ligand-binding properties of disordered regions of proteins.[49] A non-RGD containing peptide ligand was found that selectively bound to the integrin $\alpha_v\beta_6$ with 20nM affinity.[50] Remarkably, a peptide was found which binds to a range of inorganic semiconductor surfaces with high specificity, depending on the crystallographic orientation and composition of the surface.[51] A molecule consisting of multiple copies of a peptide discovered by phage display, covalently linked to a flexible backbone, prevented assembly of the anthrax toxin complex *in vitro* and blocked toxin action in an animal model.[52] Phage display has been done *in vivo*, where the phage library was injected into mice and peptides

involved in localization to specific organs were identified.[53] The utility of phage display is limited only by the researcher's creativity.

One area of science that could benefit from the power of phage display is the area of synthetic receptors. To date, no one has found a peptide that binds inside of a totally-synthetic receptor. The ability to synthesize a molecule that folds into a pocket and presents functional groups in specific orientations in three dimensional space has not been possible.

Using a building-block approach to make rigid, functionalized molecules which are potentially able to fold and form a cavity, presents an opportunity for phage display to select and identify a short peptide ligand capable of binding to an artificial receptor. This would be the first example of an artificial antibody and would represent a major step forward for the field of artificial biological receptors.

3.2 RESULTS AND DISCUSSION

Initial studies using molecular modeling on sequences of building blocks potentially able to fold and form a pocket settled on the alternating sequence of pro4(2S4S), RR, SS, RR, SS, RR as fitting this requirement. Modeling suggests that this sequence forms a tight turn, bringing both ends into close proximity (Figure 72). A tryptophan at the trailing end will serve as the UV chromophore and a Dpr at the leading end will terminate one chain by forming a DKP, and deprotection of the orthogonal side chain protecting group will initiate a new chain.

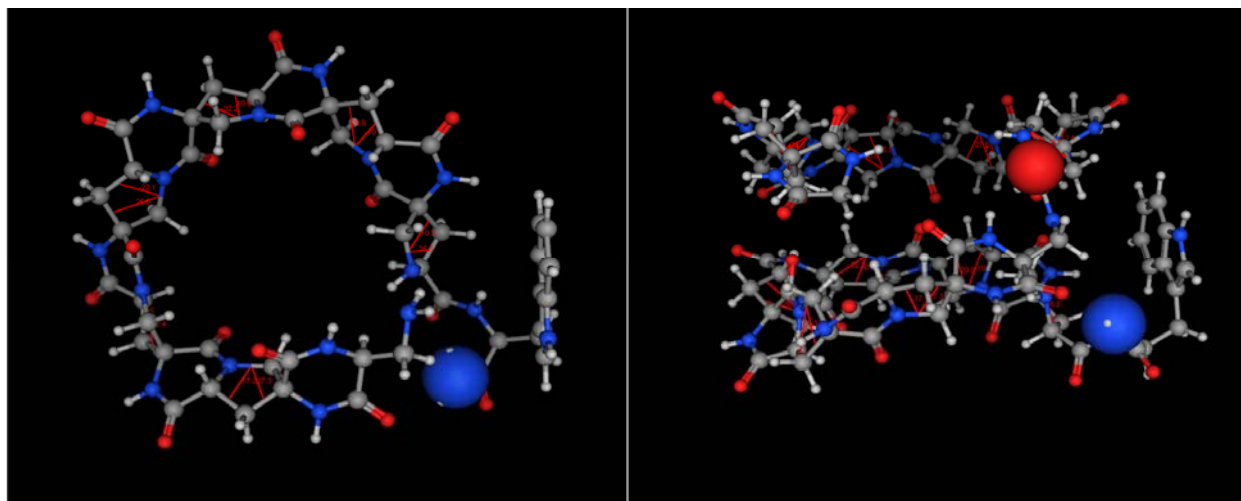


Figure 72 MOE structure (Amber 94 minimized) of LEFT- Trp-SS-RR-SS-RR-SS-RR-Dpr, RIGHT- Trp-SS-RR-SS-RR-SS-RR-Dpr-SS-RR-SS-RR-SS-RR-Dpr (BLUE sphere = Resin amide nitrogen, RED sphere = second chain, first building block amide oxygen).

The second Dpr would be functionalized with a linker, facilitating attachment to a surface. Polystyrene functionalized with various groups such as a maleimide, an amine, or a carboxy-N-hydroxysuccinimide were all commercially available through Corning Life Science. All of the above were suitable means for covalent attachment of a scaffold receptor to polystyrene for affinity selection with a phage library. However, biotin-streptavidin is a ubiquitous method for immobilizing molecules on a surface, whereas the covalent means may be a slightly over-engineered system, especially as a first attempt. The design of the scaffold and the solid-phase methods would easily allow for any mode of attachment to be employed, as the tether was the last thing attached and be orthogonal to all other functionality in the molecule.

Biotin-streptavidin was chosen for its simplicity and for the strength of the interaction. The biotinylation molecule consisted of the biotin, linked through its carboxylic acid to a diPEG spacer, and finally, a carboxylic acid which could be coupled cleanly and selectively to the last amine on the resin bound scaffold, right before cleavage. Half of the material was left unfunctionalized to provide a scaffold without biotin, used to competitively elute the bound phage from the immobilized, biotinylated receptor. Presumably this would not elute phage which were interacting with the plastic or streptavidin. Low pH was not employed, as this would likely disrupt all phage interactions. Specific elution techniques were thought to be more desired than non-specific elution. Perhaps this naively passed over because it was reasoned that low pH

would likely disrupt electrostatic interactions, while neglecting hydrogen bonds, hydrophobic interactions or van der Waals interaction. Although this seemed plausible, the function of the low pH likely serves to denature the streptavidin, or even peel off everything that was initially bound to the plastic. In retrospect low pH should have been investigated as a means for elution, because, depending on the off-rate of any phage bound to the receptor, strongly bound phage may have been de-selected by using a competitive elution pathway.

The synthesis of the first scaffold receptor on the Rink Amide resin (Figure 73), proceeded first by attachment of Trp(Boc), then an alternating series of six pro4(2S4S) and pro4(2R4R). The eighth residue was the weak acid cleavable, orthogonally protected methytrityl diaminopropionic acid (Dpr). Removal of the Fmoc quickly resulted in attack upon the methyl ester of the previous building block, consuming all of the amine to form a diketopiperazine (DKP), thus serving to terminate the chain. Removal of the methytrityl with 1%TFA proceeded quantitatively to allow for initiation of a new chain. Next a second series of six alternating SS and RR building blocks were attached as before. Finally, another Dpr(Mtt) served to terminate the second chain and, upon deprotection, served as the attachment point for the PEG-linked biotin (**52**). Half of the resin was capped with biotin, while the other half was unmodified for the purpose of competitive elution. Cleavage with 95% TFA afforded the Boc-deprotected scaffold, in yields between 10-20% (a typical yield). Also seen was the fact that the carbon dioxide remnants of the Boc group remain attached to the indole of tryptophan (M+44). Multiple lyophilizations from aqueous solutions is the standard treatment to remove this, however it was found that the carbon dioxide was labile to the 20% piperidine/ NMP conditions used to close the DKPs.

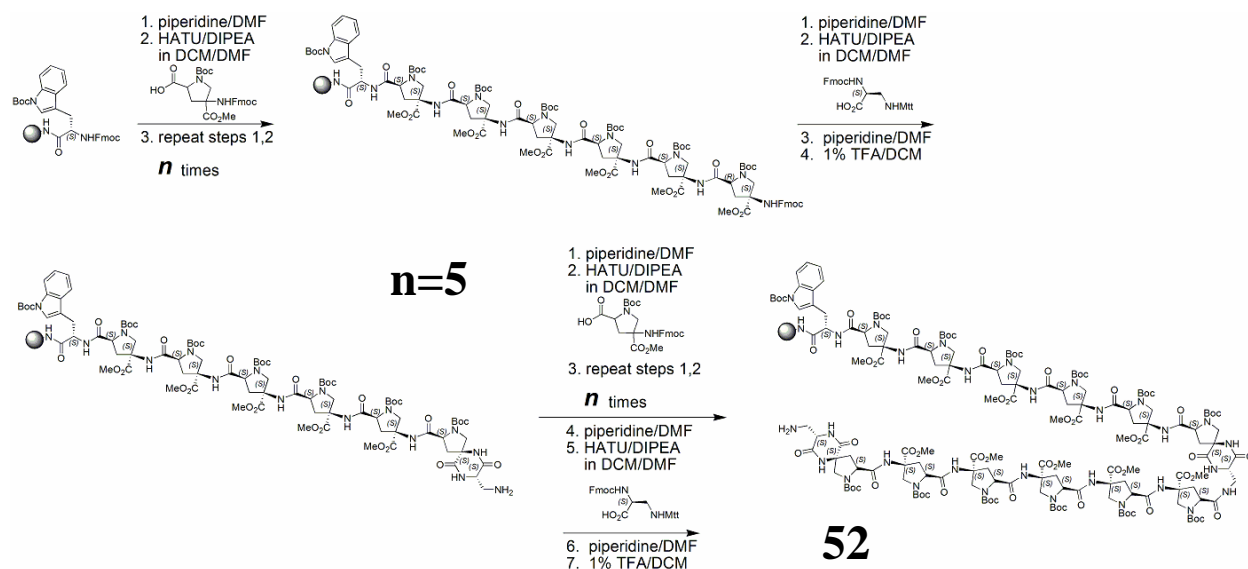


Figure 73 Figure illustrating the solid phase synthesis of the phage display receptor.

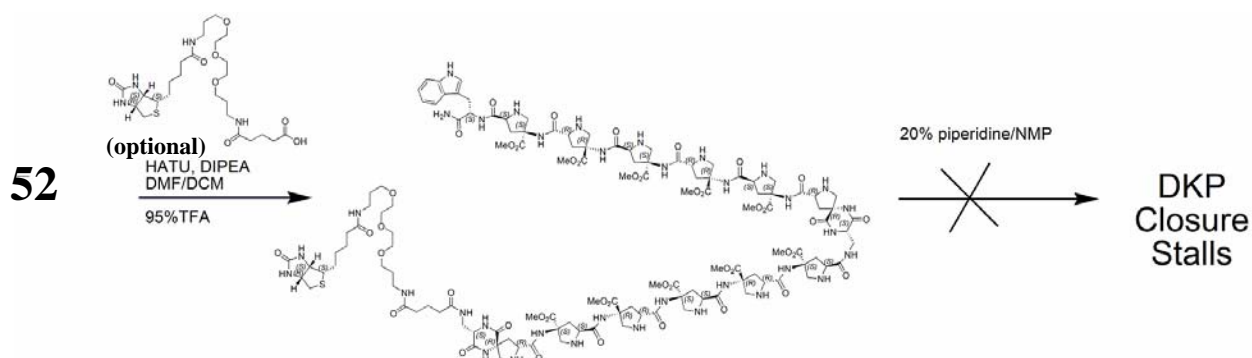


Figure 74 Acylation of 52 with biotin, followed by cleavage and failed DKPS closure.

Normal incubation of the scaffolds, with or without biotin, in 20% piperidine/ NMP for 48hrs failed to go to completion, as judged by LCMS (Figure 74). Although it was difficult to estimate the incomplete reaction yield by LC, it did appear that there was significantly less material than was initially present, as judged by the tryptophan 278nm LC absorbance. Additional time of up to one week, or elevated temperature of 50°C, had no effect on the closure. So, not only did the DKP closure reaction fail to go to completion, but material seemed to disappear as well. This exact sequence was abandoned and the second version would be truncated by one building block on both chains.

This observation of loss of material and incompleteness of the DKP closure has been seen in numerous subsequent attempts to make similar sequences containing three or more SS-RR

repeats. The current working hypothesis rationalizes these observations as being due to intramolecular, complementary, hydrogen bonding interactions (Figure 75). As the DKPs close, the hydrogen bonding interactions in one molecule become complimentary to that in another molecule, possibly producing a three-dimensional lattice with different solubility properties. Future studies are underway to potentially exploit this event via self-assembly or suppress the H-bonding using denaturants, capable of forming both H-bond acceptors and H-bond donors.

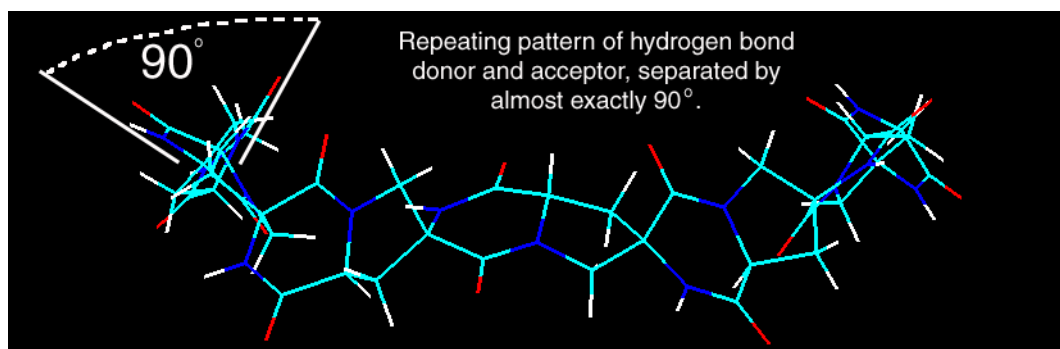


Figure 75 MOE picture, Amber 94 minimized, of SS-RR-SS-RR-SS-RR illustrating the repeating ordered nature of the DKP pattern.

A second set of potential receptors was synthesized with only 2.5 SS-RR repeats. This new sequence, resin-Trp-SS-RR-SS-RR-SS-Dpr, was made in the same fashion as illustrated in Figure 76. When it came time to initiate the second chain of five building blocks, the resin was split into two portions (**53**, in Figure 76). The difference in what was built off of these two portions consisted of two choices: -SS-RR-SS-RR-SS, as on the first chain, or its enantiomer, -RR-SS-RR-SS-RR, both being capped with another Dpr. Each of these two sequences was again split into two portions at the end, to be consisted of with or without biotin.

Cleavage yielded the product, which was subsequently closed in 20% piperidine/ NMP. After the normal 48hrs, it was apparent that the reaction was progressing sluggishly. The desired product was the major peak (~80%), but additional time of 1-3 days was necessary to drive the DKP closure to completion. In spite of the prolonged exposure to highly basic conditions, no evidence of epimerization, such as multiple peaks with the same mass or distinct shoulders, were seen in the chromatogram. The two receptors (labeled as RR (**54**) or SS (**55**) for the first point of divergence), with or without biotin were easily purified to homogeneity by preparative HPLC. Using the Trp ($\Sigma = 5600$), the concentration was determined, with a final yield in the range of 5-

10%. Two biotinylated receptors and the corresponding free receptors were successfully synthesized and purified, and would be screened for binding with a phage display library.

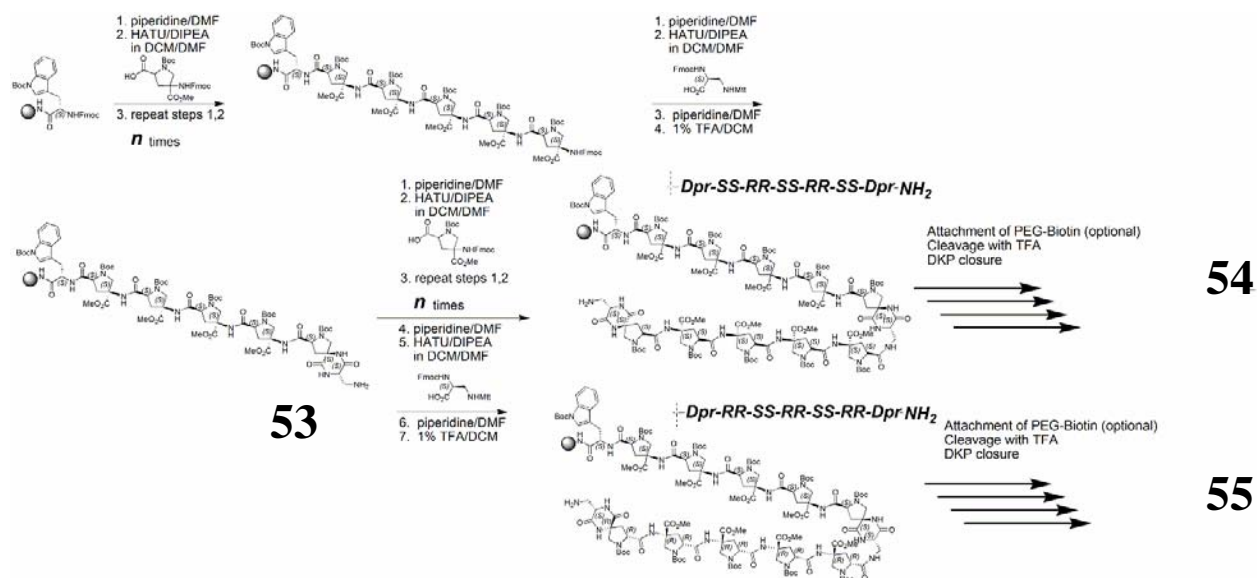


Figure 76 Solid-phase synthesis of biotinylated scaffold with DKPs that do close.

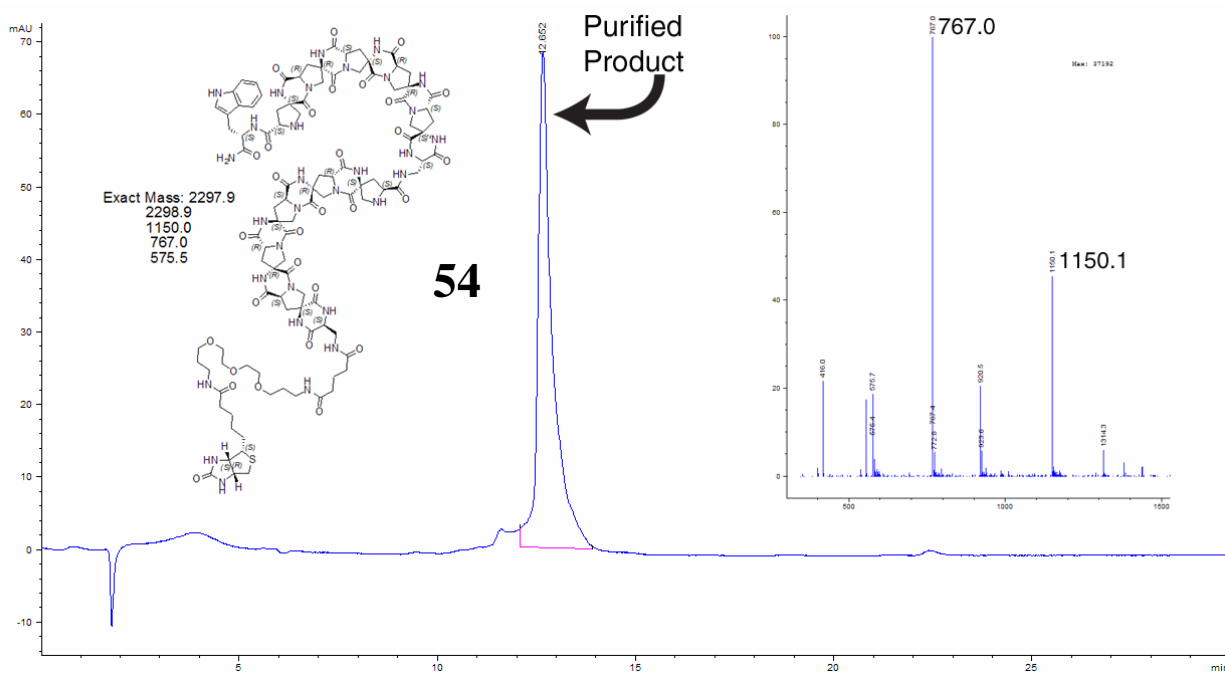


Figure 77 LC trace, MS trace and structure of the "SS-receptor."

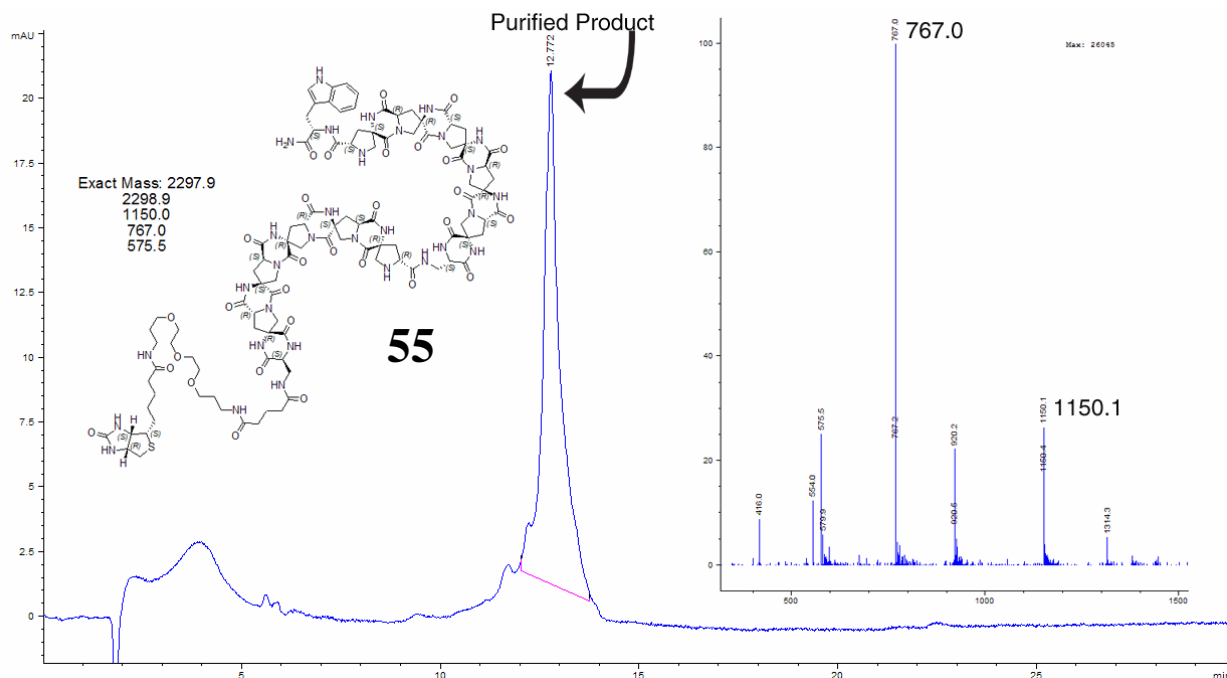


Figure 78 LC trace, MS trace and structure of the "RR-receptor."

The exact phage display procedures, modified from the NEB recommended procedure, are explicitly detailed in the experimental section, so a brief account of what was done and what the results were will be detailed here. The choice of the library was the NEB-12mer peptide-phage library, based on the fact that we were unsure how large of a peptide could potentially bind to the scaffold receptor. With a stated diversity of over 2 billion unique sequences of peptides, present at copy number of about 4 copies, this 12mer library would allow the scanning of only 1 of every 20,000 of all possible 12mers ($20^{12} / 2 \times 10^9 / 100$). In retrospect, the inadequacy of the sequence coverage should have trumped any concerns about sufficient length of the peptide, but the 12mer peptide was chosen nonetheless.

Screening of the two receptors, as well as the streptavidin control, against the 12mer phage library was carried out for three rounds and two amplifications. The phage were eluted with free receptor or with 100 μ M biotin. Titers of 10^4 to 10^5 phage were eluted and subsequent amplification produced titers of 10^{11} - 10^{12} after the first round. Similar titers were seen after the second round. The final round of selection produced titers in the range of 10^1 - 10^3 . Numerous plaques were chosen for amplification and sequencing. For the positive control phage, the streptavidin binding sequence, HPQ, was found numerous times, so the phage display selection process was successful. Out of 6 clones sequenced from the RR-receptor selection, one sequence

showed up twice. This peptide sequence was synthesized, with a fluorescein attached at the N-terminus. Using fluorescence polarization, (not shown) the binding constant was not below the 5 μ M limit of detection.

TMWHPSMIAHPQ	HFYPRFIPPSE
TMWHPSMIAHPQ	HFYPRFIPPSE
LSLEPLLIAHPQ	HIRLPTWWGAYG
NHSTPLLLGHPQ	HSWWPWPTSPTI
	SLTVPFLPLYVP
	DAWISSTLQLRY

Figure 79 Protein sequence of DNA sequenced clones. Left, (+) control, Right, RR-receptor, eluting with free receptor.

The conclusion from this first attempt at phage display was that the experiment was done properly, as judged by the presence of the HPQ sequence. However, more information was required from the experiment, because synthesizing every peptide sequence would be impractical. Interrogation of the individual phage clones was necessary to assess the binding affinity and binding properties. An Enzyme-Linked Immunosorbent Assay (ELISA) would be performed to confirm the binding properties of each phage, in a manner redundant to the phage display selection. Controls would be set up to test for plastic binders[54] and to confirm that the phage were directly interacting with the scaffold receptor. Only after this secondary inference of binding would sequencing be done, followed by peptide synthesis and solution phase binding assessment.

Following this approach, additional receptors were synthesized, this time with variation in the length of the linking diamino acid between the two scaffold segments, in the hopes that the ability of the two C-shapes to fold onto each other would be enhanced with added flexibility in the hinge region. Lysine (57) and ornithine (56) containing receptors were synthesized in an identical manner to the SS-receptor containing Dpr above. Again, incubation for at least 60hrs in piperidine/ NMP was needed to fully close the DKP. Subsequent preparative HPLC purification of the biotinylated and free receptors afforded the desired products seen in Figures 80 and 81, again with yields ranging from 5-10%. The solubility of the purified scaffolds without biotin in water was insufficient for phage elution. This was unexpected, and frankly, difficult to explain, but could be remedied in the future by attaching the identical commercially available PEG linker to the free-receptor, just omitting the biotin.

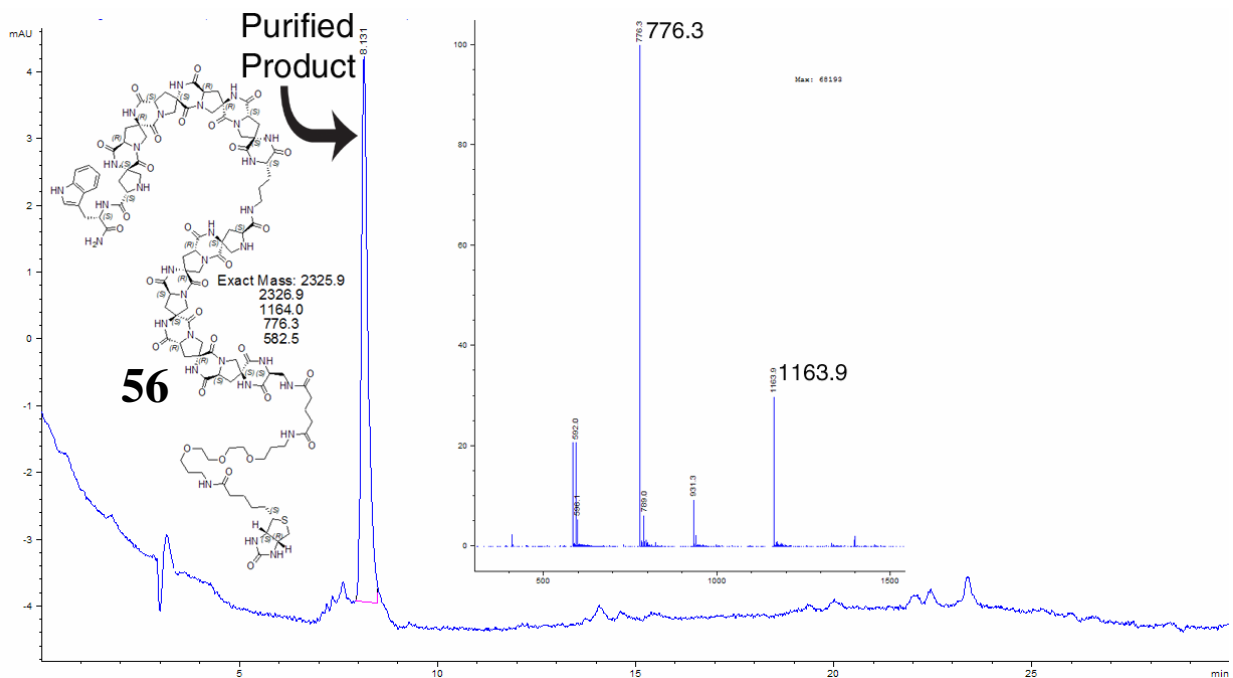


Figure 80 LC trace, MS trace and structure of the "Orn-receptor."

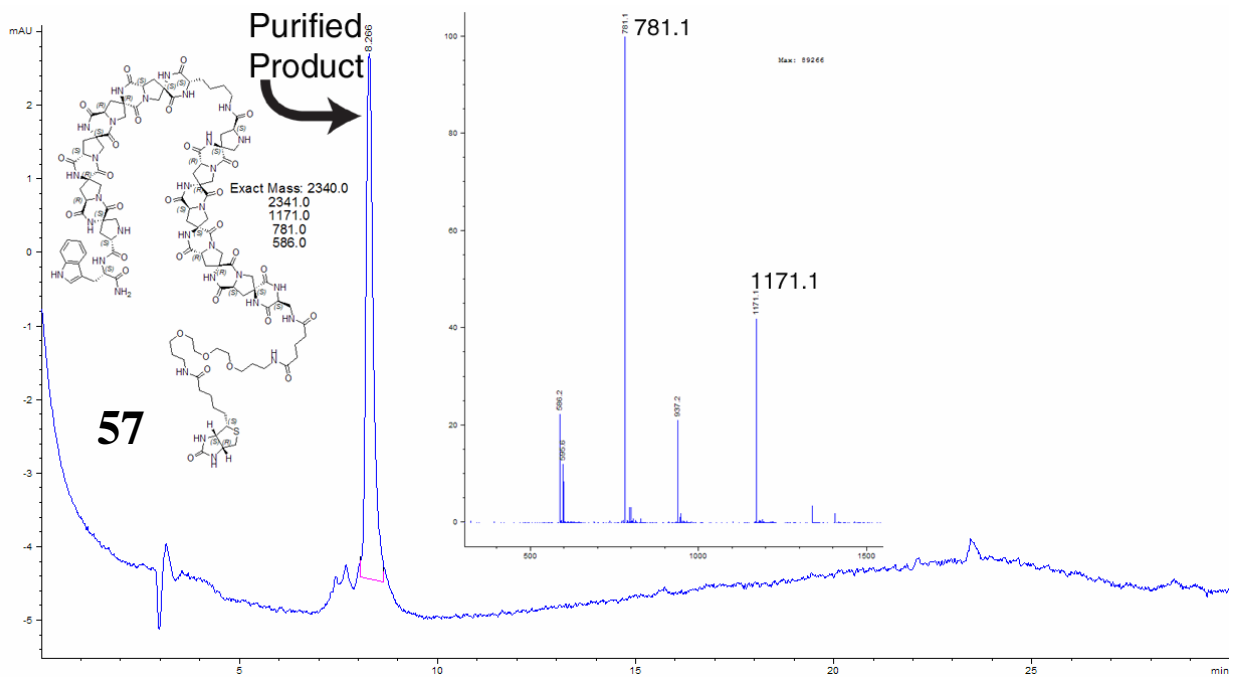


Figure 81 LC trace, MS trace and structure of the "Lys-receptor."

The second attempt at phage display was much more ambitious, as three receptors were being screened against three different libraries, for a total of 12 separate selection experiments, including the (+) controls. The 12mer library was again used, along with a 7mer library as well

as constrained 7mer library consisting of a 7mer flanked by a pair of cysteines. Incidentally, both of these 7mer libraries should have contained 100% of all possible 7-residue peptides. The Dpr(SS) receptor was eluted with free receptor, while the Lys and Orn receptors were eluted with 100mM biotin, because the free receptor was inexplicably insoluble in buffer. Typically, 10^3 - 10^4 phage were eluted during the first and second selections, while amplification of this eluent resulted in 10^{11} - 10^{12} phage. After the final selection, 10^3 - 10^5 phage were eluted. Of these plaques, ~10 clones from each positive control and ~30 clones from each of the receptors and phage library were amplified in 1mL of media in 3 x 96-deep-well plate. Again, sequencing of the positive control binding phage validated them as HPQ-streptavidin binders. The crude supernatant contained ample phage for subsequent analysis by ELISA. Analysis of each clone in the presence and absence of biotinylated receptor showed no difference in ELISA signal intensity. The conclusion was that the phage were either plastic binders or binding to something else besides the receptor. The inference was that there was insufficient selective pressure directing the phage to bind to the receptor because the receptor was not ordered enough. More rigidity was required of the scaffold receptor, which would be accomplished by exploring possible cyclization conditions.

Synthesis of the next version of a scaffold receptor utilized several modifications. First, four alternating building blocks were used instead of five, because of the difficulties in closing all of the DKPs in a timely manner. Second, by coupling an orthogonally protected Dab(ivDde) as the second residue, versatility in subsequent choice of cyclization partner was afforded. Third, by using Cbz-protected building blocks for the two trailing ends of the two scaffold sequences, which were not able to form DKPs, it was possible to attach a carboxylic acid cyclization partner in solution after the DKPs were closed. Finally, by coupling the amino acid cyclization partner last, versatility was increased.

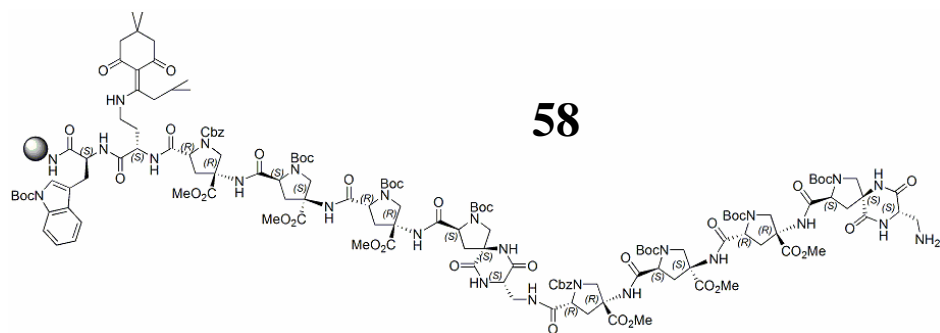


Figure 82 Versatile resin bound intermediate used for subsequent cyclization tests.

We first attempted to close a macrocycle via a thioether. The synthesis of thioether cyclized peptides are versatile and chemoselective.[55, 56] Cysteine is available with numerous protecting groups which are cleavable using a wide range of conditions, from 1% TFA (methoxytrityl), 95% TFA (trityl), triflic acid (*tert*-butyl), transition metal (acetamido), and reduction with phosphines or thiols (*S*-*tert*-butyl). Difficulties were had on previous attempts to remove an acetamido using silver salts. Therefore, we chose the *S*-*tert*-butyl as this could be deprotected at the very end of the sequence by treatment with tributylphosphine. Starting from the intermediate in Figure 83 (**58**), Fmoc-Cys(*St*Bu) was attached to the leading end (Figure 84), the Fmoc removed, and acetylated, the ivDde was removed, the scaffold was cleaved, the DKPs closed, and the product was treated with bromoacetic anhydride (**59**), with a selective reaction facilitated by the presence of the Cbz groups in blue in Figure 84. Purification gave the desired product, which was dissolved in 20%ACN/water and treated with 10mg/mL *tris*-carboxyethyl phosphine (TCEP), a water soluble phosphine reducing agent. Exposure to this reagent failed to remove the *S*-*tert*-butyl disulfide protecting group, even after a few days time.

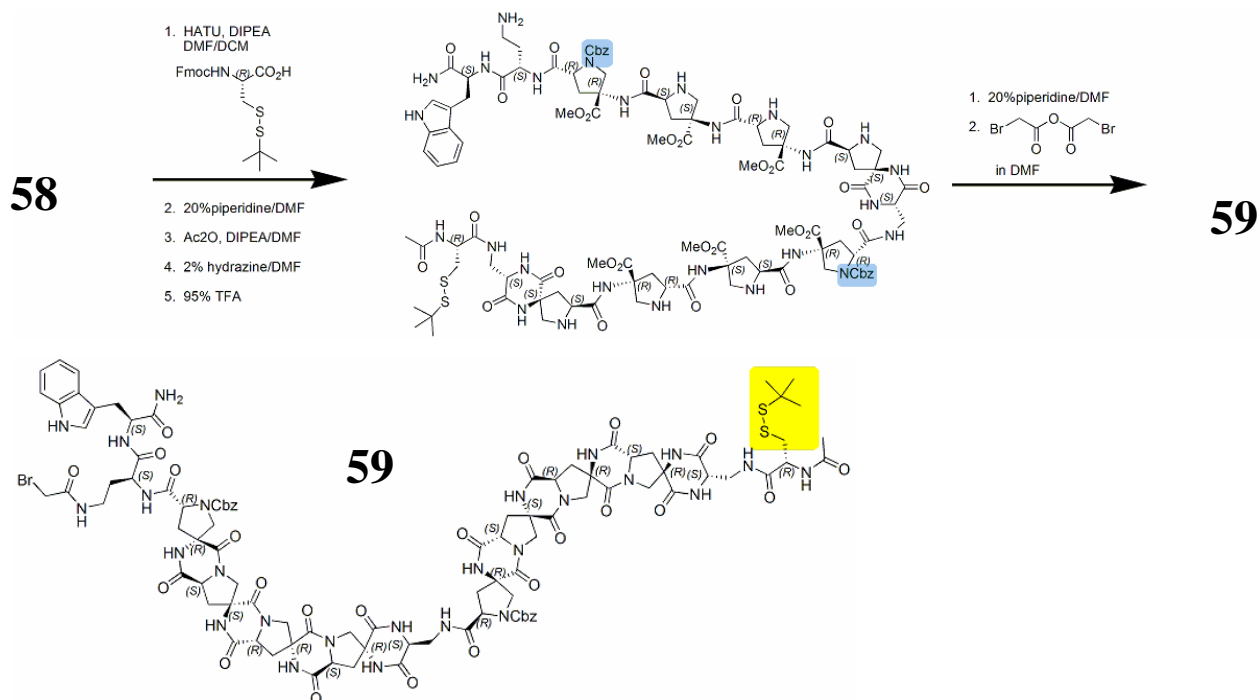


Figure 83 The desired thioether substrate was formed, but the protecting group (yellow) could not be removed.

At this point, a paper documenting an on-resin, head-to-tail cyclodimerization utilizing the Huisgen cyclization between an alkyne and an azide was found.[57] Finn and coworkers synthesized a C-terminal propargyl glycine followed by a >10 residue peptide, culminating with azidovaleric acid. In the presence of copper(I) and base, the peptide was cleanly cyclodimerized. No starting material or cyclomonomer were found. Modifying the resin bound intermediate **58** with propargyl glycine and azidovaleric acid[58] (**61**) (Figure 84) produced a substrate similar to that of Finn, although the locations of the azide and alkyne were reversed. Subjecting this azido-alkyne containing scaffold to the conditions used by Finn produced a new peak **60** with the same mass, as would be expected.

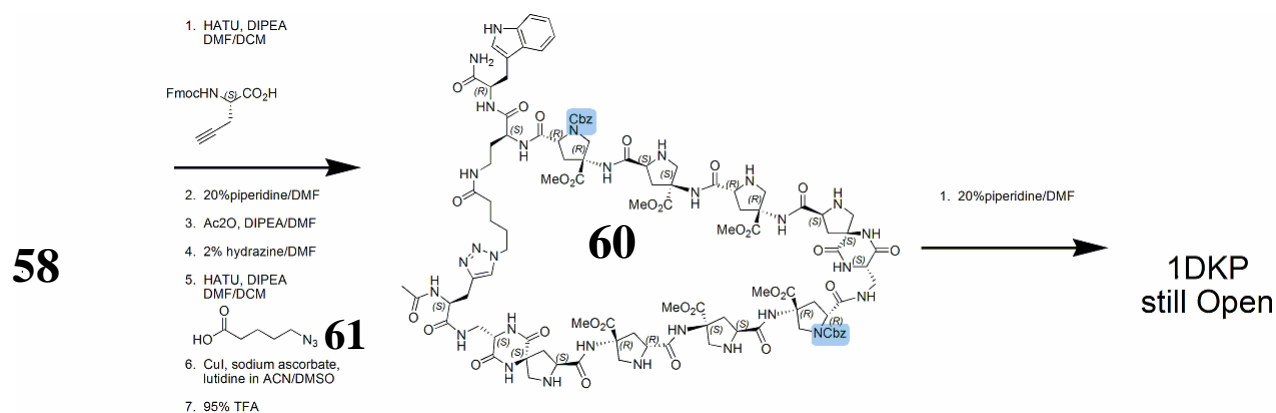


Figure 84 Synthesis of Huisgen macrocycle.

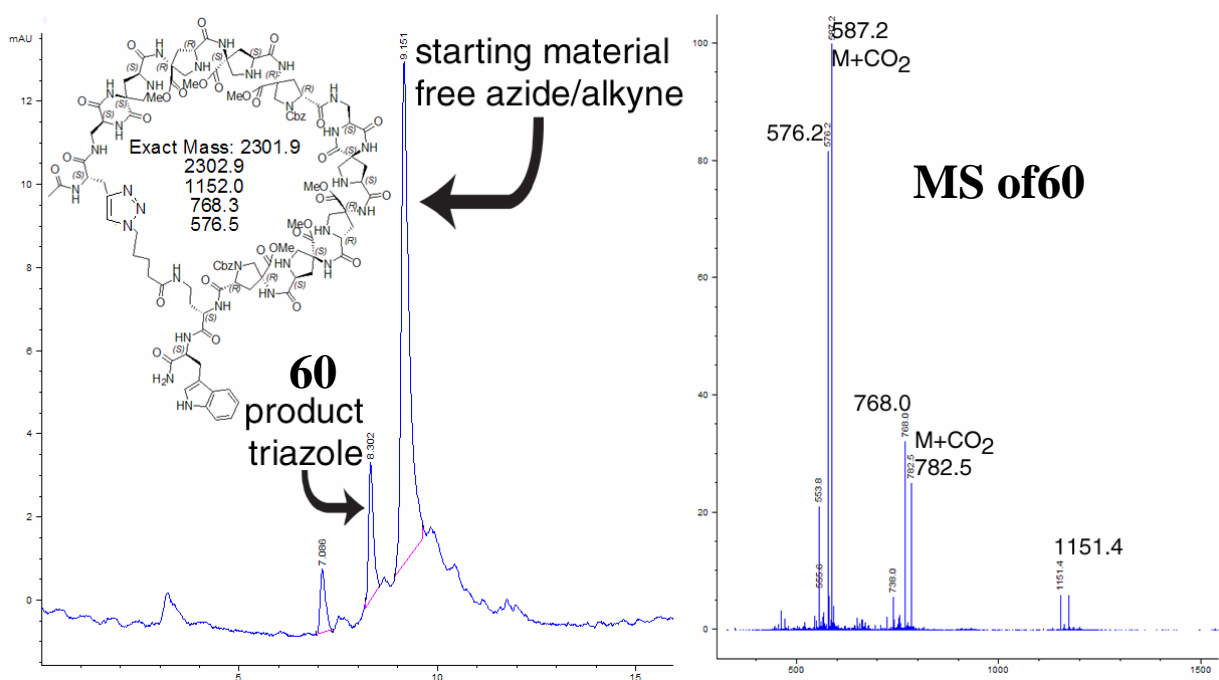


Figure 85 First attempt at Huisgens cyclization, producing cyclized product in low overall yield.

The two peaks were purified using preparative HPLC. The low-res MS (Figure 85) of both peaks after purification look similar, with the noticeable absence of charge states indicative of a cyclodimer, such as $(2M+3)/3$ or $(2M+5)/5$, where M = mass of the monomer. Additionally, close inspection of the $(M+1)/1$ reveals an isotope pattern differing by integer mass units, not $\frac{1}{2}$ as would be expected for a dimer. Both isolated peaks were subjected to typical DKP closing conditions. The starting material peak closed in 36hrs, while the product triazole peak appeared to stall with 1 open DKP. The interpretation was complicated by the Cbz groups (shown in blue in Figure 84), which masked the amines and reducing the MS signal. Removal of the Cbz groups with triflic acid, after cyclization, produced triflate salts that also complicated interpretation. The purpose of the Cbz groups was to allow a selective reaction in solution with bromoacetic anhydride in the sequence of forming a thioether, which was not a requirement for the Huisgens cyclization. The thioether would be shelved and this copper catalyzed cyclization further explored.

Free of the need to use Cbz groups, the next proposed macrocycle would contain an extra “arm” that could fold on top of the macrocycle to shield the pocket (Figure 86). A Dab(ivDde) would serve as a future attachment point for the PEG-linked biotin. The synthesis of this shielded macrocycle started with Dab, then propargyl glycine and Dpr(Mtt). The UV tag, nitrophenylalanine, was attached after a series of three alternating building blocks. The Mtt was then removed and an additional four alternating building blocks were added. Another Dpr(Mtt) followed by azidovaleric acid (**61**) completed the synthesis (**62**).

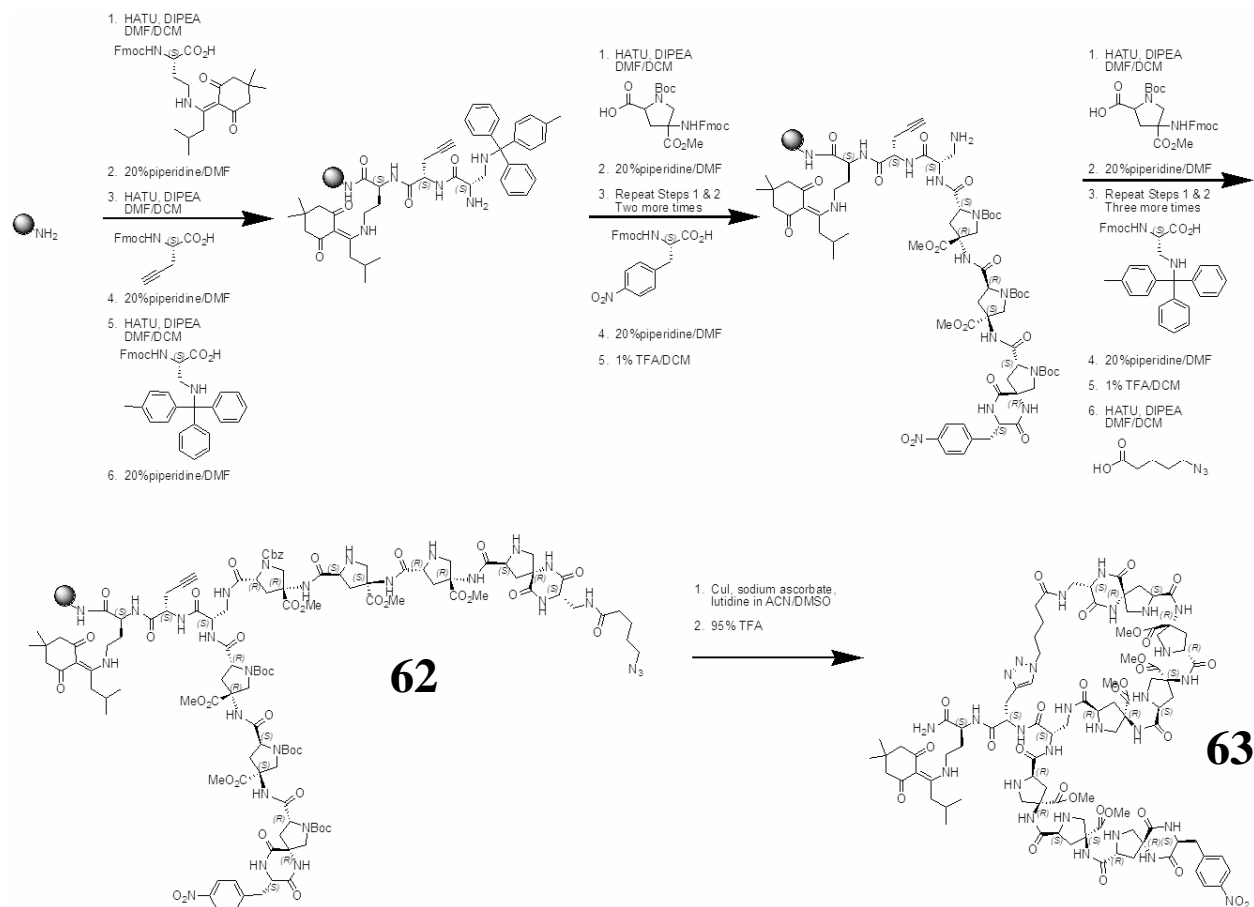


Figure 86 Synthesis outline of a "shielded" macrocycle.

This molecule **62** was subjected to the on-resin copper(I) cyclization conditions, followed by cleavage to produce the desired product (**63**) as the major peak, as seen in Figure 87. Again, the mass spectrum was consistent with a cyclomonomer. Treatment of the product with phosphines for a few days gave no change in the chromatogram, suggesting the absence of a free azide.

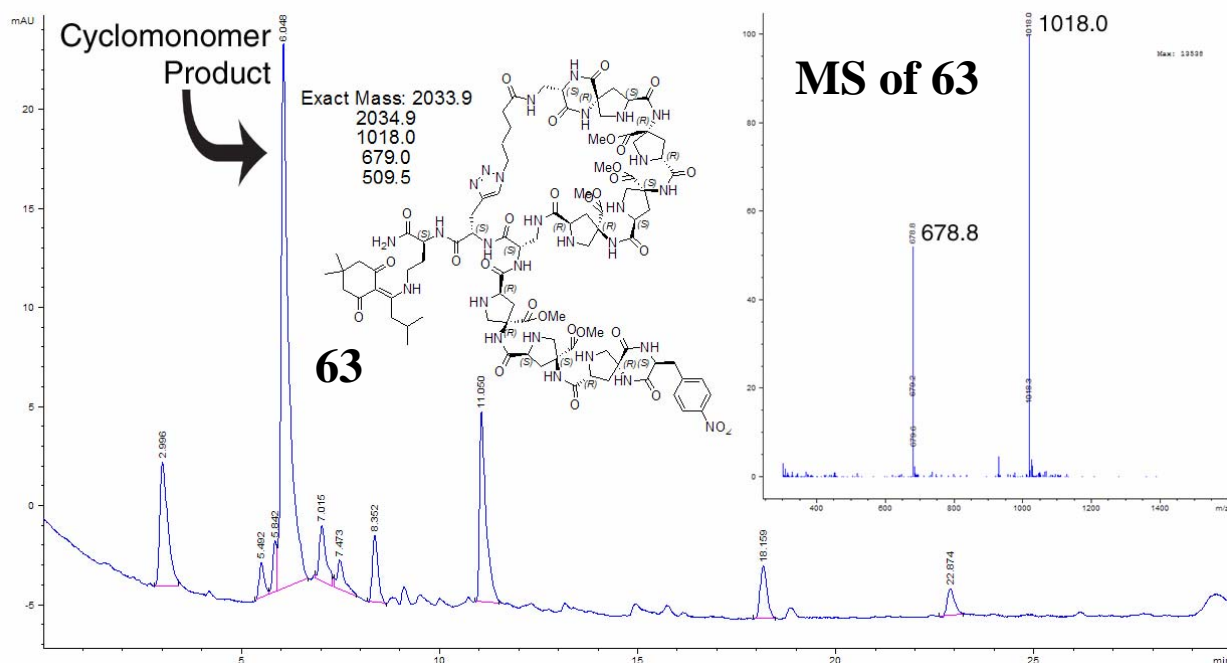
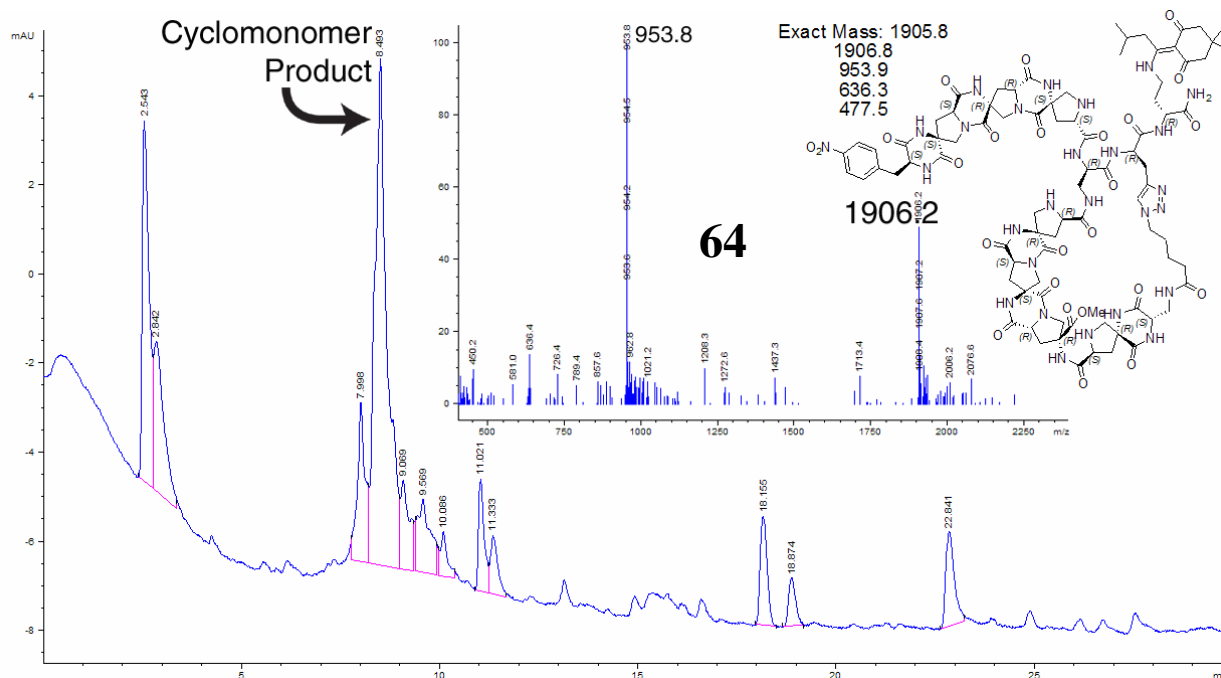


Figure 87 Shielded macrocycle (major peak), cleavage product.

The crude cleavage product **63** was subjected to 20% piperidine/NMP for 36hrs to produce **64**. This time there was no ambiguity in this cyclization result, as the final product still contained one open DKP (Figure 88). There was a total absence of fully closed product, even after an additional two days. This result along with the lack of a reaction with phosphines imply that the copper catalyzed cyclization was clean and efficient, however the resulting ring was too strained to be able to close all of the DKPs. The consensus now is that the DKPs of a cyclomonomer are difficult to close fully, perhaps because of strain or sterics. The solution is to close the DKPs first, and then subject that product to macrocyclization conditions.



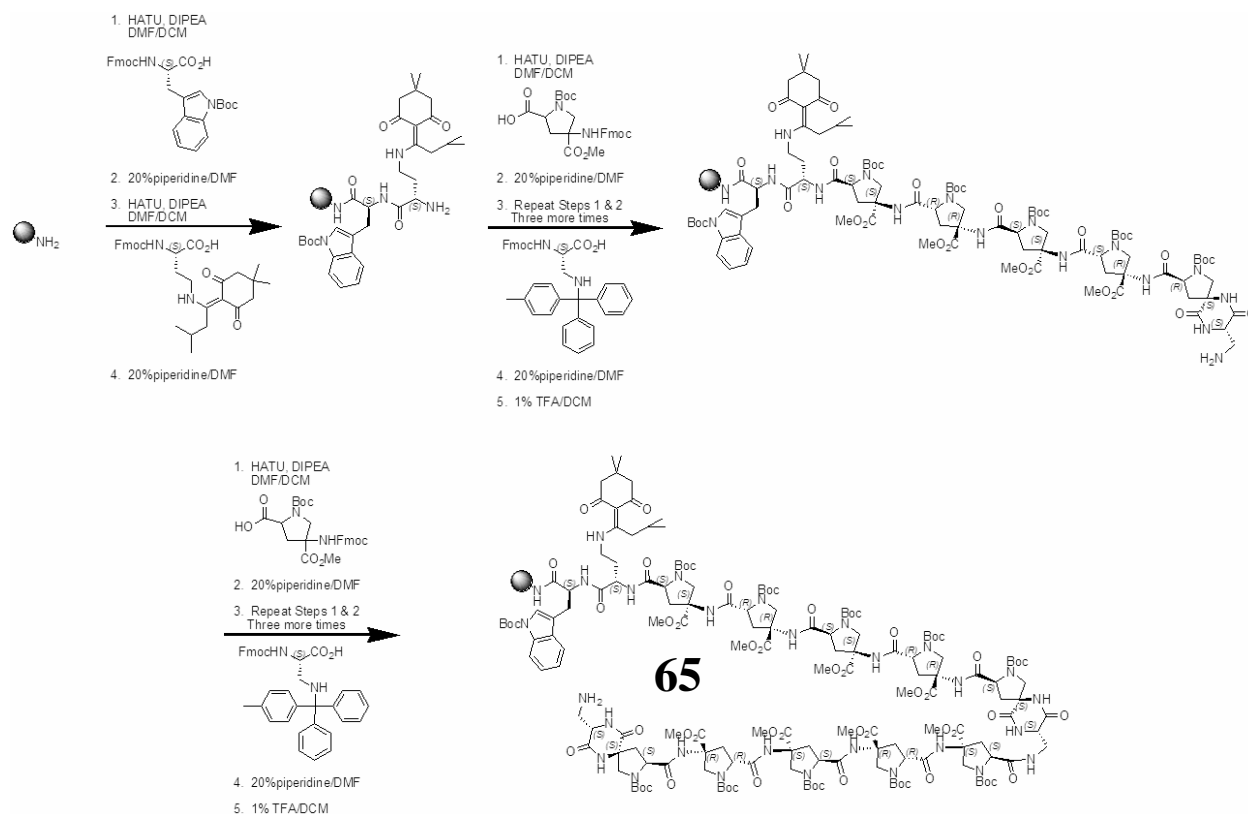


Figure 89 Versatile intermediate used for subsequent cyclization tests

The intermediate was readied for cyclization by the following steps (Figure 90). First, the Fmoc-amino acid was attached to the leading-end amine. Next, the Boc groups were removed using trimethylsilyl triflate (TMSOTf) and 2,6-lutidine in DCM.[42] The DKPs were closed in 12hrs at 37°C. At this point, the 3 remaining amines, and also the Trp indole needed to be protected or masked. Initial trials were done by acetylation, however, this lead to difficulties in MS interpretation. A superior solution was to re-protects the amine with Boc-anhydride and base. The ivDde was then removed and the carboxylic acid cyclization partner was attached to form **66**. Cyclization conditions followed by cleavage yielded the final product which was analyzed by LCMS.

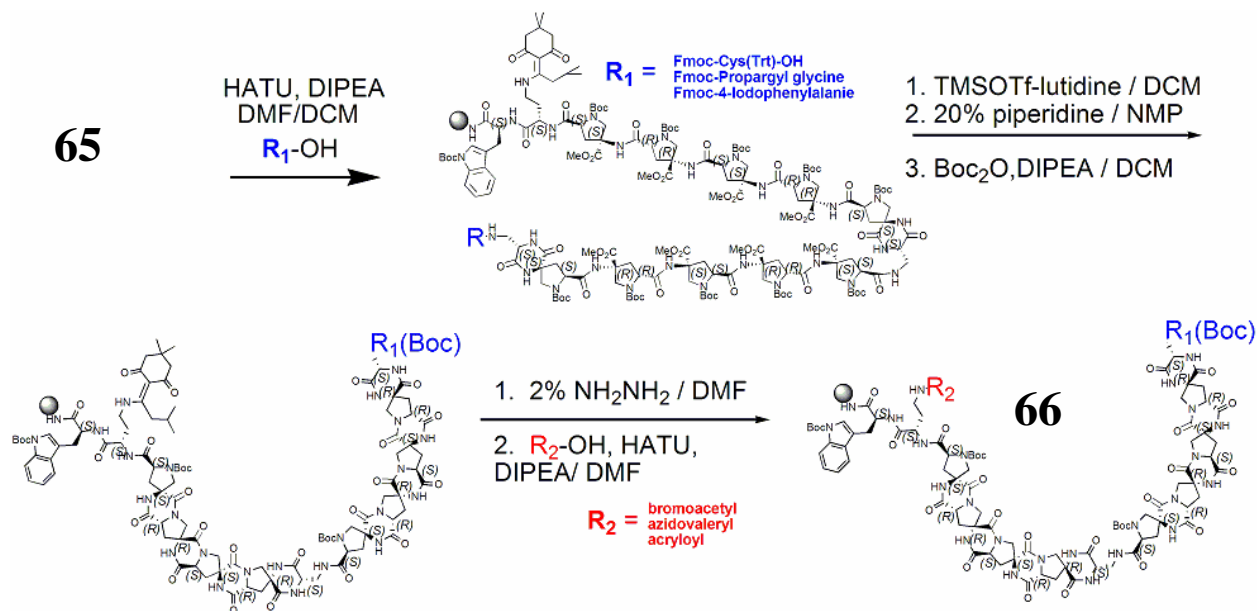


Figure 90 General synthetic scheme for the synthesis of the cyclization substrate.

For the synthesis of the thioether macrocycle ($R_2 =$ bromoacetyl), the cysteine derivative Fmoc-Cys(Trt)-OH (R_1) was chosen because this was the only protecting group that had been successfully and reliably deprotected in the past. The final Cys protecting group was actually unknown, as it was uncertain whether the Boc-deprotection conditions would also remove the ~50% TFA cleavable Trt group. If it was removed it would surely be re-protected with a Boc group, which would also be labile to the 95% TFA final cleavage solution.[59] Either way, the final DKP closed product would contain a bromoacetyl and a free thiol, all ready for the thioether formation. **66** was cleaved from the resin to produce **67** (Figure 91).

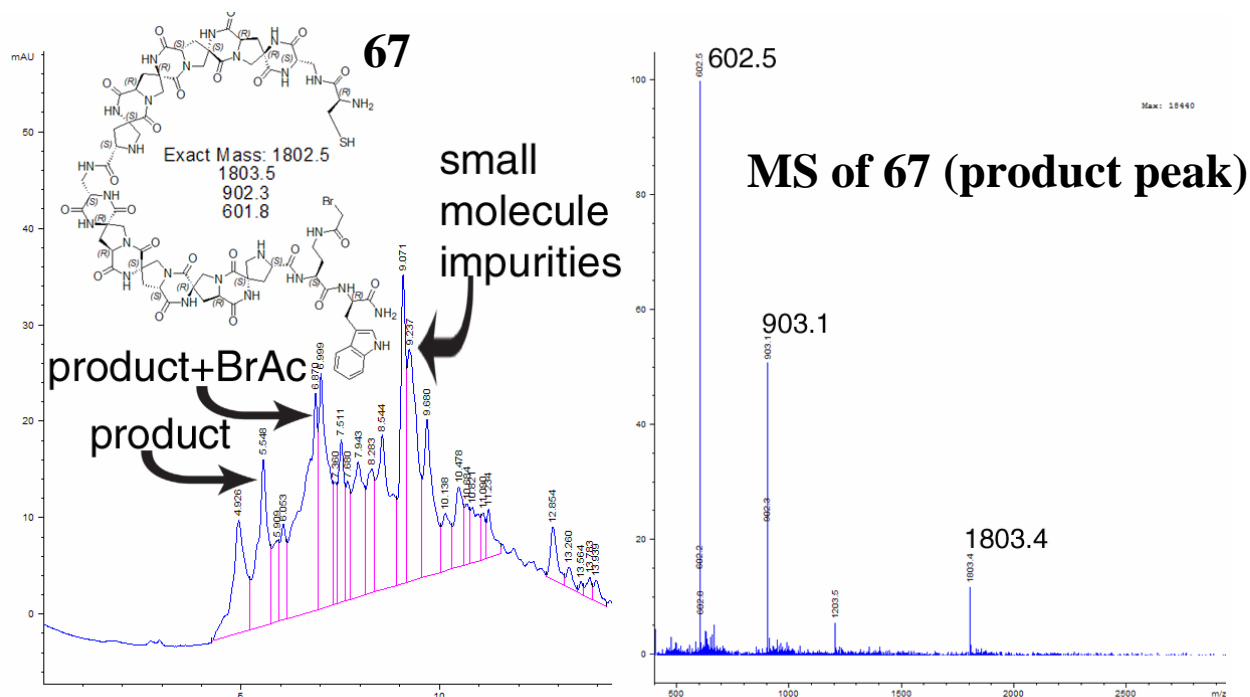


Figure 91 Successful synthesis of a thioether substrate (the second peak).

The desired product was synthesized, containing the free thiol and bromoacetyl. Additional peaks were identified corresponding to incorporation of an extra bromoacetyl. This likely was due to incomplete Boc protection. This could be remedied by washing the beads down that may have stuck to the sides of the reactor during the protection, followed by another Boc protection. Incubation of the crude substrate in 10%ACN/water for 1 week resulted in no formation of thioether macrocycle. Bray and coworkers were able to cyclize a 5-residue peptide using acidic or basic conditions in a variety of solvents.[55] It would appear from the literature that the formation of a thioether is robust, however we cannot explain why thioether formation does not occur in our molecule.

Synthesis of the Huisgens substrate proceeded as described in Figure 90 by coupling Fmoc-propargyl glycine (R_1 in Figure 90) and azidovaleric acid (R_2 in Figure 90). Subjecting this to the copper(I) cyclization conditions similar to that used in Figure 86 should have afforded the desired macrocycle upon cleavage. However, interpretation of this result was complicated by the fact the cleavage product before and after alleged cyclization had identical retention times. Treatment of the expected product with phosphines resulted in no change in mass or retention

time, however this is a negative result and we can not be certain about the cyclization. The best that can be said about the Huisgens cyclization is that further testing and optimization is needed.

An alternative cyclization procedure involving the palladium catalyzed Heck reaction between an olefin and aromatic iodide was found in the literature, and seemed robust and high-yielding.[60] Preparation of the cyclization substrate was described in Figure 90. Treatment with 0.4eq Pd(OAc)₂, PPh₃, and Bu₄NCl in DMF:water:Et₃N (20:2:1) for 12hrs at room temperature, followed by cleavage yielded the final cyclized product **68** (Figure 92, 93).

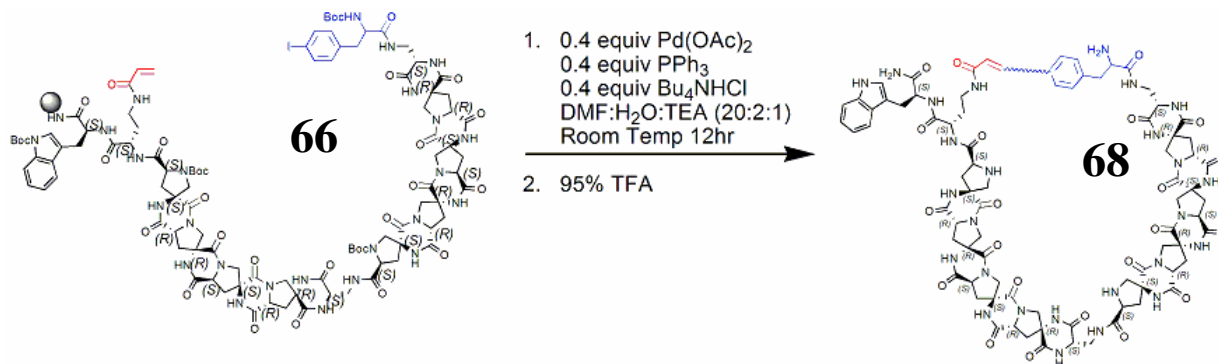


Figure 92 Heck macrocyclization, followed by cleavage.

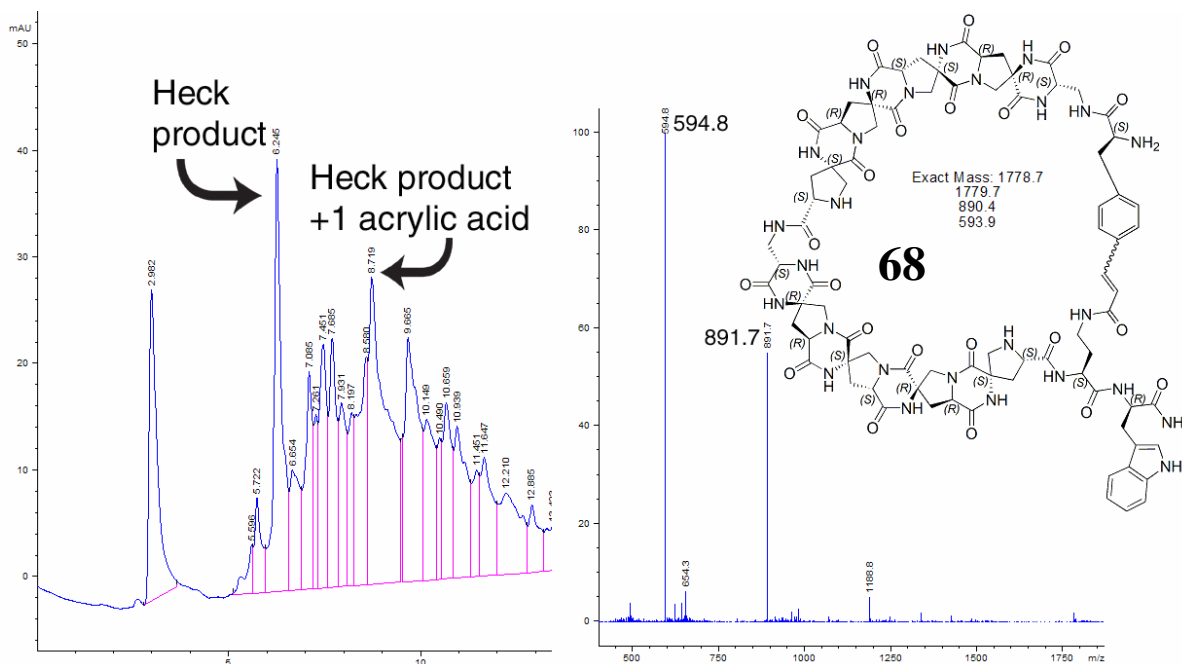


Figure 93 LCMS confirmation of a successful Heck macrocyclization.

This reaction went to >95% completion based on absence of aryl iodide substrate, and resulted in >40:1 ratio of two isomeric olefins. Estimates of the combined yield of all steps

involved in the synthesis of the cyclized scaffold, based on an HPLC calibration curve, appear to be typical (~10%). Again, the major impurities were cyclized products with multiple acrylic acids, derived from incomplete Boc re-protection, and could easily be prevented in the future. It should be noted that the Heck reaction worked the first time, and three subsequent times, all going to completion in less than 12hr at room temperature. Also, the Heck reaction was tolerant to water and was run open to the atmosphere, proving to be a particularly easy reaction with no optimization. The Heck reaction has been successfully implemented for making cyclic scaffolds, having all DKPs closed, and warrants further development as a promising tool for making molecules that can act as receptors, ligands, or sensors.

3.3 CONCLUSIONS

Although the search for a peptide that binds to a synthetic receptor has been unsuccessful thus far, there is a great deal of promise for the future of this project. The techniques and methods of phage display have proven to be easy to master, as judged by the successful selection of the HPQ phage for streptavidin. We believe that the first two attempts at phage display failed because the receptors were too flexible or floppy. Because of the inadequacies of the first generations of scaffold receptors, conditions for making large cyclic scaffolds have been explored, and the Heck reaction has been identified as an excellent way to macrocyclize a rigidified scaffold, having all DKPs closed. Large cyclic scaffolds capable of folding and forming a pocket should now be possible, so the potential exists that a specific peptide could be recognized by this scaffold receptor, thus propelling the field of artificial antibodies forward.

3.4 EXPERIMENTAL

General: Dichloromethane was distilled from CaH_2 . All other reagents were used as received, unless stated otherwise. Column chromatography was performed using ICN Silitech 32-63 D (60 Å) grade silica gel and TLC analysis was performed on EM Science Silica Gel 60 F₂₅₄ plates

(250 μ m thickness). NMR spectroscopy was performed using a Bruker 300 MHz instruments. Chemical shifts were reported in parts per million downfield relative to trimethylsilane and categorized as br = broad, s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, q = quartet, and m = multiplet. Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. N,N-diisopropylethylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and N-Hydroxybenzotriazole hydrate (HOBt) were obtained from Novabiochem. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and an inert atmosphere over the reaction. HPLC-MS analysis was performed on a Hewlett-Packard Series 1050 instrument equipped with a Waters Xterra MS C₁₈ column (3.5 μ m packing, 4.6 mm x 150 mm) and a diode-array detector, while the MSD-ESI was Series 1100. Preparative purification was done using a Varian Prostar 500 equipped with a Waters Xterra Prep MS-C₁₈ column (5 μ m packing, 10 mm x 100 mm), equipped with a dual wavelength detector.

Solid phase synthesis of receptor Version 1 (SS,RR,SS,RR,SS,RR)₂ (52).

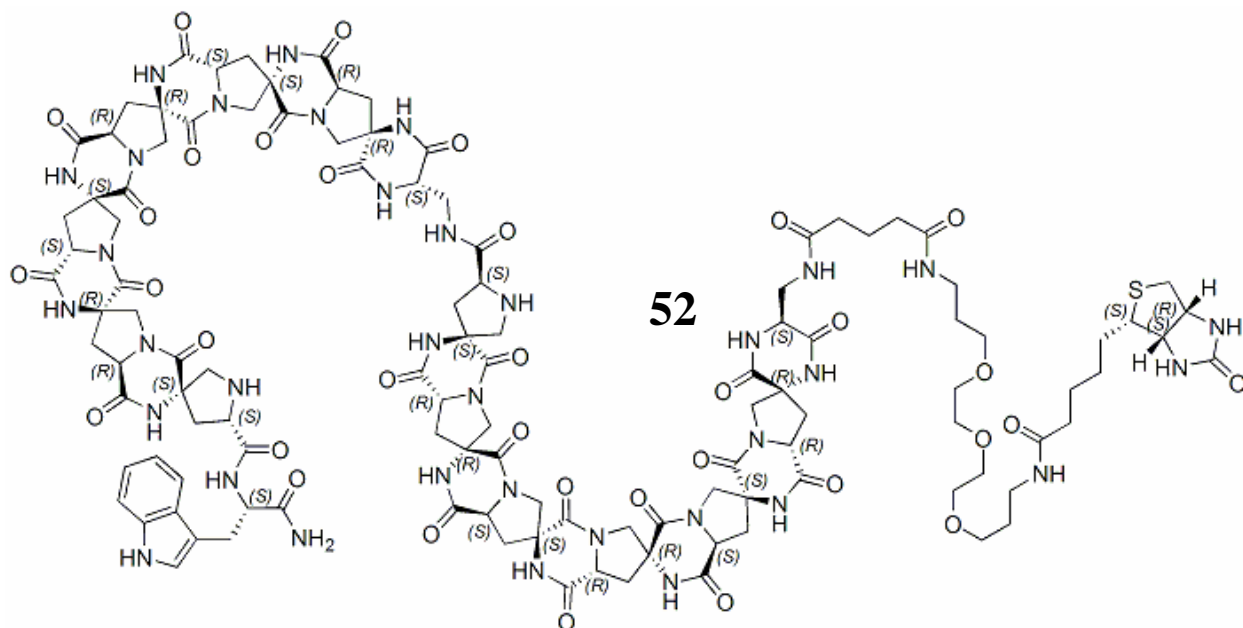


Figure 94 Biotin labeled receptor, containing two 6-building block sections

To a 50mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (40mg, 25.2 μ mol loading). The resin was swollen for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 2mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 66.4mg (126 μ mol) of Fmoc-L-Trp(Boc)-OH (Novabiochem) and 47.9mg of HATU (126 μ mol) in 630 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 43.9 μ L (252 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 25.7mg (50.4 μ mol) of Boc-protected pro4(2S4S) building block[17] and 19.2mg of HATU (50.4 μ mol) in 252 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 17.6 μ L (100.8 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. This process of coupling/deprotection was

repeated five additional times, by alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Trp-SS-RR-SS-RR-SS-RR-free amine.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 73.4mg (126 μ mol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 47.9mg of HATU (126 μ mol) in 630 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 21.9 μ L (252 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

The second arm of the receptor was made in same manner as the first, consisting of alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Trp-SS-RR-SS-RR-SS-RR-Dpr-SS-RR-SS-RR-SS-RR-free amine, followed by coupling of Fmoc-Dpr(Mtt)-OH, removal of Fmoc for 2hr, removal of Mtt with 1% TFA, and neutralization with 5% DIPEA, as above.

The resin was then portioned into halves. Half was prepared for cleavage and half was acylated with biotin. The resin was swelled in DMF for 30min. In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 35.3mg (63.0 μ mol) of N-Biotinyl-NH-(PEG)₂-COOH (Novabiochem) and 24.0mg of HATU (63.0 μ mol) in 315 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μ L (126 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. Both halves of the resin were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds, with and without biotin, were cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and

the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

The diketopiperazines were closed by incubation for 48hr in 0.5mL of 20% piperidine in NMP. In this case, it was concluded by LCMS that the product was not fully closed, so the reaction was incubated at 60°C for 3 days. Then, the product was dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour, before being dissolved in 25% ACN in water with 0.5% formic acid and analyzed by LCMS.

Solid phase synthesis of receptor Version 2 (SS,RR,SS,RR,SS)-(SS,RR,SS,RR,SS) (54) or (SS,RR,SS,RR,SS)-(RR,SS,RR,SS,RR) (55).

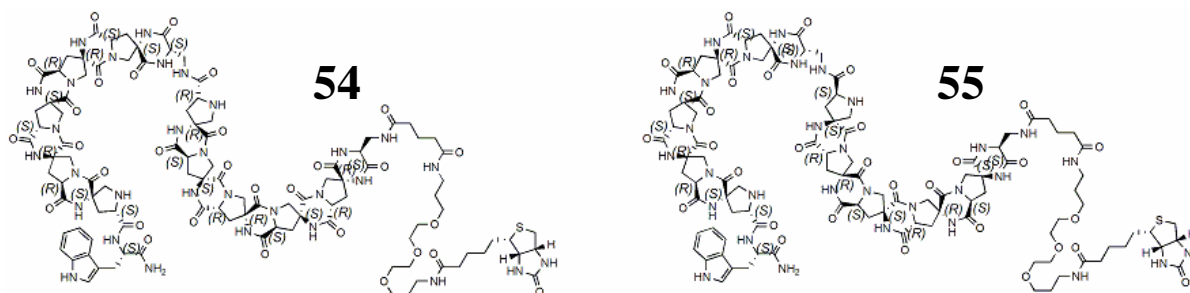


Figure 95 Biotin labeled receptor, containing two 5-building block sections, with different stereochemistries.

To a 50mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (80mg, 50.4 μ mol loading). The resin was swollen for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 4mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 132.8mg (252 μ mol) of Fmoc-L-Trp(Boc)-OH (Novabiochem) and 95.8mg of HATU (252 μ mol) in 1260 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 87.8 μ L (504 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 51.4mg (100.8 μ mol) of Boc-protected pro4(2S4S) building block and 38.4mg of HATU (100.8 μ mol) in 504 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 35.1 μ L (201.6 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. This process of coupling/deprotection was repeated four additional times, by alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Trp-SS-RR-SS-RR-SS- free amine.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 146.8mg (252 μ mol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 95.8mg of HATU (252 μ mol) in 1260 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 87.8 μ L (504 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

The resin was split into halves. The difference between the two was whether the 6th building block was Boc-protected pro4(2S4S) or Boc-protected pro4(2R4R). This produced resin-Trp-SS-RR-SS-RR-SS-Dpr-SS-RR-SS-RR-SS-free amine or resin-Trp-SS-RR-SS-RR-SS-Dpr-RR-SS-RR-SS-RR-free amine, followed by coupling of Fmoc-Dpr(Mtt)-OH, removal of Fmoc for 2hr, removal of Mtt with 1% TFA, and neutralization with 5% DIPEA, as above.

Each resin was then again portioned into halves. Half was prepared for cleavage and half was acylated with biotin. The resin was swelled in DMF for 30min. In 2 x 1.5 mL microcentrifuge tube (for each of the two receptors), the coupling solution was made by dissolving 35.3mg (63.0 μ mol) of N-Biotinyl-NH-(PEG)₂-COOH (Novabiochem) and 24.0mg of HATU (63.0 μ mol) in 315 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μ L (126 μ mol) of DIPEA was added to make the active ester, as

above, followed by washing, a second coupling, washing, and Fmoc release. Both halves of the resin were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds, with and without biotin, were cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

The diketopiperazines were closed by incubation for 36hr in 0.5mL of 20% piperidine in NMP. Then, the product was dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour, before being dissolved in 25% ACN in water with 0.5% formic acid. The final products (with and without biotin) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. Fractions containing product were pooled, lyophilized, and resuspended in *tris*-buffered saline (TBS). From the absorbance of the Trp, the concentration was calculated ($\epsilon = 5600 \text{ M}^{-1} \text{ cm}^{-1}$). The stock solutions were stored at 4°C.

Solid phase synthesis of receptor Version 3 (SS,RR,SS,RR,SS)-X-(SS,RR,SS,RR,SS), X=Orn (56) or Lys (57).

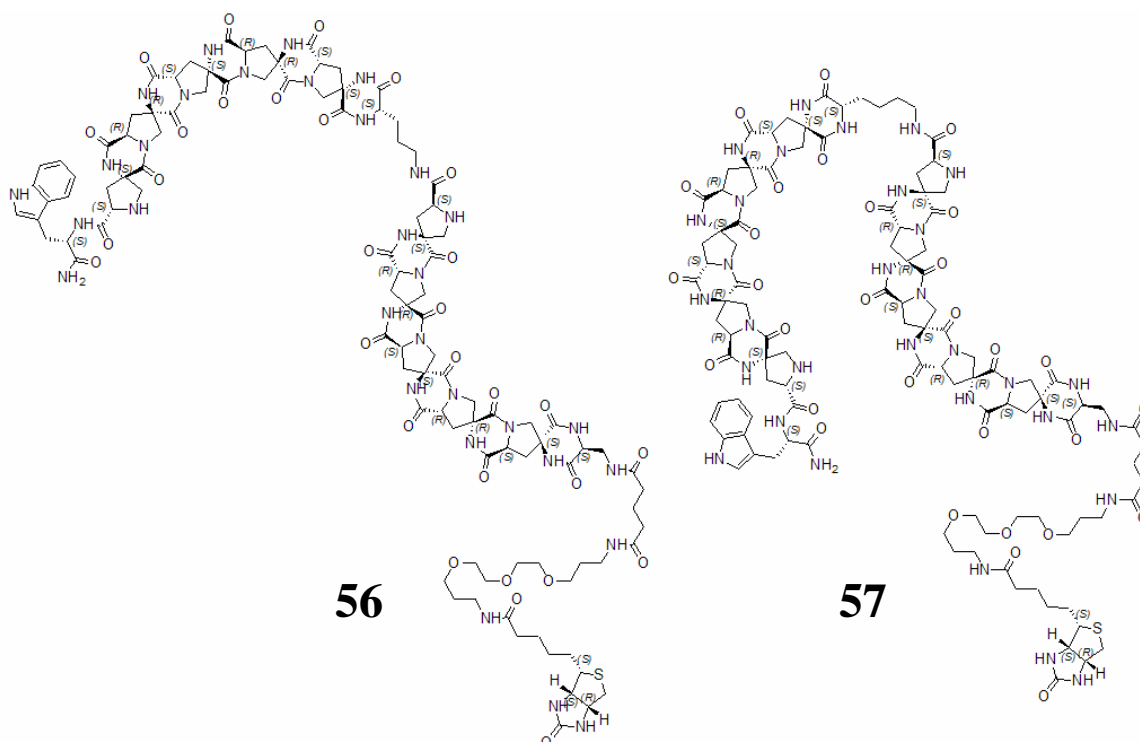


Figure 96 Biotin labeled receptor, containing two 5-building block sections, with Orn or Lys spacer.

To a 50mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (80mg, 50.4 μ mol loading). The resin was swollen for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 4mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 132.8mg (252 μ mol) of Fmoc-L-Trp(Boc)-OH (Novabiochem) and 95.8mg of HATU (252 μ mol) in 1260 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 87.8 μ L (504 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 51.4mg (100.8 μ mol) of Boc-protected pro4(2S4S) building block and 38.4mg of HATU (100.8 μ mol) in 504 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 35.1 μ L

(201.6 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. This process of coupling/deprotection was repeated four additional times, by alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Trp-SS-RR-SS-RR-SS- free amine.

The resin was split into halves. The difference between the two was whether the bridging amino acid was Fmoc-Orn(Mtt)-OH or Fmoc-Lys(Mtt)-OH. In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 76.9mg of Orn or 78.7mg of Lys (126 μ mol) of Fmoc-L-AA(Mtt)-OH (Novabiochem) and 47.9mg of HATU (126 μ mol) in 630 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 43.9 μ L (252 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

The two resins were then elongated with five alternating building blocks. This produced resin-Trp-SS-RR-SS-RR-SS-Orn-SS-RR-SS-RR-SS-free amine or resin-Trp-SS-RR-SS-RR-SS-Lys-SS-RR-SS-RR-SS-free amine, followed by coupling of Fmoc-Dpr(Mtt)-OH, removal of Fmoc for 2hr, removal of Mtt with 1% TFA, and neutralization with 5% DIPEA, similar to above.

Each resin was then again portioned into halves. Half was prepared for cleavage and half was acylated with biotin. The resin was swelled in DMF for 30min. In 2 x 1.5 mL microcentrifuge tube (for each of the two receptors), the coupling solution was made by dissolving 35.3mg (63.0 μ mol) of N-Biotinyl-NH-(PEG)₂-COOH (Novabiochem) and 24.0mg of HATU (63.0 μ mol) in 315 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μ L (126 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. Both halves of the

resin were extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffolds, with and without biotin, were cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

The diketopiperazines were closed by incubation for 36hr in 0.5mL of 20% piperidine in NMP. Then, the product was dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour, before being dissolved in 25% ACN in water with 0.5% formic acid. The final products (with and without biotin) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. Fractions containing product were pooled, lyophilized, and resuspended in *tris*-buffered saline (TBS). From the absorbance of the Trp, the concentration was calculated ($\epsilon = 5600 \text{ M}^{-1} \text{ cm}^{-1}$). The stock solutions were stored at 4°C.

Solid phase synthesis of receptor Version 4, macrocyclization via thioether or Huisgens cyclization (58).

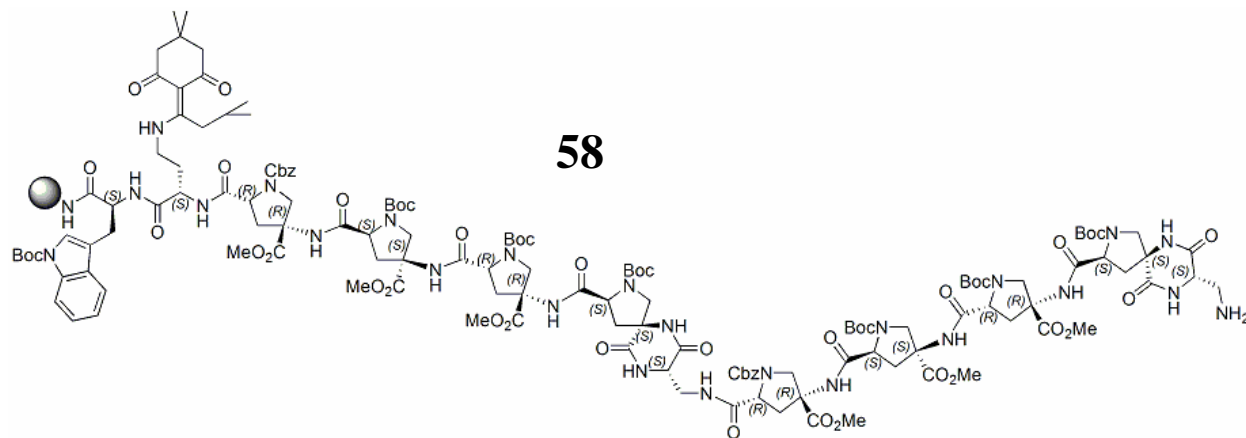


Figure 97 Common intermediate for macrocycle synthesis

To a 10mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (20mg, 12.6 μ mol loading). The resin was swollen for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20%

piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 33.2mg (63 μ mol) of Fmoc-L-Trp(Boc)-OH (Novabiochem) and 24.0mg of HATU (63 μ mol) in 315 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 21.9 μ L (126 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 34.4mg (63 μ mol) of Fmoc-L-Dab(ivDde)-OH (Novabiochem) and 24.0mg of HATU (63 μ mol) in 315 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 21.9 μ L (126 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 13.2mg (25.2 μ mol) of Cbz-protected pro4(2R4R) building block and 9.6mg of HATU (25.2 μ mol) in 126 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 8.8 μ L (50.4 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. This process of coupling/deprotection was repeated three additional times, by alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Trp-RR(Cbz)-SS-RR-SS- free amine.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 18.4mg (31.5 μ mol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 12.0mg of HATU (31.5 μ mol) in 160 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 5.5 μ L (63.0 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

The resin was then elongated with five alternating building blocks, as described above. This produced resin-Trp-Dab-RR(Cbz)-SS-RR-SS-Dpr-RR(Cbz)-SS-RR-SS-free amine, followed by coupling of Fmoc-Dpr(Mtt)-OH, removal of Fmoc for 2hr, removal of Mtt with 1% TFA, and neutralization with 5% DIPEA, similar to above.

The resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*, and then stored in the desiccator.

Synthesis of thioether macrocycles (68).

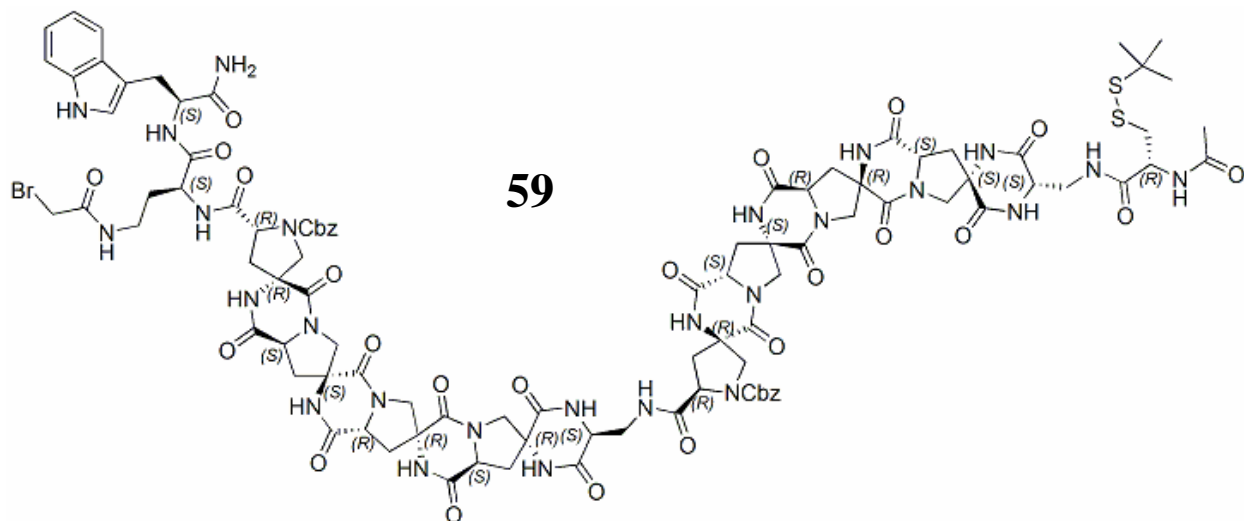


Figure 98 Bromoacetylated scaffold, with the cysteine still protected.

A few (~3mg) beads were placed in a 1mL polypropylene solid phase peptide synthesis reaction vessel. After being swelled in DMF for 30min, a solution of activated ester, consisting of estimated 20eq Fmoc-Cys(StBu)-OH (Novabiochem), 20eq HATU, 40eq DIPEA at 0.2M in DMF, was added and allowed to react for. The beads were washed 3 x 2min with DMF, and the coupling was repeated. The Fmoc was removed, and the free amine was capped with acetic anhydride: DMF: DIPEA (100:400:20). The Dab side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-

5 minutes was sufficient to completely unmask the side chain. The resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

The diketopiperazines were closed by incubation for 36hr in 0.5mL of 20% piperidine in NMP. Then, the product was dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour, before being dissolved in 25% ACN in water with 0.5% formic acid and analyzed by LCMS.

The bromoacetyl group was attached to the only free amine by dissolving 10mg bromoacetic anhydride (Aldrich) in 200 μ L DMF, and adding to the ether precipitated pellet. The reaction was stirred for 30min, and precipitated into ether as described above. The dried pellet was dissolved in 25% ACN in water, 0.5% formic acid and analyzed by LCMS.

Synthesis of Huisgens macrocycle (60).

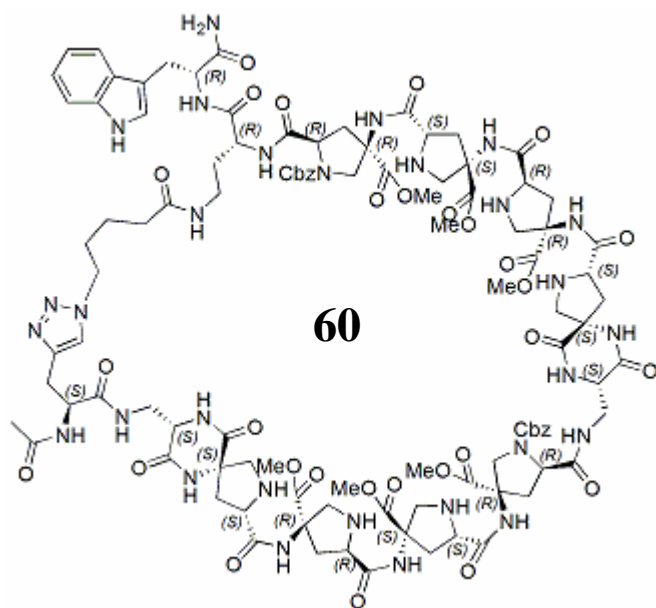


Figure 99 Macrocycle via Huisgens dipolar cycloaddition reaction.

methyl 5-azidovalerate[58] (Caution—low molecular weight azides are potentially EXPLOSIVE)

To a stirred solution of 1.15g (6.0mmol) of methyl 5-bromovalerate (Aldrich) in 20mL of DMF in a 50mL round bottom, 430mg (6.6mmol) of sodium azide was added, and the solution was stirred under dry nitrogen overnight. The solution was then poured into 200mL of water, and extracted with 3 x 100mL EtOAc. The organic layers were pooled, washed with 2 x 50mL brine, and dried over sodium sulfate. The solvent was removed in vacuo, and dried in vacuo for 2hrs (product *is* volatile). The resulting product (720mg, 76% yield) was a colorless oil and was used without further purification.

5-azidovaleric acid (61). [58]

The substrate (700mg, 3.7mmol) was dissolved in 5mL MeOH and 5mL of 0.5M LiOH was added, and the reaction was stirred for 4hrs. The reaction was quenched with 3mL of 1M HCl, and extracted with 3 x 50mL EtOAc. The pooled organic layers were washed with 2 x 20mL brine and dried over sodium sulfate. The solvent was removed in vacuo to yield 610mg product (94%) as a colorless oil. The product was dissolved in 5mL dry dichloromethane and stored like this to minimize explosive conditions. ¹H NMR (300MHz, 25°C, CDCl₃) δ 11.2 (br, 1H), 3.28 (t, 2H), 2.38 (t, 2H), 1.65 (m, 4H).

A few (~3mg) beads were placed in a 1mL polypropylene solid phase peptide synthesis reaction vessel. After being swelled in DMF for 30min, a solution of activated ester, consisting of estimated 20eq Fmoc-L-propargyl glycine-OH (Fluka), 20eq HATU, 40eq DIPEA at 0.2M in DMF, was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated. The Fmoc was removed, and the free amine was capped with acetic anhydride: DMF: DIPEA (100:400:20) for 10min. The Dab side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the side chain. A solution of ~8eq 5-azidovaleric acid, 8eq HATU, 16eq DIPEA at 0.2M in DMF, was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated.

The on-resin macrocyclization conditions were similar to those used by Finn for an on-resin head-to-tail, cyclodimerization.[57] Solutions of 4.8mg CuI (Avacodo) in 2.5mL of 20%

DMSO in ACN and 12.5mg of sodium ascorbate (Aldrich) in 0.25mL water were prepared, as well as 12 μ L of 2,6-lutidine. A volume fraction of each, based on the number of moles of Fmoc released from the propargyl glycine (Ppg), divided by the number of moles used in the paper (50 μ mol), was used to compute the volumes of CuI, NaAsc, and lutidine that were needed. The CuI was added first, followed by the lutidine and finally the NaAsc. The reaction and beads were stirred for 16hrs at room temperature. Following this, the beads were washed extensively with acetonitrile and DMF, then water and 0.1M EDTA, and acetonitrile and DMF again, repeated at least three times. Then, the resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

The diketopiperazines were closed by incubation for 36hr in 0.5mL of 20% piperidine in NMP. Then, the product was dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour, before being dissolved in 25% ACN in water with 0.5% formic acid and analyzed by LCMS.

Solid phase synthesis of receptor Version 5, Huisgens macrocycle (63).

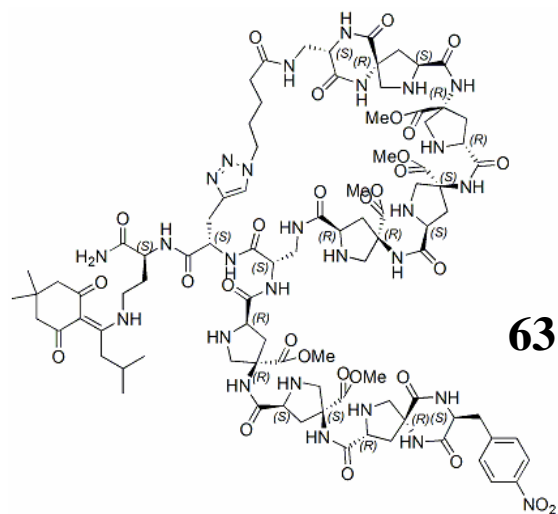


Figure 100 Macrocycle, with an additional arm, possibly capable of shielding the pocket.

To a 10mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (10mg, 6.3 μ mol loading). The resin was swollen for 1hr in DMF. The terminal Fmoc-protected amine was deprotected in 1mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 10.3mg (18.9 μ mol) of Fmoc-L-Dab(ivDde)-OH (Novabiochem) and 7.2mg of HATU (18.9 μ mol) in 100 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 6.6 μ L (37.8 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 6.3mg (18.9 μ mol) of Fmoc-L-Ppg-OH (Novabiochem) and 7.2mg of HATU (18.9 μ mol) in 100 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 6.6 μ L (37.8 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 11.0mg (18.9 μ mol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 7.2mg of HATU (18.9 μ mol) in 100 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 6.6 μ L (37.8 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 6.4mg (12.6 μ mol) of Boc-protected pro4(2R4R) building block and 4.8mg of HATU (12.6 μ mol) in 75 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 4.4 μ L (25.2 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. This process of coupling/deprotection was repeated two additional times, by alternating between Boc-protected pro4(2R4R) building block and Boc-protected pro4(2S4S) building block, yielding the sequence, resin-Dab-Ppg-Dpr-RR-SS-RR-free amine.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 16.6mg (31.5 μ mol) of Fmoc-L-4-Nitrophenylalanine-OH (Novabiochem) and 12.0mg of HATU (31.5 μ mol) in 160 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μ L (63 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 6.4mg (12.6 μ mol) of Boc-protected pro4(2S4S) building block and 4.8mg of HATU (12.6 μ mol) in 75 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 4.4 μ L (25.2 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. This process of coupling/deprotection was repeated three additional times, by alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Dab-Ppg-Dpr-(-RR-SS-RR-SR-free amine)-SS-RR-SS-NO₂Phe.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 11.0mg (18.9 μ mol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 7.2mg of HATU (12.6 μ mol) in 100 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 6.6 μ L (37.8 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely

unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 4.5mg (31.5 μ mol) of 5-azidovaleric acid and 12.0mg of HATU (31.5 μ mol) in 160 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μ L (63.0 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, and washing.

The on-resin macrocyclization conditions were similar to those used by Finn for an on-resin head-to-tail, cyclodimerization.[57] Solutions of 4.8mg CuI (Avacodo) in 2.5mL of 20% DMSO in ACN and 12.5mg of sodium ascorbate (Aldrich) in 0.25mL water were prepared, as well as 12 μ L of 2,6-lutidine. A volume fraction of each, based on the number of moles of Fmoc released from the propargyl glycine (Ppg), divided by the number of moles used in the paper (50 μ mol), was used to compute the volumes of CuI, NaAsc, and lutidine that were needed. The CuI was added first, followed by the lutidine and finally the NaAsc. The reaction and beads were stirred for 16hrs at room temperature. Following this, the beads were washed extensively with acetonitrile and DMF, then water and 0.1M EDTA, and acetonitrile and DMF again, repeated at least three times. Then, the resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

The diketopiperazines were closed by incubation for 36hr in 0.5mL of 20% piperidine in NMP. Then, the product was dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour, before being dissolved in 25% ACN in water with 0.5% formic acid and analyzed by LCMS.

Solid phase synthesis of receptor Version 6, macrocyclization via thioether, Huisgens cyclization, or Heck reaction (65).

repeated three additional times, by alternating between Boc-protected pro4(2S4S) building block and Boc-protected pro4(2R4R) building block, yielding the sequence, resin-Trp-SS-RR-SS-RR-free amine.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 31.5mg (54.0 μ mol) of Fmoc-L-Dpr(Mtt)-OH (Novabiochem) and 20.5mg of HATU (54.0 μ mol) in 270 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 18.8 μ L (108 μ mol) of DIPEA was added to make the active ester, as above, followed by washing, a second coupling, washing, and Fmoc release. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DCM with slight bubbling for 30min. The Dpr side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the side chain amine. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation.

The resin was then elongated with four alternating building blocks, as described above. This produced resin-Trp-Dab-SS-RR-SS-RR-Dpr-SS-RR-SS-RR-free amine, followed by coupling of Fmoc-Dpr(Mtt)-OH, removal of Fmoc for 2hr, removal of Mtt with 1% TFA, and neutralization with 5% DIPEA, similar to above.

The resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*, and then stored in the desiccator.

Synthesis of Macrocycle via a Heck reaction (68).

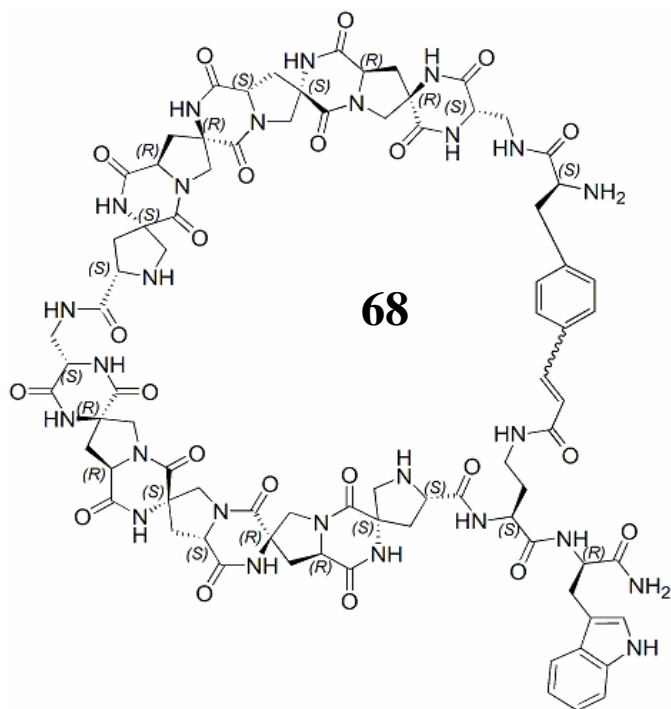


Figure 102 Macrocycle made with a Heck reaction

A few beads (~3mgs) of resin were swelled in DMF for 30min. In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 10eq of Fmoc-L-4-Iodophenylalanine-OH (Synthetech) and 10eq of HATU at 0.2M of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20eq of DIPEA was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated. The Fmoc was removed and washed with DMF, IPA, DMF, IPA, DCM, IPA, and DCM.

Because the solid support was the ordinary Rink Amide resin, the Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 5mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min, with slight bubbling, the Boc groups were removed while still attached to the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on the resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 12hrs at 35°C -40°C.

The Boc groups were then reattached to the 2 trailing end amines and N-terminus using a solution of ~10mg of Boc-anhydride in DCM with 30 μ L of DIPEA, with stirring overnight. The resin was then washed with DCM, MeOH, DCM, IPA, DMF, IPA, DMF, and DMF.

The Dab side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the side chain. A solution of ~20eq acrylic acid, 20eq HATU, 40eq DIPEA at 0.2M in DMF, was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated.

A solution of 5mg Pd(OAc)₂ (Aldrich), 3mg tetrabutylammonium chloride (Aldrich) and 6mg of triphenylphosphine (Aldrich) in 2mL of DMF: water: triethylamine (20:2:1) was prepared (all reagents are 0.4eq). The volume fraction of the Heck reagents was calculated, based on the number of moles of Fmoc released from the Iodophenylalanine (Iph), divided by the concentration, which was 36 μ L per μ mol of Fmoc released, similar to the conditions used by others.[60] The reaction was allowed to stir for 12hrs at room temperature. It was then washed extensively with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

Synthesis of Macrocycle via a thioether (67).

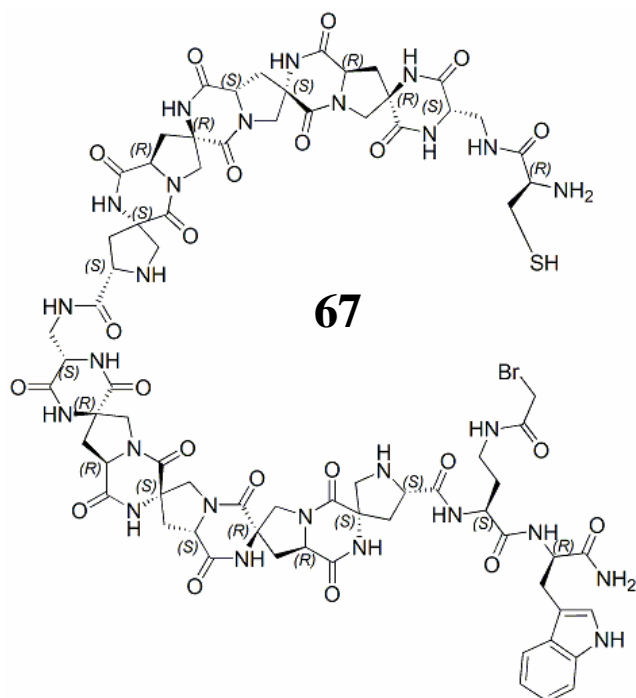


Figure 103 Thioether substrate used for attempted thioether synthesis

A few beads (~3mgs) of resin were swelled in DMF for 30min. In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 10eq of Fmoc-L-Cys(Trt)-OH (Novabiochem) and 10eq of HATU at 0.2M of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20eq of DIPEA was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated. The Fmoc was removed and washed with DMF, IPA, DMF, IPA, DCM, IPA, and DCM.

The Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 5mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf-from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min, with slight bubbling, the Boc groups were removed from the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on the resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 12hrs at 35°C -40°C.

The Boc groups were then reattached to the 2 trailing end amines and N-terminus, and, likely, the cysteine sulfur as well, using a solution of ~10mg of Boc-anhydride in DCM with

30 μ L of DIPEA, with stirring overnight. The resin was then washed with DCM, MeOH, DCM, IPA, DMF, IPA, DMF, and DMF.

The Dab side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the side chain. A solution of ~10mg bromoacetic anhydride in 200 μ L of DMF was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated. The beads were then washed extensively with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

Synthesis of Macrocycle via a Huisgen cyclization

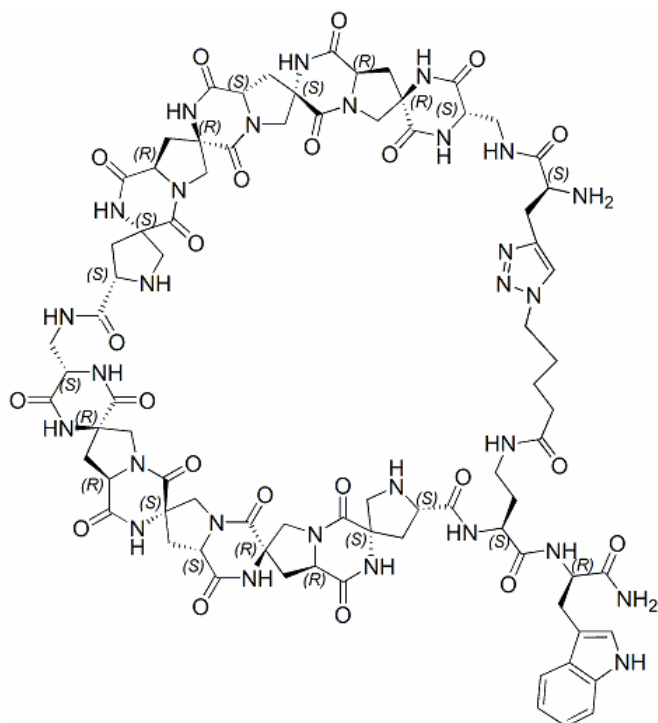


Figure 104 Macrocycle made with a Huisgen cycloaddition.

A few beads (~3mgs) of resin were swelled in DMF for 30min. In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 10eq of Fmoc-L-Propargyl

glycine-OH (Fluka) and 10eq of HATU at 0.2M of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 20eq of DIPEA was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated. The Fmoc was removed and washed with DMF, IPA, DMF, IPA, DCM, IPA, and DCM.

The Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 5mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf-from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min, with slight bubbling, the Boc groups were removed from the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on the resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 12hrs at 35°C -40°C.

The Boc groups were then reattached to the 2 trailing end amines and N-terminus, using a solution of ~10mg of Boc-anhydride in DCM with 30μL of DIPEA, with stirring overnight. The resin was then washed with DCM, MeOH, DCM, IPA, DMF, IPA, DMF, and DMF.

The Dab side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the side chain. A solution of ~20eq 5-azidovaleric acid, 20eq HATU, 40eq DIPEA at 0.2M in DMF, was added and allowed to react for 30min. The beads were washed 3 x 2min with DMF, and the coupling was repeated.

The on-resin macrocyclization conditions were similar to those used by Finn for an on-resin head-to-tail, cyclodimerization.[57] Solutions of 4.8mg CuI (Avacodo) in 2.5mL of 20% DMSO in ACN and 12.5mg of sodium ascorbate (Aldrich) in 0.25mL water were prepared, as well as 12μL of 2,6-lutidine. A volume fraction of each, based on the number of moles of Fmoc released from the propargyl glycine (Ppg), divided by the number of moles used in the paper (50μmol), was used to compute the volumes of CuI, NaAsc, and lutidine that were needed. The CuI was added first, followed by the lutidine and finally the NaAsc. The reaction and beads were stirred for 16hrs at room temperature. Following this, the beads were washed extensively with acetonitrile and DMF, then water and 0.1M EDTA, and acetonitrile and DMF again, repeated at least three times. Then, the resin was extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then dried overnight, *in vacuo*.

The scaffold was cleaved in 2mL of 2.5% water, 2.5% triisopropylsilane in trifluoroacetic acid, with stirring for 2hours. The solutions were drained and the beads were then washed with 2 x 2mL additional TFA. The cleavage solutions were pooled into a 13mm glass tube and the solvent was removed in a SpeedVac.

Phage Display Procedure

The phage display experiments were done using a kit from New England Biolabs (NEB). The procedures used were similar to that found in the Phage Display Peptide Library Kit Manual, available at www.neb.com/nebecomm/products/productE8110.asp. However, some changes were made, and the amended procedure is found below.

Media and Solutions:

LB Medium: Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl. Aliquot into many 25mL or a few 10mL portions in 50mL Falcon tubes, cap loosely. Autoclave, tighten cap, store at room temperature.

LB/IPTG/Xgal Plates: LB medium + 15 g/L agar. Autoclave, cool to < 70°C, add 1 ml IPTG/Xgal (Mix 1.25 g IPTG (isopropyl -D-thiogalactoside) and 1 g Xgal (5-Bromo-4-chloro-3-indolyl-D-galactoside) in 25 ml DMF. Solution can be stored at -20°C in the dark) and pour, 20mL per plate. Store plates at 4°C in the dark.

LB-Tet Plates: LB medium + 15 g/l Agar. Autoclave, cool to <70°C, add 20mg Tetracycline and pour, 20mL per plate. Store plates at 4°C in the dark. Do not use plates if brown or black.

Agarose Top: Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g MgCl₂•6H₂O, 7 g agarose. Dispense into 100 ml aliquots in orange-cap bottles. Autoclave only on the morning of the experiments, retrieve hot, and store in water bath, set at 45°C, until needed.

Blocking buffer: 0.1 M NaHCO₃(pH 8.6), 5 mg/ml BSA, 0.02% NaN₃. Filter sterilize, store at 4°C. The blocking buffer should contain 0.1 µg/ml streptavidin in order to complex any biotin in the BSA.

TBS: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl (make at least 10 liters). Autoclave, store at room temperature.

PEG/NaCl: 20% (w/v) polyethylene glycol-8000, 2.5 M NaCl (difficult to dissolve-may even be cloudy after heating). Autoclave, store at room temperature.

Streptavidin Stock Solution: Dissolve 1.5 mg Lyophilized Streptavidin (supplied) in 1 ml 10 mM sodium phosphate (pH 7.2), 100 mM NaCl, 0.02% NaN₃. Store at 4°C.

Strain Maintenance

1. The supplied E. coli host strain ER2738 is a robust F⁺-strain with a rapid growth rate and is particularly well-suited for M13 propagation.

2. Since M13 is a male-specific phage, it is recommended that all cultures for M13 propagation be inoculated from colonies grown on media selective for presence of the F-factor, rather than directly from the supplied glycerol culture. The F-factor of ER2738 contains a mini-transposon which confers tetracycline resistance, so cells harboring the F-factor can be selected by plating and propagating on tetracycline- containing media.

3. Streak out ER2738 from the included glycerol culture onto an LB-Tet plate. Invert and incubate at 37°C overnight and store wrapped with parafilm at 4°C in the dark for a maximum of 1 week.

4. ER2738 cultures for infection can be grown either in LB or LB-Tet media. Loss of F-factor in nonselective media is insignificant as long as cultures are not serially diluted repeatedly.

Avoiding Phage Contamination

The M13 coat protein pIII mediates infectivity by binding to the F-pilus of the recipient bacterium. Display of foreign peptides as N-terminal fusions to pIII appears to attenuate infectivity of the library phage relative to wild-type M13. As a result, there is a strong in vivo selection for any contaminating wild-type phage during the amplification steps between rounds of panning. In the absence of a correspondingly strong in vitro binding selection, even vanishingly small levels of contamination can result in a majority of the phage pool being wild-type phage after three rounds of panning.

1. The potential for contamination with environmental bacteriophage can be minimized by using aerosol-resistant pipette tips for all protocols described in the Manual.

2. Since the library phage are derived from the common cloning vector M13mp19, which carries the lacZ gene, phage plaques appear blue when plated on media containing Xgal and IPTG.

Environmental filamentous phage will typically yield white plaques when plated on the same media. These plaques are also larger and "fuzzier" than the library phage plaques. We strongly recommend plating on LB/IPTG/Xgal plates for all titering steps and, if white plaques are evident, picking ONLY blue plaques for sequencing.

Phage Titering

The number of plaques will increase linearly with added phage only when the multiplicity of infection (MOI) is much less than 1 (i.e., cells are in excess). For this reason, it is recommended that phage stocks be titered by diluting prior to infection, rather than by diluting cells infected at a high MOI. Plating at low MOI will also ensure that each plaque contains only one DNA sequence.

1. Inoculate 5-10 ml of LB-TET with a single colony of ER2738 and incubate with shaking until mid-log phase (OD₆₀₀~ 0.5; about 12hrs).
2. Once cells are growing, autoclave Agarose Top, remove hot, and store in water bath at 45°C until ready for use.
3. Pre-warm LB/IPTG/Xgal plate per expected dilution at 37°C until ready for use.
4. Prepare 10-fold serial dilutions of phage in LB. Suggested dilution ranges: for amplified phage culture supernatants, 10⁸ to 10¹¹; for unamplified panning eluents, 10¹-10⁴. Use a fresh pipette tip for each dilution.
5. Once culture has reached mid-log phase, dispense 200 µl culture into 15mL conical tubes, 1 for each phage dilution.
6. Add 10 µl of each dilution to each tube, vortex quickly, and incubate at room temperature for 1-5 minutes.
7. After this time, remove 5 caps at a time, and transfer 3mL of Top-Agar to each tube, **quickly** cap and vortex and **quickly** pour onto a pre-warmed LB/IPTG/Xgal plate. Spread Top-Agar evenly by tilting plate.
8. Allow plates to cool 5 minutes, invert and incubate overnight at 37°C.
9. Inspect plates and count plaques on plates having ~10² plaques. Multiply each number by the dilution factor for that plate to get phage titer in plaque forming units (pfu) per 10 µl.

Control Panning Experiment

Follow the below procedure using streptavidin as the target adding 0.1 µg /ml streptavidin to the blocking solution to complex to any biotin in the BSA. Elute bound phage with 0.1 mM biotin in TBS for at least 30 minutes. After 3 rounds of enrichment/amplification, the consensus sequence for streptavidin-binding peptides should contain the motif His-Pro-Gln (HPQ).

Day One

Panning is carried out in a 96-well microtiter plates (ReactiBind, Pierce #15042). Alternatively, pre-coated streptavidin plates could be used (Sigma #M5432).

1. Prepare a solution of 100 µg/ml of streptavidin in 0.1 M NaHCO₃ (pH 8.6).
2. Add 150 µl to each well.
3. Incubate overnight at 4°C in a humidified container (e.g., a sealable plastic box lined with damp paper towels). Store plates at 4°C in humidified container until needed.

Day Two

4. Inoculate 10 ml LB medium (add Tet) with streaked-plate ER2738 (plating culture for titering). Incubate 37°C for 8-10hrs with vigorous shaking.
5. Pour off the coating solution from the microplate and firmly slap it face down onto a clean paper towel to remove residual solution. Fill each plate or well completely with Blocking Buffer. Incubate at least 1 hour at 4°C.
6. Discard the blocking solution as in step 5. Wash each plate rapidly 6X with TBST (TBS + 0.1% [v/v] Tween-20). Coat bottom and sides of plate or well by swirling, pour off the solution, and slap the plate face down on a clean paper towel each time. Work quickly to avoid drying out the plates.
- 7a. While plates are blocking, pre-complex phage with biotinylated target. Combine in microfuge tube 0.1-10 µg biotinylated target (depending on amount available) and 1.5 x 10¹¹ pfu of the input phage (=10 µl of original library) in 400 µl TBST. Incubate at room temperature for 60 minutes.
- 7b. Add phage-target solution to the washed blocked plate. Incubate at room temperature for 30 minutes.

7c. Add biotin to a final concentration of 0.1 mM (to displace potential HPQ phage) and incubate an additional 5 minutes. The off rate for the biotinylated target is sufficiently slow that the target will not be displaced by the biotin.

8. Discard nonbinding phage by pouring off and slapping plate face-down onto a clean paper towel.

9. Wash plates 10 times with TBST as in step 6. Use a clean section of paper towel each time to prevent cross-contamination.

10. Elute bound phage with 100 μ l of a solution of the "biotin-less" ligand (\sim 100 μ g/ml in TBS) to compete the bound phage away from the immobilized target on the plate. Rock gently for 60 minutes at room temperature. Pipette eluent into a sterile microcentrifuge tube.

10a. Alternatively, a general buffer for nonspecific disruption of binding interactions (likely lifts everything off of the plate or totally denatures the streptavidin) is 150 μ l of 0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA. Rock gently for no more than 10 minutes. Pipette eluent into a microcentrifuge tube. Neutralize with 150 μ l (15 μ l for microtiter wells) 1 M Tris-HCl (pH 9.1). The pH neutralization here is absolutely critical.

11. Titer a small amount (\sim 1 μ l) of the eluent as described in General M13 Methods, above. The remaining eluent can be stored overnight at 4°C at this point and amplified the next day.

12. Set up an overnight culture of ER2738 (from streaked plate) in LB-Tet.

Day Three

13. The next day dilute the overnight culture 1:100 in 20 ml LB-TET in a 250 ml Erlenmeyer flask and add $\frac{1}{2}$ the volume unamplified eluent (store the rest at 4°C in case of emergency). Incubate at 37°C with vigorous shaking for 4.5 hours (time critical here).

14. Transfer the culture to a centrifuge tube and spin 10 minutes at 5,000 rpm in large, floor-centrifuge at 4°C. Transfer the supernatant to a fresh tube and re-spin to ensure all cells removed (SAVE SUPERNATANT-phage is in the liquid layer).

15. Pipette the upper 80% of the supernatant to a fresh tube (purple cap=higher strength) and add 1/6 volume of PEG/NaCl. Allow phage to precipitate at 4°C for at least 2hrs.

16. Spin PEG precipitation 15 minutes at 10,000 rpm at 4°C. Decant supernatant, re-spin briefly, and remove residual supernatant with a pipette.

17. Suspend the pellet in 1 ml TBS. Transfer the suspension to a microcentrifuge tube and spin for 5 minutes at 4°C to pellet residual cells.

18. Transfer the supernatant to a fresh microcentrifuge tube and re-precipitate with 1/6 volume of PEG/NaCl. Incubate on ice 30 minutes. Microcentrifuge for 10 minutes at 4°C. Discard supernatant, re-spin briefly, and remove residual supernatant with a micropipette.

19. Suspend the pellet in 200 µl TBS, 0.02% NaN₃. Microcentrifuge for 1 minute to pellet any remaining insoluble matter. Transfer the supernatant to a fresh tube. This is the amplified eluent.

20. Titer the amplified eluent as described in General M13 Method on LB/IPTG/Xgal plates. Store at 4°C.

21. Coat a plate for the second round of panning, as in day one.

Days Four and Five

22. Count blue plaques and determine phage titer. Use this value to calculate an input volume corresponding to 1-2 x 10¹¹ pfu. If the titer is too low, succeeding rounds of panning can be carried out with as little as 10⁹ pfu of input phage.

23. Carry out a second round of panning: repeat steps 4-12 and raising the Tween concentration in the wash steps to 0.5% (v/v).

24. Titer the resulting second round amplified eluent on LB/IPTG/Xgal plates. Amplify ½ the volume eluent and precipitate phage as in steps 13-20.

25. Coat a plate for the third round of panning, as in day one.

Days Six

22. Count blue plaques and determine phage titer. Use this value to calculate an input volume corresponding to 1-2 x 10¹¹ pfu. If the titer is too low, succeeding rounds of panning can be carried out with as little as 10⁹ pfu of input phage.

23. Carry out a second round of panning: repeat steps 4-12 and raising the Tween concentration in the wash steps to 0.5% (v/v).

24. Titer the resulting second round amplified eluent on LB/IPTG/Xgal plates. Plaques from this titrating can be used for sequencing or ELISA assays: time the procedure so that plates are incubated no longer than 18 hours, as deletions may occur if plates are grown longer. Store the remaining eluent at 4°C.

Assaying titered phage for binding to receptor by ELISA

1. The night before ELISA is to be done, inoculate 20 ml of LB-Tet medium with streaked ER2738 and incubate at 37°C overnight. Also coat two plates (FisherBrand 21-377-204) for every deep-welled plate with 100µl of 100 µg/ml of streptavidin in 0.1 M NaHCO₃ (pH 8.6). Incubate at 4°C overnight in an air-tight humidified box (e.g., a sealable plastic box lined with wet paper towels).

2. The amplified crude, phage-containing supernatant can be used for the ELISA assay. For the highest throughput, use a deep-welled (2mL, VWR-47749-874) 96-well plate to amplify the titered 3rd elution plaques from the last step, step 24. Dilute the overnight culture, 1:100 with fresh LB-Tet, and, use a flame-sterilized metal wire cell-picker to barely touch the tip to a blue colony and transfer to 1mL of 1:100 diluted cell culture. More explicitly, flame sterilize the tip, pierce the agar to cool, then immediately touch the tip to a colony, which was numbered using a marker to write on the petri dish (don't "sweep" the colony- need to conserve for multiple uses, potentially). Repeat many times. At least 50-200 colonies should be investigated, per library, per receptor.

3. Incubate deep-well plate at incubate at 37°C with vigorous aeration for 4 1/2 hours, although be careful to secure the plate to the shaker, and be sure not to shake too hard and get cross-talk between wells.

4. After 4.5 hrs transfer plate to benchtop centrifuge and use the 96-well plate rotor to pellet cells at 2000rpm for 20-30min at 4°C.

5. While the phage is amplifying, shake out excess streptavidin solution and slap plate face-down onto a paper towel. Fill each well completely with Blocking Buffer. Additionally, a second microtiter plate/per deep-welled plate should also be blocked in order to test for binding of each selected sequence to BSA-coated plastic. Incubate at 4°C, 1-2 hours.

6. Shake out the blocking buffer and wash each plate 6 times with TTBS (the percentage of Tween should be the same as the concentration used in the panning wash steps), slapping the plate face-down onto a clean section of paper towel each time.

7. At this point there are three blocked plate. One has only been blocked and will be used to test for BSA-plastic binding. Two others have been coated with streptavidin and blocked. One will

be used to test for streptavidin binding, by omitting the biotinylated-receptor, and the other plate will be the actual experiment, containing all important components.

8. Add 50 μ l of phage supernatant from the deep-well amplification plate to each of the three blocked microtiter plates, using the multichannel pipettor. Then add biotinylated receptor, 0.1 μ g in 50 μ l TTBS, to each well of the experimental plate. Add 50 μ l TTBS to the other two plates (BSA/phage only and BSA-streptavidin/phage only). Incubate at room temperature for 1-2 hours with agitation.

9. Wash plate 6 times with 1X TBS/Tween as in step 6.

10. Dilute HRP-conjugated anti-M13 antibody (Pharmacia # 27-9411-01) 1:5,000 in blocking buffer. Add 200 μ l of diluted conjugate to each well and incubate at room temperature for 1 hour with agitation.

11. Wash 6 times with 1X TBS/Tween as in step 6.

12. Prepare the HRP substrate solution as follows: a stock solution of ABTS can be prepared in advance by dissolving 22 mg ABTS (Sigma #A1888) in 100 ml of 50 mM sodium citrate, pH 4.0. Filter sterilize and store at 4°C. Immediately prior to the detection step, add 36 μ l 30% H₂O₂ to 21 ml of ABTS stock solution per plate to be analyzed.

13. Add 200 μ l substrate solution to each well, incubate at room temperature for 10-60 minutes.

14. Read plates using a microplate reader set at 405-415 nm.

Plaque amplification

1. The night before, inoculate 20 ml of LB-Tet medium with streaked ER2738 and incubate at 37°C overnight.

2. Dilute the ER2738 overnight culture 1:100 in LB-Tet. Dispense 2 ml diluted culture into a 15mL falcon culture tubes with the clear caps that rest or snap, one for each clone to be characterized.

3. Using a sterile metal cell-picker, touch a blue plaque and transfer to a tube containing diluted culture. Important: pick plaques from plates having no more than ~100 plaques. This will ensure that each plaque contains a single DNA sequence.

4. Incubate tubes at 37°C with shaking for 4.5-5 hours.

5. Snap caps and centrifuge cultures for 20 min, 3000rpm at 4°C. Transfer the upper 80% of the supernatant (1.6 mL) to a fresh tube. This is the amplified phage stock and can be stored at 4°C

for several weeks with little loss of titer. For long-term storage, dilute 1:1 with sterile glycerol and store at -20°C.

6. Add 300 µl PEG/NaCl. Invert to mix, and let stand at room temperature 10 minutes.

7. Centrifuge 10 minutes, discard supernatant.

8. Re-spin briefly. Carefully pipette away any remaining supernatant.

9. Suspend pellet thoroughly in 100 µl Iodide Buffer and add 250 µl ethanol. Incubate 10 minutes at room temperature. Short incubation at room temperature will preferentially precipitate single-stranded phage DNA, leaving most phage protein in solution.

10. Spin 10 minutes, discard supernatant. Wash pellet in 70% ethanol, dry briefly under vacuum.

7. Suspend pellet in 30 µl deionized water (DO NOT USE TE buffer-EDTA bad for sequencing enzymes)

11. 10 µl of the resuspended template + 3pmol of -96 primer (for automated sequencing) in fresh 1.5mL tube. Label with template name, Schafmeister, tube number, starting with 1 (will correspond to number on order form to be faxed over before 9am the next day- see website for more info <http://www.pitt.edu/~rsup/DNAstaticSplash.html>). That night, deliver to fridge marked DNA sequencing, found in hallway of 11th floor outside room W1105 BSTWR, in the service corridor.

FYI -- The sequence being read corresponds to the anticodon strand of the template. Download BioEdit (free program found on web, also on scratch drive, Bird folder) and download (http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/sequences/m13ke.txt) the M13KE vector nucleotide sequence from NEB. Open the text file in BioEdit, transform into complimentary strand then use the Sequence and Map from the PhD manual to search for sequence found in the figure in the manual. Alternatively, a file on the scratch drive, Bird folder, filename "gIII 12mer DNA sequence complement 5-3" contains the sequence of the primer and the sequence, as it is extended. The M and N nucleotides correspond to the nucleotides encoding the 12mer peptide. Delete the appropriate number of nucleotides if using shorter peptides. Actual sequence can be opened and overlaid for comparison, such that the constant regions on both sides of the peptide nucleotides are identical (it may be necessary to delete or insert

nucleotides to get the flanking regions to perfectly overlap. Take the reverse-complement for the coding strand and translate for the peptide sequence.

4. STUDIES OF BIVALENT INTERACTIONS: MATCHED AND MISMATCHED BIVALENT PAIRS

4.1 INTRODUCTION

The interaction between vancomycin and D-Ala-D-Ala is one of the most well studied and characterized non-protein receptor-ligand interactions. This is because of the importance of vancomycin in the treatment of post-operative patients infected with particularly virulent strains of *Staphylococcus aureas* and enterococci, which are often resistant to all other antibiotics. Vancomycin and other glycopeptide antibiotics act against gram-positive bacteria, having an outer peptidoglycan cell-wall layer, while inactivity in gram-negative stems from the ability of glycopeptides to cross the lipopolysachharide layer outside of the peptidoglycan cell-wall.

The mechanism of action of vancomycin differs from that of other antibiotics like penicillin that also target the cell-wall (Figure 105). Whereas penicillin binds and inhibits the transglycosidase/transpeptidase (TG/TP) involved in the cell-wall cross-linking, vancomycin binds to the substrate of the TG/TP.

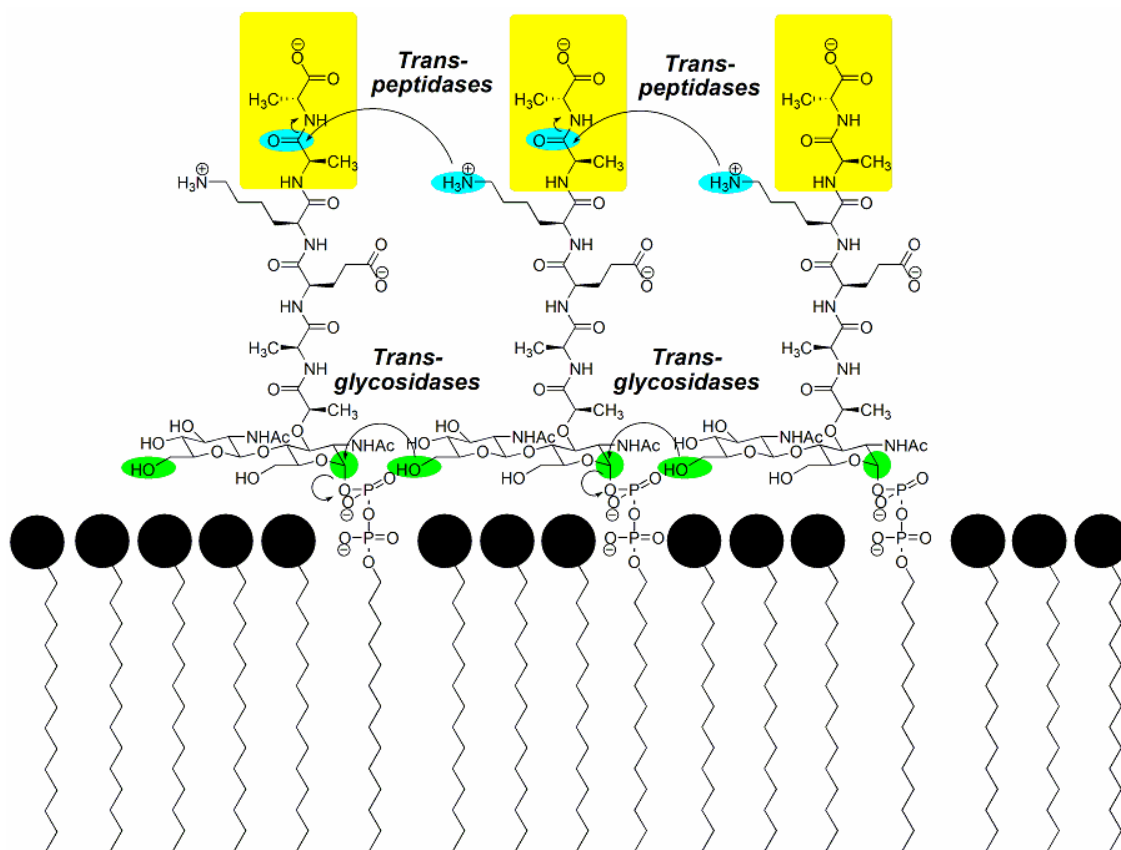


Figure 105 Mechanism of cell-wall formation. Yellow indicates D-Ala-D-Ala ligand for vancomycin.

The terminal D-Ala-D-Ala dipeptide of the parent pentapeptide linked to the lipodisaccharide membrane anchor is the ligand for vancomycin, while the terminal D-Ala is the leaving group resulting from the intermolecular cross-linking of adjacent L-Lys and penultimate D-Ala. The steric bulk of the vancomycin bound to D-Ala-D-Ala prevents the TG/TP from binding to its D-Ala substrate.[61] This leads to incomplete cell-wall cross-linking, causing holes to form which end up bursting the cell from the osmotic pressure inside the cell.

The poly-aromatic backbone of vancomycin serves to precisely pre-arrange the peptide backbone of vancomycin (**69**) for hydrogen bonding interactions with the terminal D-Ala-D-Ala as seen in Figure 106. There exists considerable uncertainty as to the exact function of the disaccharide portion of vancomycin. Affinity can be modulated by adding or subtracting substituents to the disaccharide, (results suggest that this affinity modulation occurs through alternate pathways, perhaps dimerization) from interaction with the cross-linked oligosaccharide cell-wall layer, or through interaction with TG/TP.[62, 63]

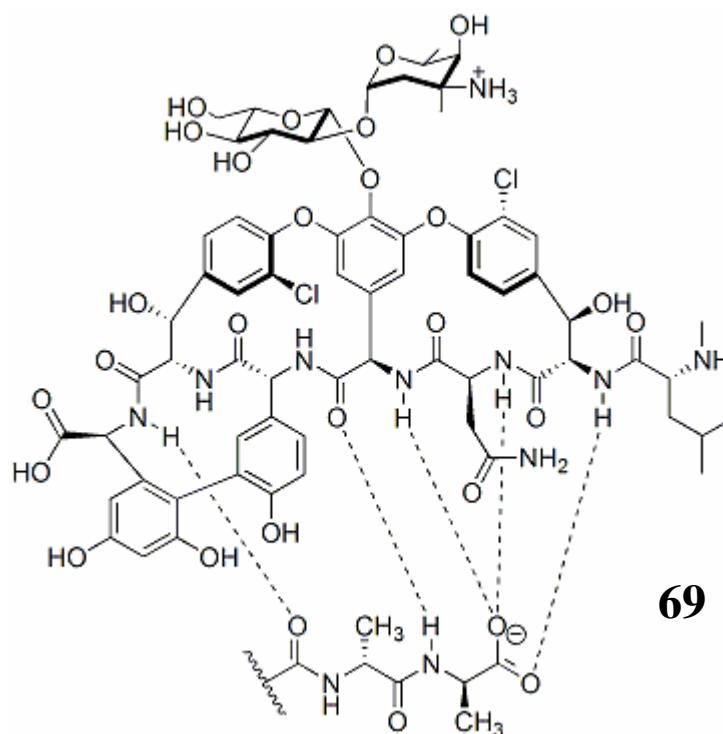


Figure 106 Hydrogen bonding interactions in the vancomycin D-Ala-D-Ala complex.

These hydrogen bonding interactions are crucial for high affinity and specificity in the complex. For example, when a single H-bond is lost, in the case of mutating the D-Ala-D-Ala amide linkage to a D-Ala-D-lactate ester linkage, the cost is 3 orders of magnitude in binding affinity. This is the most common mechanism of resistance to vancomycin.[64] Two things are apparent when looking at the crystal structure of the complex (Figure 107). First, the proximity and geometry between the atoms involved in the recognition network of hydrogen bonds is very well-defined. Second, the way in which the vancomycin folds over the D-Ala-D-Ala allows for this exquisite recognition of complimentary interactions.[65]

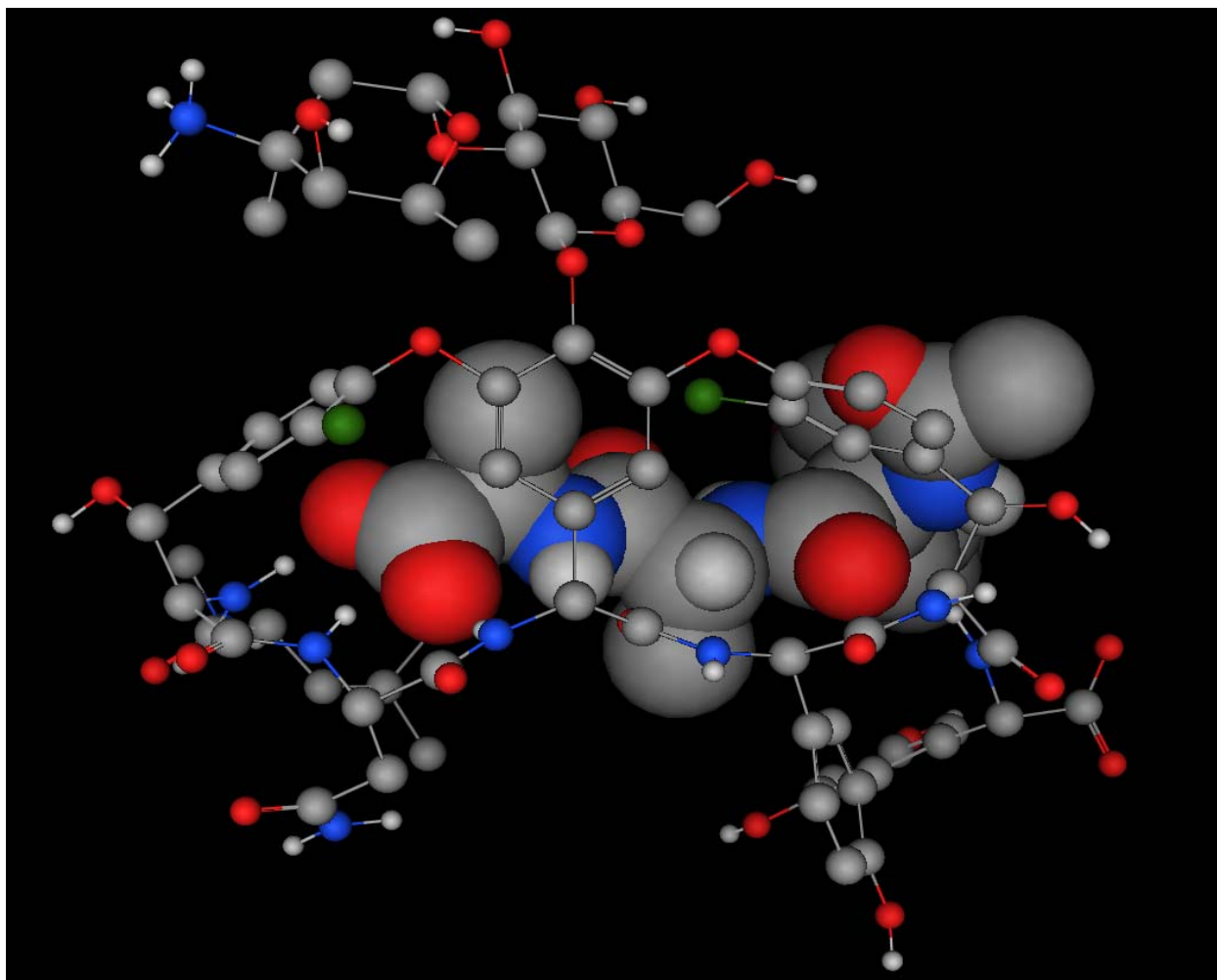


Figure 107 Crystal structure of a vancomycin (ball & stick)-D-Ala-D-Ala (space filling) complex.

The numerous analogues of vancomycin have been made, but the effects on activity and binding affinity are outside of the relevance of this discussion, except for Whitesides' *tris*-Van (**70**) / *tris*-D-Ala-D-Ala (**71**) system (Figure 108).[22, 25] Compared to the monovalent K_d ($1\mu\text{M}$), the trivalent system has a K_d of 4×10^{-17} , as measured by HPLC via a competition with monovalent D-Ala-D-Ala. The magnitude of the strength of this interaction is almost difficult to comprehend: it is 25 times stronger than the benchmark for high affinity, avidin-biotin. Whitesides remarks that the trivalent K_d only differs from the monovalent (K_d)³ by a factor of 40, which is likely due to the fact there is little loss of conformational entropy from the linker and the vancomycin itself, as both were initially described as relatively rigid.[25]

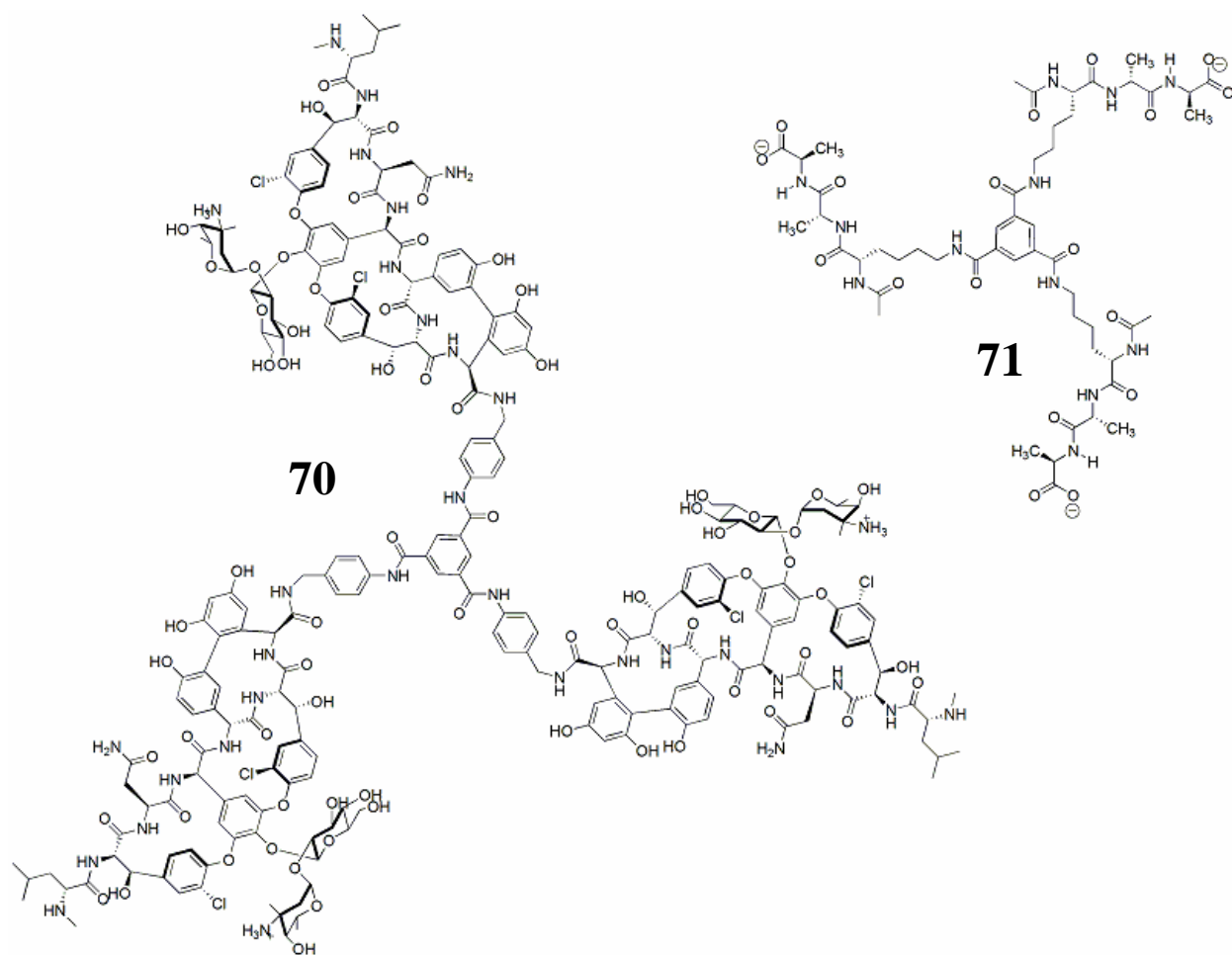


Figure 108 Trivalent vancomycin and trivalent D-Ala-D-Ala structures.

However, Whitesides concluded that there was indeed entropy loss of 13.3 kcal/mol upon binding, due to the 9 rotatable bonds in the 3-Van and the 18 rotatable bonds in the 3-D-Ala that had to be frozen before complexation.[22] Jencks estimated the entropic cost of each rotatable bond to be ~1.2 kcal/mol.[21] Whitesides states that the difference in his calculated values and the value using Jencks' formula arise from the difference due the different types of rotatable bonds being frozen, both between Jencks' and Whitesides' systems, and within Whitesides' system. [22]

There are brilliant contributions being made to improving the affinity and antibiotic activity of vancomycin. However, there are other features of this interaction that can be exploited. Using a building block approach to make rigid linkers of varying lengths which hold two ligands or two receptors a given distance apart will usher in a new field of applied bivalent interactions. The design and synthesis of bivalent pairs (Figure 109), where the distances

between the ligand (D) and receptor (V) are either matched or mismatched would be a novel application of bivalent interactions and building block approaches towards functional, nanoscale molecules. Matched pairs of D_2 and V_2 would have complimentary distances between each other, while mismatched pairs of D_2 and V_2 would have distances that were either too long or too short to interact as strongly as a matched pair. As a result, one would expect that a matched pair of D_2 and V_2 would bind to each other, even in the presence of D_2 and V_2 whose spacing was not similar to the matched pair of D_2 and V_2 . Additionally, if there were two or more matched pairs in solution, one would expect that those pairs that were of similar spacing would preferentially interact with each other, resulting in multiple matched bivalent pairs.

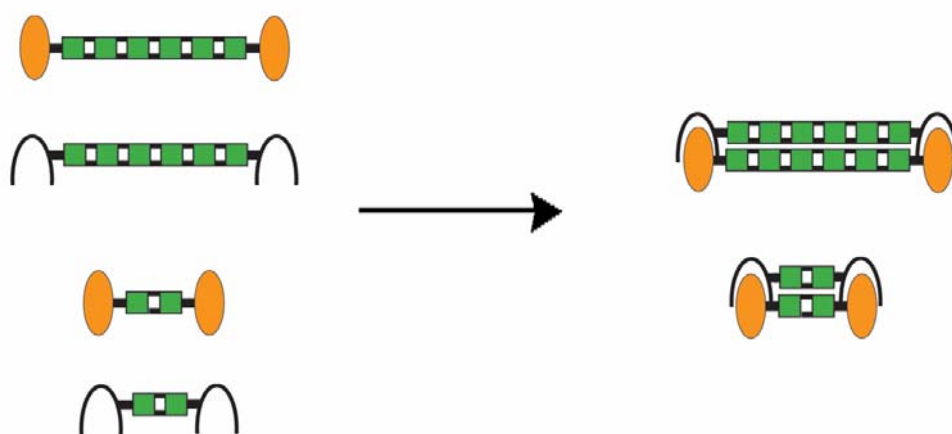


Figure 109 Two sets of two complimentary pairs should preferentially interact with each other.

This may have application in separation of complex mixtures. For instance, one could imagine a mixture of compounds, each individually fused to a bivalent ligand of having differing spacing between the ligands (Figure 110). Using immobilized bivalent receptors of differing length, each individually attached to a resin that was confined in a “tea bag” or lantern of differing color, one could separate the mixture of compounds by allowing the different bivalent pairs to commingle. After sufficient time for equilibration, the mixture could be separated, or at least enriched, by using the preferred interactions between matched bivalent pairs having similar spacing between pairs of ligands and pairs of receptors.

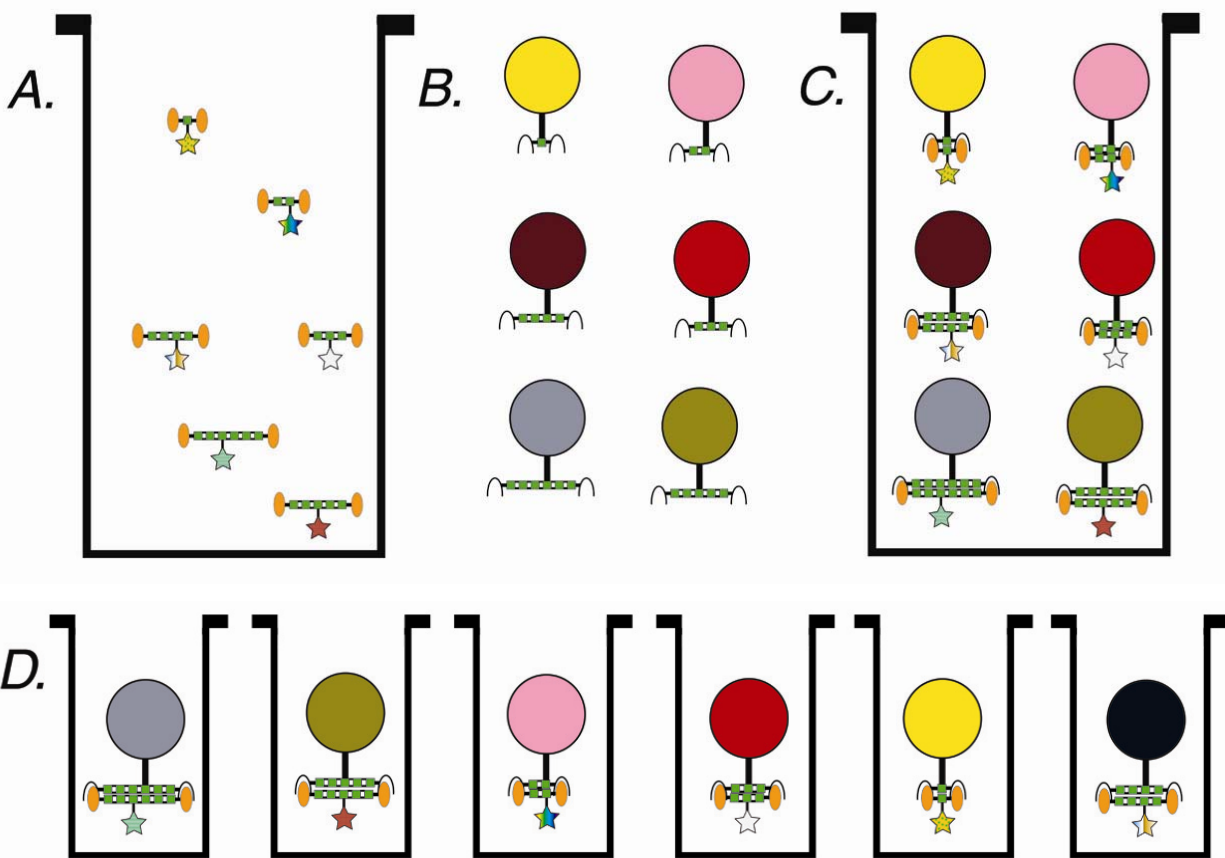


Figure 110 Illustration of proposed mixture separation (A) mixture of "stars" attached to bivalent ligands of differing length (B) bivalent receptors of differing lengths, immobilized on "colored beads" (C) complexation of mixture of ligands with immobilized receptors, after equilibration (D) visual segregation of colored beads facilitates separation of the initial mixture.

4.2 RESULTS AND DISCUSSION

The goal of this project was to synthesize bivalent D-Ala-D-Ala and bivalent vancomycin *bis*-amino acid scaffold spacers varying from short to long. The first part of this discussion will address the synthesis of D-Ala-D-Ala component and subsequent incorporation into a scaffold. The second part will deal with the synthesis of the bivalent vancomycin-containing scaffolds, from commercially available vancomycin.

A series of reactions were necessary to synthesize the D-Ala-D-Ala component needed for solid-phase incorporation into bivalent D-Ala-D-Ala molecules. Using commercially

available protected amino acids, a solution phase coupling reaction was utilized to assemble the protected dipeptide, followed by deprotection at the N-terminus. The liberated amine was then used to ring open an anhydride to yield a carboxy-terminating linker attached to D-Ala-D-Ala-OtBu, ready for incorporation into a scaffold on the solid-phase.

Initial attempts utilized the Fmoc-D-Ala as the coupling partner (Figure 111). This group was chosen initially based previous experiences with it and because it contains a very bright chromophore which facilitates ease of purification. The first step, making the dipeptide, proceeded cleanly on a gram-scale using HOBt/HBTU in DMF. A slight excess of the D-Ala-OtBu and HOBt/HBTU was used to ensure that all Fmoc-D-Ala was consumed, resulting in only one species containing an Fmoc group (**72**). After reaction was complete, as judged by LC/MS, the contents were poured in 20 volumes of water. This served multiple purposes. First, it served to stop the reaction, and second, it served to remove the DMF. Most helpful though, the HOBt related species were highly water soluble, while the fully protected dipeptide had zero water solubility. Filtration served to isolate the product away from the coupling reagents and the DMF. The product was then dried *in vacuo*, and loaded onto celite for purification on the Isco. A gradient of hexanes-to-EtOAc was used to remove any slight impurities.

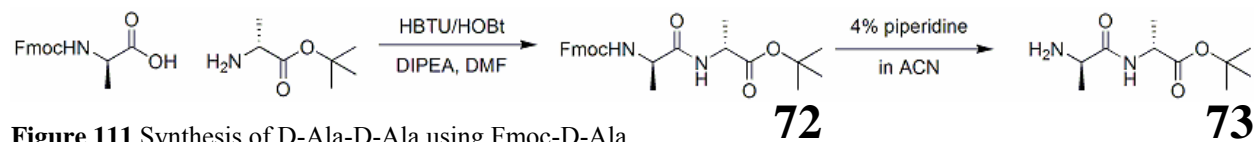


Figure 111 Synthesis of D-Ala-D-Ala using Fmoc-D-Ala

The assembly of the dipeptide proved to be easily accomplished, however problems were encountered in subsequent steps. Removal of the Fmoc group was done using 4% piperidine in acetonitrile, for 30min (**73**). After LCMS confirmed completion, the solvent was removed *in vacuo* to yield a white powder, which was then dried overnight *in vacuo*, at 45°C. The dried powder was then used to ring open an anhydride, in this case phthalic anhydride. Problems were encountered because it was difficult to remove all of the piperidine from the previous reaction. The piperidine-fullvene adduct which is stable in a solution of piperidine likely reverted back to piperidine and fullvene via an elimination reaction. This liberated piperidine or any trace piperidine reacted on a similar time-scale to the desired D-Ala-D-Ala-OtBu substrate to consume the anhydride which was used sparingly. In order to get complete reaction to form the desired product, excess of the anhydride was required. This resulted in a number of carboxylic acid-

containing products being formed (Figure 112), all of which eluted on an HPLC chromatogram within two minutes of each other. It is known that mixtures of carboxylic acids are very difficult to separate, either on normal or reversed phase, owing to the carboxylic acid being the polarity dictating functional group.

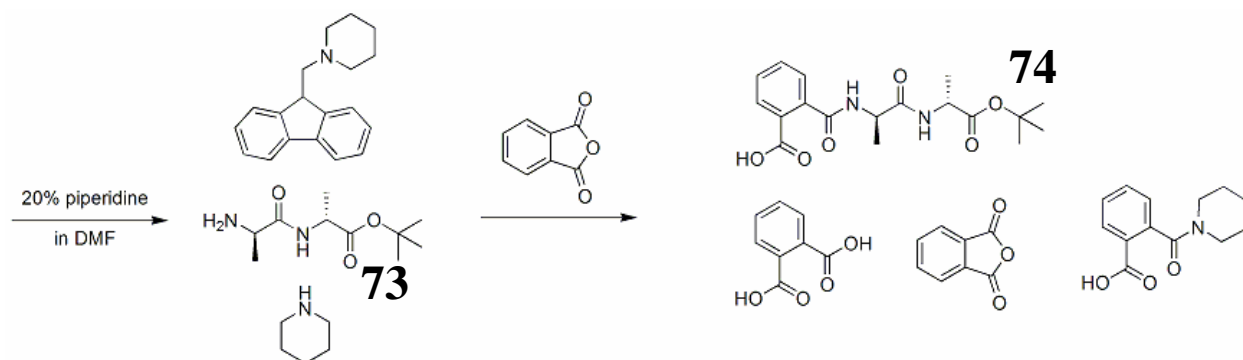


Figure 112 Multiple products formed because piperidine is needed to remove the Fmoc group

An alternative synthesis (Figure 113) was easily implemented, simply by replacing the Fmoc-D-Ala with Cbz-D-Ala. The dipeptide **75** was synthesized in an identical fashion, only the purification was slightly challenged by the fact the Cbz absorbs UV light more than 1,000 times less at 254nm. Using a smaller column (40g as opposed to 330g) allowed for clean separation and a lower flow rate, thus increasing the sensitivity. Removal of the Cbz group was easily accomplished using palladium-on-charcoal and a hydrogen balloon, with the chosen solvent being EtOAc. Previous experiences had shown that hydrogenations proceed cleanly in 12hrs when EtOAc is the solvent. Although it was known that protic solvents systems, especially AcOH:MeOH:water, drastically accelerate the reaction, this would have caused the formation of salts upon removal of the protecting groups. Additionally, acetic acid should be avoided in any step near the end of a synthetic product destined for incorporation onto the solid-phase, as even trace amounts of acetic acid will react very quickly with the amine, to the detriment of the desired product. However, because clean hydrogenolysis in EtOAc occurred, no acceleration was needed. Since the byproducts were CO₂ and toluene, pure product **73** was afforded by removal of the solvent *in vacuo*, yielding an oil used without further purification. Reaction of the amine and the anhydride (0.85 equivalents, relative to the amine) in distilled dichloromethane occurred within 2hours to form the desired product (**74**) quantitatively, which was purified using the Isco system and a gradient of chloroform to 10% MeOH/chloroform.

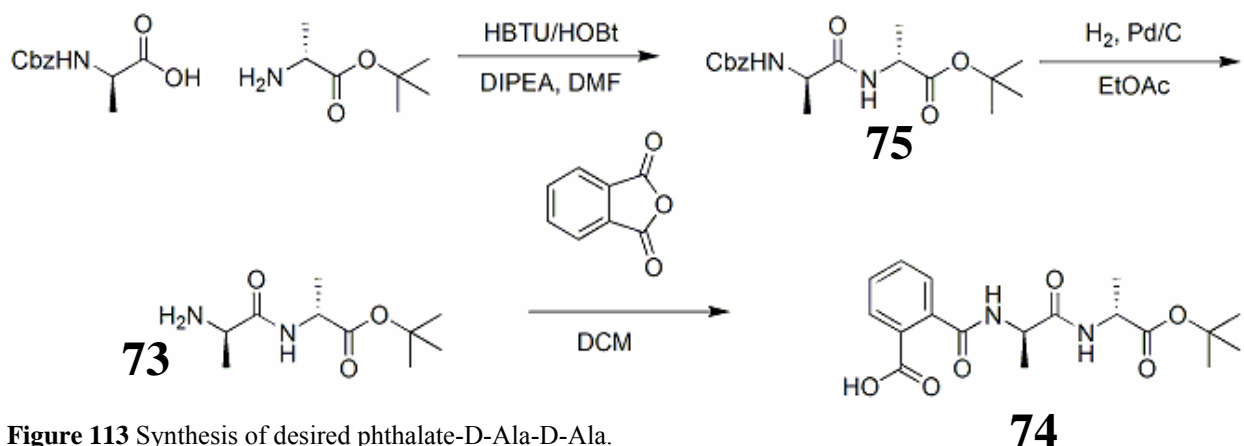


Figure 113 Synthesis of desired phthalate-D-Ala-D-Ala.

A second anhydride (diglycolic anhydride) was used to form an amide of the form, HO₂C-CH₂-O-CH₂-CONH-. However, this linker was deemed less useful, because of the lack of a chromophore from which an accurate concentration could be measured.

With the *ortho*-phthalate-D-alanine-D-alanine-*tert*-butyl ester ligand in hand, the next step was the synthesis of the scaffolds of varying length. It was important to have rigidity, resulting from DKPs at both ends of this bivalent scaffold. A DKP at the leading end is easy to accomplish: remove the Fmoc of an amino acid that follows a building block and DKP immediately forms. However, no one in our group had been able to form a DKP at the trailing end (Figure 114). In order to accomplish this, a few things are required. First a carboxylic acid at the trailing end needs to be present. Many resins produce a free acid after cleavage. Second the resin would need to be cleaved with dilute acid to produce a fully protected scaffold, containing a free acid. Then the free acid could be selectively methylated with TMSCHN₂, while all other sensitive functionality remained protected. The trityl resin was chosen, as it met all of the above requirements. After formation of the methyl ester at the trailing end, all protecting could then be removed, and the DKPs closed, including the trailing end DKP between the first building block and the newly formed methyl ester. This proposed scheme was validated on a model substrate and found to work exactly as planned. It was then broadly applied to the synthesis of both the *bis*-vancomycin and the *bis*-D-Ala-D-Ala bivalent molecules.

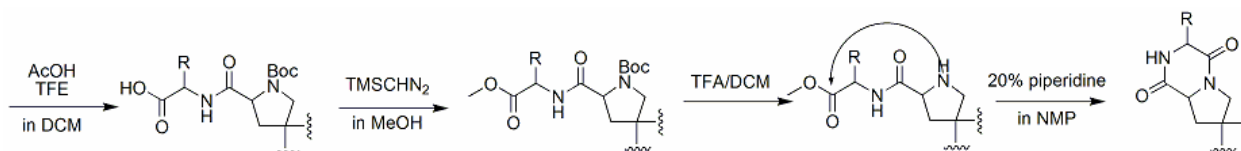


Figure 114 Proposed trailing end-DKP formation.

The chlorotriptyl chloride resin was the solid-support for the synthesis of a series of different length scaffolds, having a D-Ala-D-Ala at either end. The orthogonal protecting group isovaleryl-dimethyl-dioxocyclohexylidene (ivDde), cleavable by 2% hydrazine in DMF, was chosen for diaminobutanoic acid (Dab) side chain. One cannot use the diaminopropionic acid because it was known to be susceptible to intramolecular migration of the ivDde group when the Fmoc was removed, from the N- β to N- α .

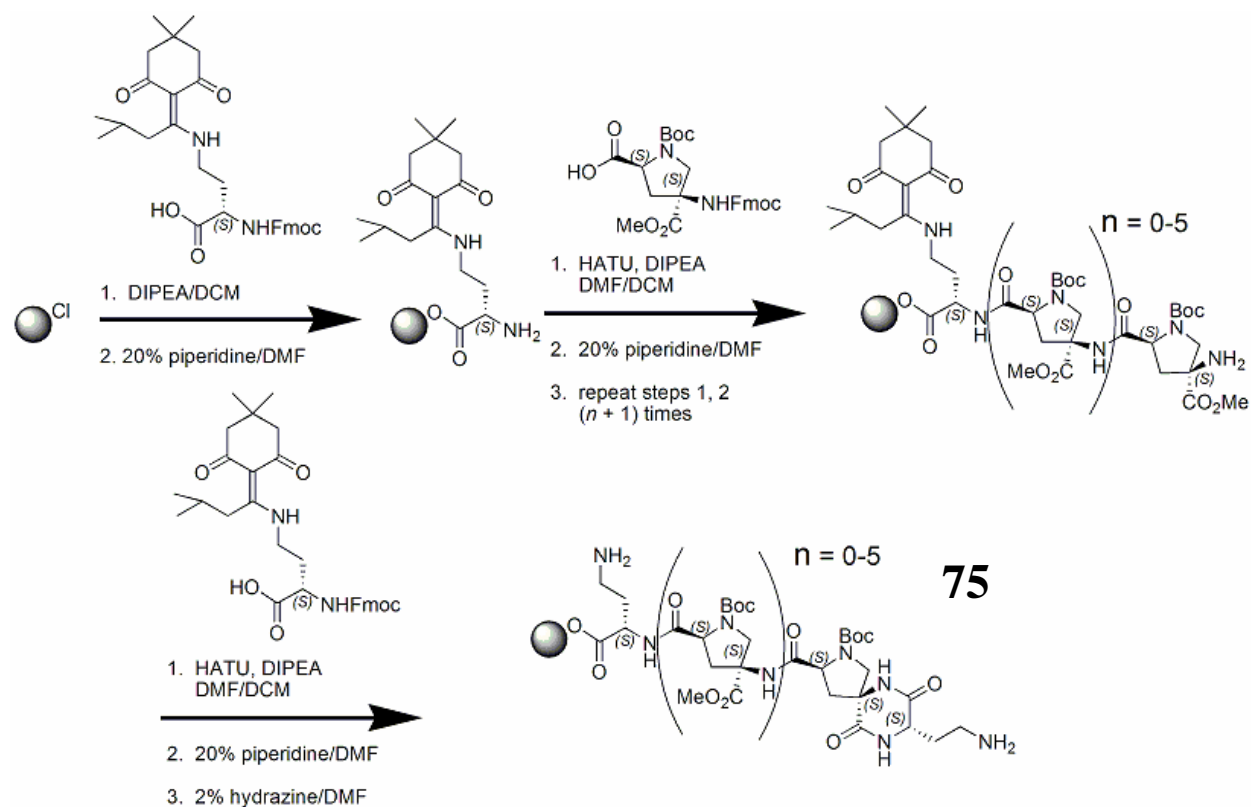


Figure 115 Solid phase synthesis of the intermediate used to make bivalent D-Ala-D-Ala.

This first residue was attached to the triptyl resin (Figure 115) by deprotonation of the carboxyl with DIPEA and then subsequent nucleophilic attack on the triphenylmethyl position of the resin. Attachment of the first building block, pro4(2S4S) with HATU followed by Fmoc removal with piperidine served to elongate the chain of building blocks. After each Fmoc removal, an appropriately sized portion of resin would have to be removed so that the varying lengths could be made. By washing the resin with DCM, followed by drying with aspiration for 5min, this allowed for removal of the dried resin from the reactor and placement in a weigh boat.

Removal of a fraction of the resin (first = 1/6th, second = 1/5th, third = 1/4th, etc.) allowed for a series of equimolar amounts of varying lengths to be produced. After completion of the series of lengths of 1-6 building blocks, the final Fmoc-Dab(ivDde) was attached using HATU and the Fmoc removal was allowed to go for 2hrs to ensure complete closure. After this, the ivDde was removed with hydrazine to give the resin-bound intermediate **75**.

At this point the resins were each split into 3 equal portions, with one portion being set aside in the desiccator in case anything went wrong. The first portion was to contain a β -Ala linker between the ligand and scaffold. This was done in order to evaluate the effect that added flexibility may have on the formation of matched bivalent pairs. This was done by first coupling Fmoc- β -Ala to **75**, followed by Fmoc removal (Figure 116). Next the *ortho*-phthalate-D-alanine-D-alanine-*tert*-butyl ester (**74**) was attached to the resin using HATU.

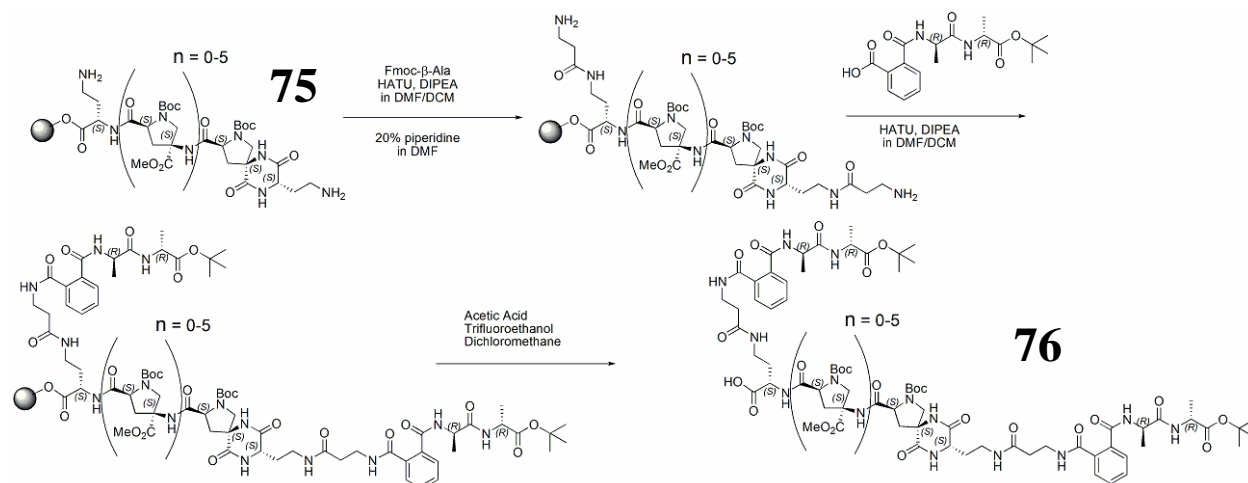


Figure 116 Synthesis and cleavage of bivalent- β -Ala-D-Ala-D-Ala. Bivalent D-Ala-D-Ala was made using the same steps, except omission of the β -Ala coupling and Fmoc removal step.

The second portion had the initial β -Ala coupling step omitted followed by coupling of **74** to **75** and cleavage to generate product **77** in an analogous way (Figure 117).

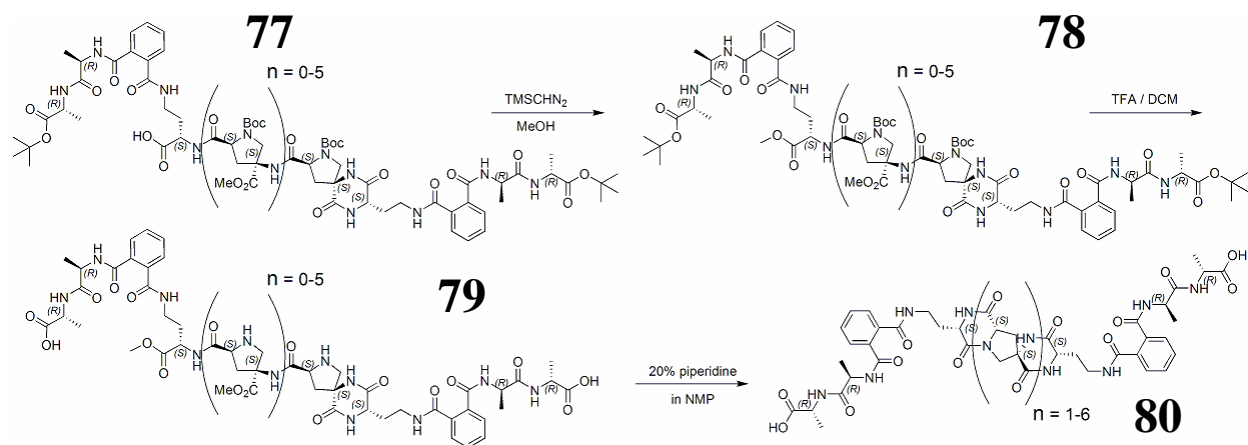


Figure 117 Synthetic steps of methyl esterification, acid-labile deprotection, and DKP closure to yield final bivalent D-Ala-D-Ala molecules **78**.

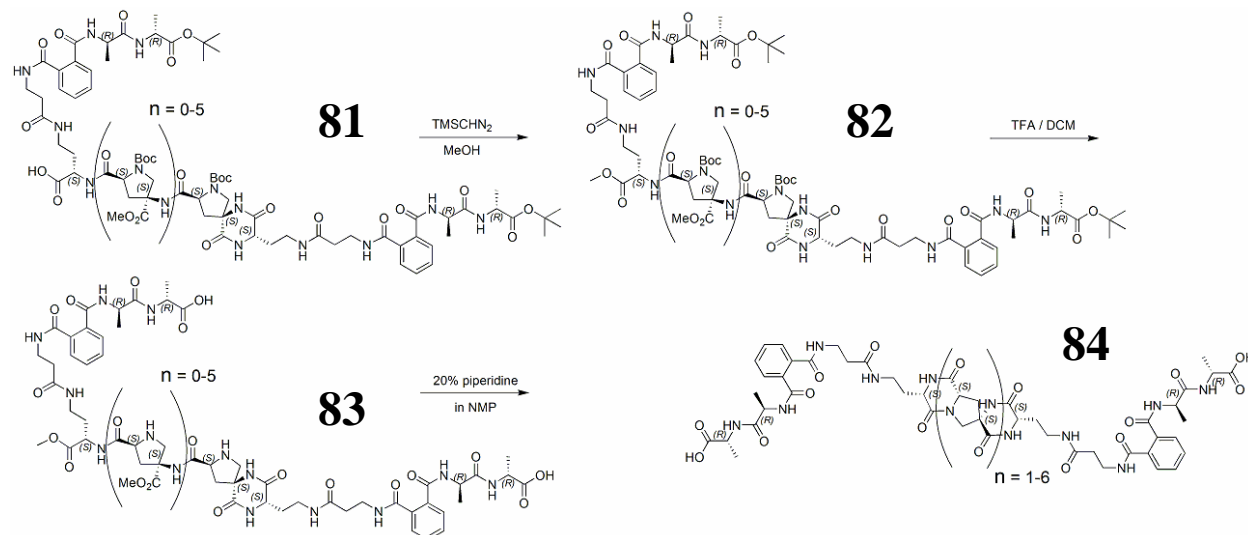


Figure 118 Synthetic steps of methyl esterification, acid-labile deprotection, and DKP closure to yield final bivalent β -Ala-D-Ala-D-Ala molecules. Analogous steps were used to synthesize the bivalent D-Ala-D-Ala molecules.

Cleavage was carried out in 0.5mL of AcOH:trifluorethanol:DCM (1:1:3) for 2hrs, and then the resin beads were again treated with 0.5mL of fresh cleavage cocktail, for 3 x 20min additional time to give **77/81**. The cleavage solutions were pooled and the solvent was removed in a SpeedVac. Addition of DCM:hexanes (3:1) to the oily residue followed by removal of the solvent in the SpeedVac, and repeating this two more times, the oily residue turned into a white pellet. This indicated that all of the AcOH had been removed, which would prove to be important for the success of the next step.

Dissolving the pellet in MeOH and treatment of the pellet with 25eq (based on initial resin loading and amounts) of trimethylsilyldiazomethane (TMSCHN₂) in ether, resulted in the

conversion of the carboxylic acid to the methyl ester **78/82** (Figure 117/118), with no apparent side reactions. Quenching of the excess TMSCHN₂ with TFA, followed by removal of the solvent in the SpeedVac resulted in an oil that was subsequently treated with TFA:DCM (1:1) to remove the Boc groups (**79/83**). Solvent was again removed in the SpeedVac. Closure of the diketopiperazines was carried out in 200μL of 20% piperidine in N-methyl pyrrolidone (NMP) at room temperature for 36hrs. Precipitation into ether, centrifugation, ether wash and centrifugation gave the final product **80/84**, which was resuspended in 0.5mL of 12.5% ACN, water with 0.25% formic acid.

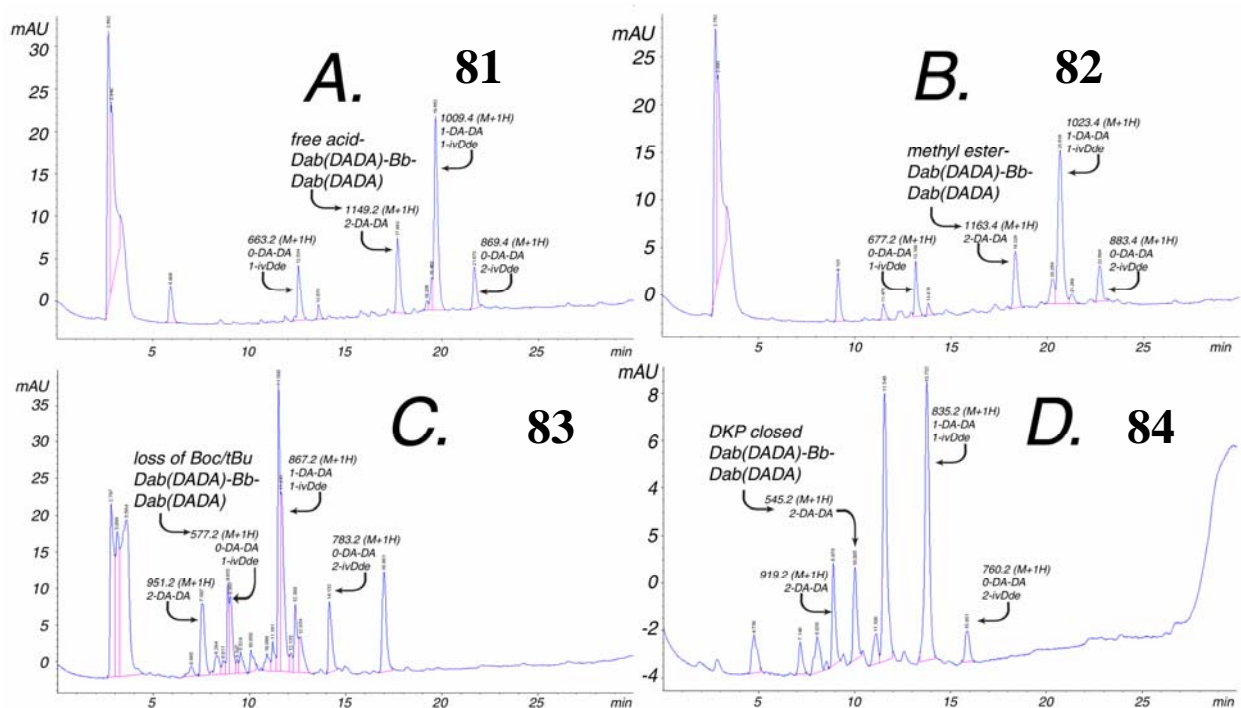


Figure 119 Representative HPLC chromatograms of 1mer-DA-DA (A) free acid, cleavage product (B) methyl ester product (C) TFA deprotection product (D) final crude DKP product.

Each of the 12 samples **80/84** was purified on the preparative HPLC, as a single injection. Fractions suspected of containing the desired product were reinjected onto the LCMS. In all cases, the desired product was obtained in acceptable amounts and in purity in excess of 90% (Figure 120/121). Fractions containing product were lyophilized, and resuspended in 0.5mL 25% ACN in water. 5μL was injected onto the analytical HPLC in order to quantify the amount of the bivalent D-Ala-D-Ala molecules, as described below. The samples were again lyophilized and stored at 4°C.

Quantitation of the amounts of material was done using an HPLC calibration curve made by injection of known amounts of a phthalic diamide. Phthalic anhydride was ring opened by glycine methyl ester, and another equivalent of glycine methyl ester was used to acylate the liberated free acid. The phthalic diamide was purified to homogeneity using the preparative HPLC. Appropriate fractions were pooled, lyophilized, and weighed to make a standard solution of known concentration.

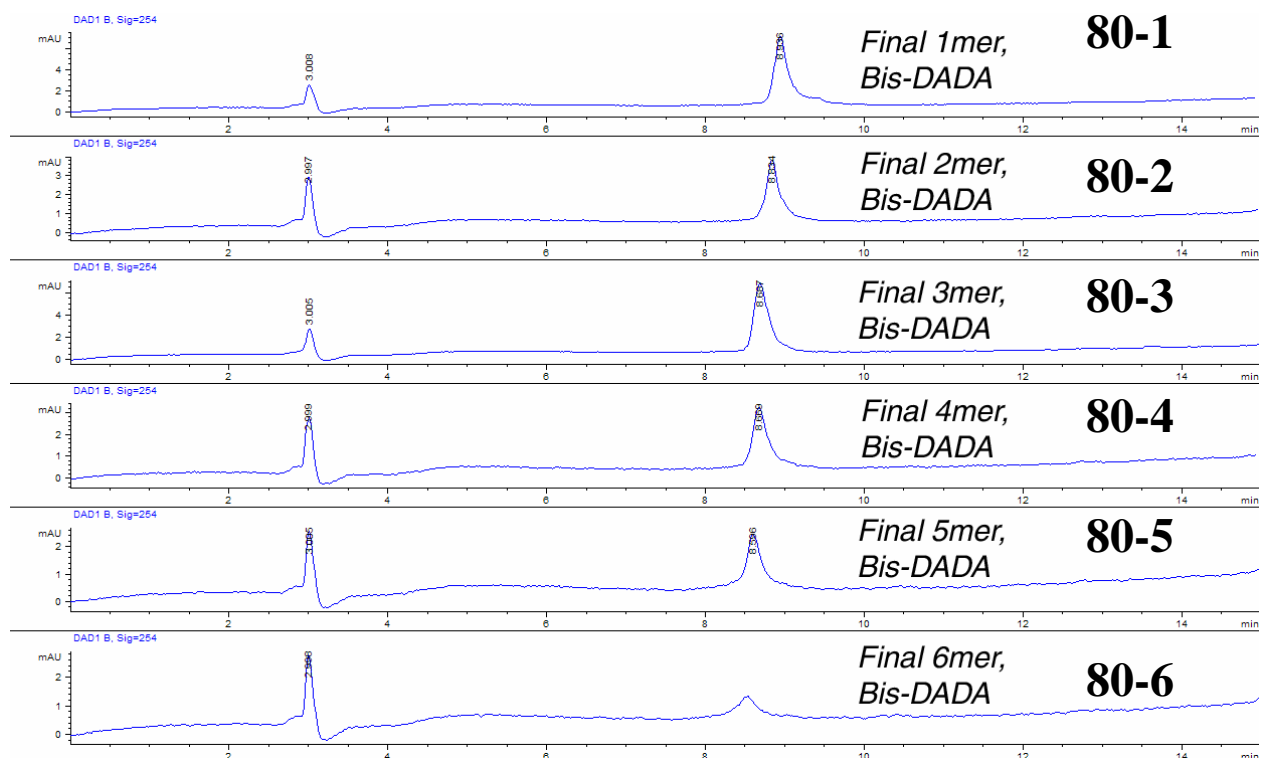


Figure 120 Final purified bivalent-D-Ala-D-Ala, 1-6mers

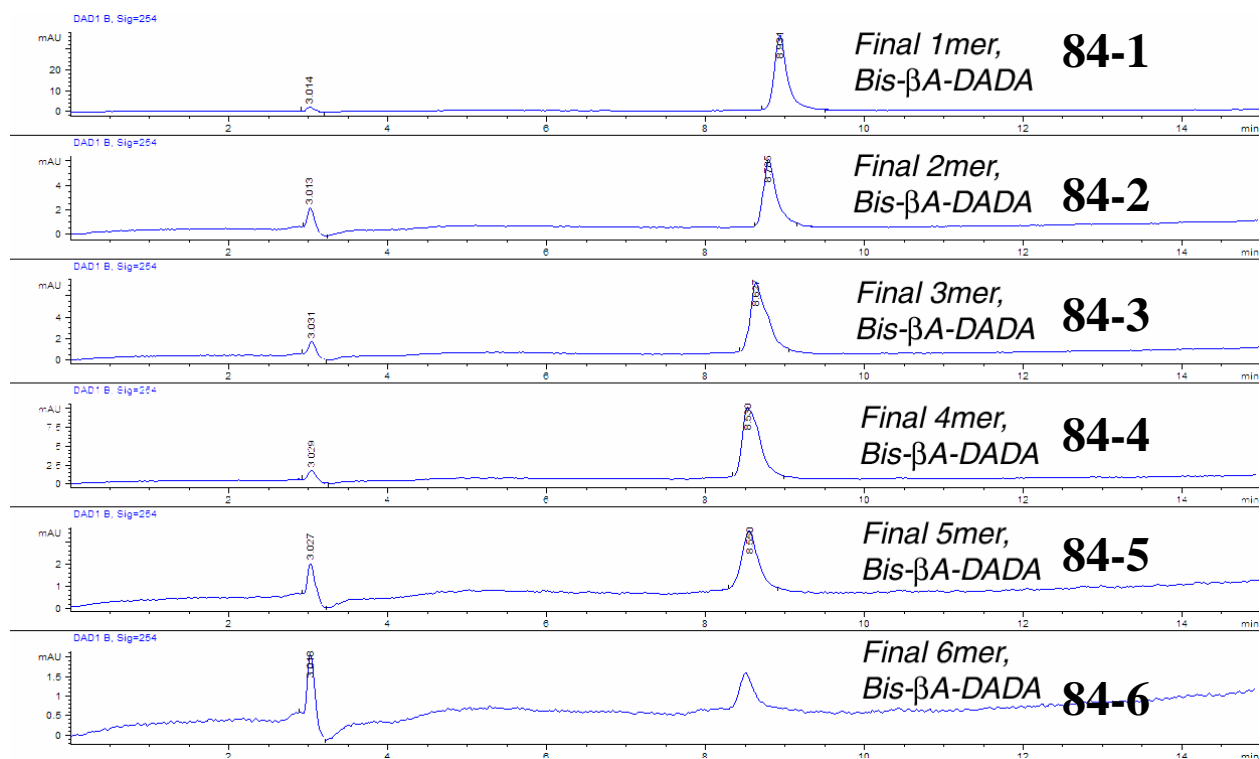


Figure 121 Final purified bivalent-β-Ala-D-Ala-D-Ala, 1-6mers

After analysis of the crude cleavage product by HPLC (Figure 119-A), it became clear that there was a problem with the removal of the ivDde groups. But after careful inspection, it was obvious that the difference in peak height was not a direct measurement of the relative amounts. This is because the ivDde chromophore has a much larger extinction coefficient than the substituted benzene ring of the phthalic diamide, although no experiments were done to identify the molar absorptivity of the ivDde. Previous experiences with the ivDde suggest that it is difficult to totally remove this protecting group. This suggests that the peaks corresponding to ivDde protected amines were minor contaminants. In subsequent experiments involving other projects, it was found that the ivDde could be more completely removed by increasing the hydrazine concentration from 2% to 4%. Also, some amount of product that failed to be acylated by the D-Ala-D-Ala can be identified throughout the series of manipulations. This is surprising, as an excess of D-Ala-D-Ala **74** and coupling reagent were used. However, this coupling reaction which failed to go to completion may be an anomaly, as the amount of unreacted amine in most of the other 11 sequences is much less or nonexistent. Nevertheless, the desired products were successfully synthesized and purified, although the yields for some of the longest sequences

could stand to be improved, this was more than enough material for subsequent analysis of binding by capillary electrophoresis.

Synthesis of the *bis*-vancomycin scaffolds also required some development. Coupling reactions involving the carboxylic acid of the vancomycin and an external amine have been utilized by Whitesides in his synthesis of his bivalent and trivalent vancomycin derivatives,[22, 25, 66] by Griffin in his synthesis of vancomycin carboxamide derivatives and vancomycin dimmers,[67, 68] and by Williams, who also studied vancomycin dimers.[69, 70] The general consensus of the various approaches is that when a mild coupling reagent, like HOBt/HBTU is used, reaction between the vancomycin carboxylic acid and a primary amine is favored, while in the case of more reactive coupling reagents, like PyBOP (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate) or PyBrOP (bromo-tripyrrolidinophosphonium hexafluorophosphate), the secondary amine and neopentyl amine of vancomycin become involved as possible sites of acylation. So, HOBt/HBTU was obviously the reagent of choice to accomplish a selective coupling between the scaffold diamine and two separate vancomycin molecules. Additionally, carrying out the coupling reaction in an ice bath will increase the selectivity for only the most reactive amines, improving the yield of desired product.

Initial tests of the efficacy of coupling vancomycin with HOBt/HBTU to a derivatized Rink amide resin proved to be successful, however upon cleavage with 95% TFA, total deglycosylation occurred to produce the undesired vancomycin aglycone, as anticipated. The sugar portion of vancomycin is not directly involved in the recognition of the D-Ala-D-Ala, although it surely provides added water solubility to polyaromatic core. We decided to first try other approaches for incorporation of the vancomycin into a scaffold, with solid-phase synthesis of aglycone-scaffolds as a backup plan.

Some conclusions from initial experiments on possible ways to attach vancomycin to a scaffold on a weak-acid cleavage resin follows. First, vancomycin failed to react with the first amino acid attached to the trityl resin. Diaminobutanoic acid was attached to the trityl resin and its side chain was deprotected. The vancomycin failed to acylate that amine, while tyrosine succeeded. This was interpreted as failure due to the steric clash of the bulky trityl resin with the equally bulky vancomycin. Secondly, a polymer-bound-HOBt was tested for its ability to be acylated with activated vancomycin, and then transfer that vancomycin to an amine in solution.

This approach failed primarily due to difficulties in completely washing away vancomycin which was not initially attached to the resin bound HOBt. Because these two approaches failed, the only to cleanly attach vancomycin to our scaffolds would be a solution-phase coupling. On that thought, one final test involved the feasibility of a post-reaction scavenging. The ability of the chloro-trityl chloride resin to scavenge excess carboxylic acid was tested with vancomycin. This was found to be possible, however times in excess of two day were needed to removed only 50% of the vancomycin. This may be useful in the future, but for now, a simple solution phase coupling reaction between the scaffold diamine and vancomycin was approach we would take.

The synthesis of the diamine scaffolds, of length 1-6 building blocks, proceeded using an approach similar to scaffold backbone of the *bis*-D-Ala-D-Ala, however, there were a few differences. Since there would be no on-resin derivatization of the side chain of the terminal amino acids, the shorter, diaminopropionic acid (Dpr) would be used instead of Dab. Also, the side chains needed to remain protected for the selective methyl ester formation with TMSCHN₂, so the Boc group was chosen as the side chain protecting group for Dpr.

It should be noted that an alternative approach employing Dab(ivDde) was attempted. This approach envisioned that the steps would involve cleavage, TMSCHN₂, then removal of ivDde using hydrazine, followed by coupling of vancomycin. It was hypothesized that the presence of the Boc groups on the building blocks would aid in the purification of the bis-vancomycin product away from vancomycin, as the polarity difference would be larger due to the non-polarity of the *tert*-butyl protecting groups. This attempt failed because the hydrazine was not easily removed from the reaction mixture. When the deprotection mixture was added to ether, in an attempt to precipitate the product, it was found that the hydrazine was not miscible with the ether. So, the hydrazine was removed in the SpeedVac. High temperatures (50°C) and long times (20hr) were needed to totally remove the high-boiling hydrazine. These conditions caused extensive epimerization of the oligomer, as judged by LCMS (Figure 122).

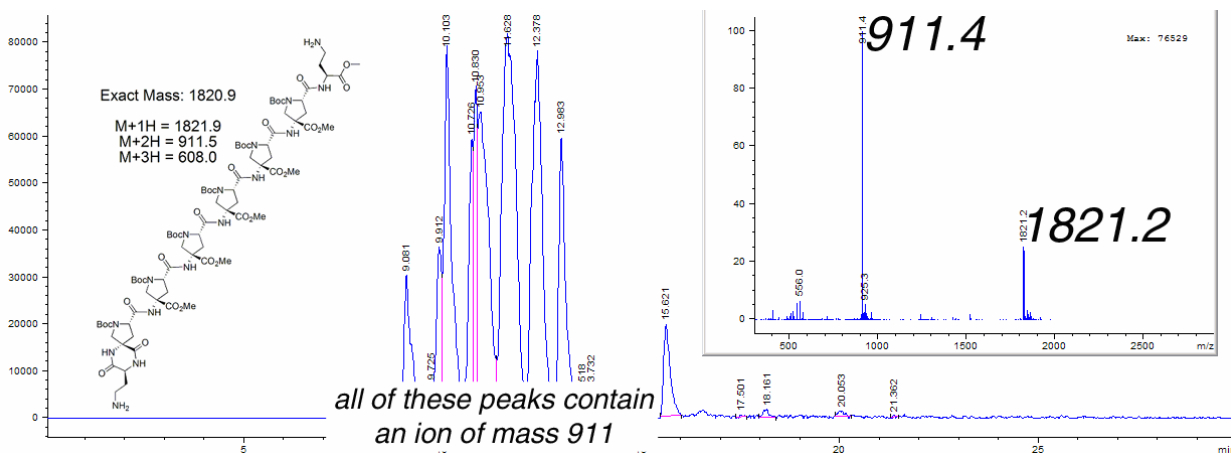


Figure 122 Extracted ion chromatogram illustrating the epimerization of the scaffold during removal of hydrazine after a solution-phase ivDde deprotection.

The second difference in the scaffold synthesis was that the leading-end Fmoc group was left on, because there were no hydrazine treatments at the end of this synthesis. Also this was the only intense chromophore in the molecule and facilitated characterization of all species, until the second-to-last step, when the DKPs were closed. When the Fmoc was removed, quantifying the amount of material in solution was achieved by using an HPLC calibration curve. This accuracy in measuring the concentration of diamine for the vancomycin coupling step ensured that precisely 5eq of vancomycin, relative to diamine, were used, greatly facilitating purification of the final *bis*-vancomycin product.

The final approach for the synthesis of *bis*-vancomycin scaffolds utilized, first the solid-phase synthesis of a scaffold diamine (Figure 122), followed by the solution-phase incorporation of vancomycin using HOBt/HBTU. The diamine was synthesized on the trityl resin, by first reacting the carboxylate salt of Fmoc-Dpr(Boc)-OH with the resin in DCM. Fmoc removal resulted in an amine that was acylated with pro4(2S4S) building block and HATU. After Fmoc removal, the resin was appropriately portioned, and the process of acylation, Fmoc removal, and portioning was repeated, resulting in six equimolar amounts of scaffolds containing a resin-bound Dpr(Boc), followed by one to six building blocks, all terminating in a deprotected amine. These amines were then acylated with Fmoc-Dpr(Boc)-OH and HATU, to yield the final product (**85**), which was washed, dried and then cleaved (Figure 124, **86**).

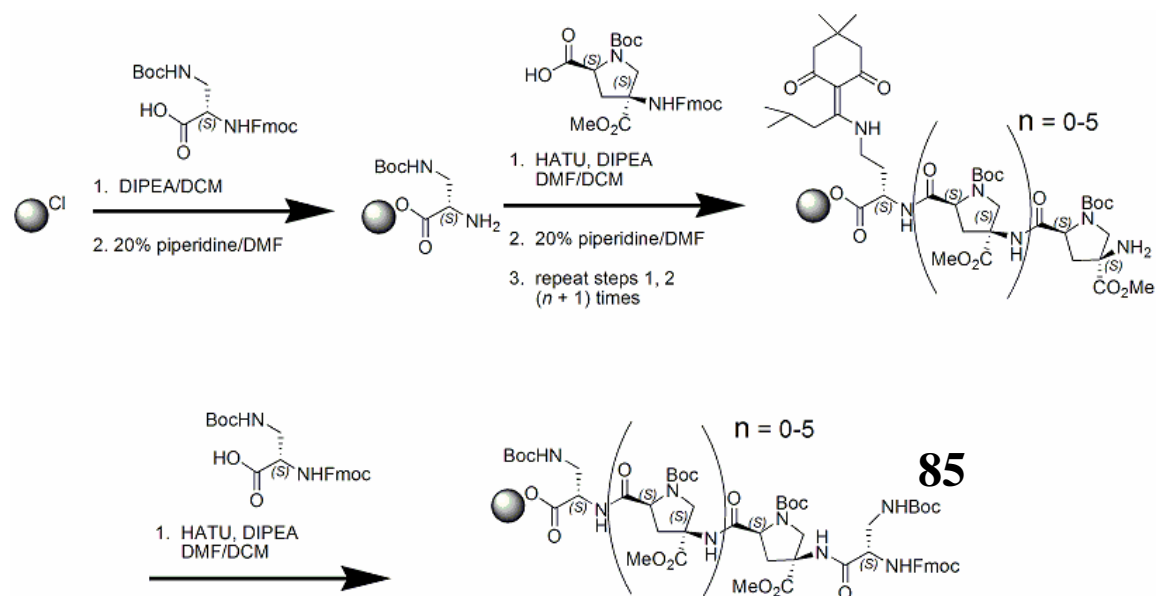


Figure 123 Solid phase synthesis of the scaffold diamines used to make bivalent vancomycins.

After cleavage (Figure 124, **86**), the liberated trailing end carboxylic acid was esterified with TMSCHN₂ to form **87**. The Boc groups were then removed using TFA in DCM to yield **88**. Each of the six sequences was then aliquoted into 4 equal volumes, and the solvent removed in the SpeedVac, and stored in a desiccator, until needed. When a vancomycin was to be attached, first the DKPs were closed in 200 μ L of 20% piperidine in NMP (**89**). It was especially important to use small volumes for this step, so that precipitation could be done into a minimum volume of ether (~1.5 mL). In the case of the shorter sequences, the insolubility in ether was not as pronounced as the longer sequences, due to smaller mass and smaller numbers of amide bonds. In order to recover the desired product from the ether precipitations, it was therefore critical to use small volumes of piperidine/NMP and correspondingly small volumes of ether. The resulting pellet was washed at least twice with fresh ether in order to remove as much piperidine as possible, because it too could be a substrate for the vancomycin coupling reaction.

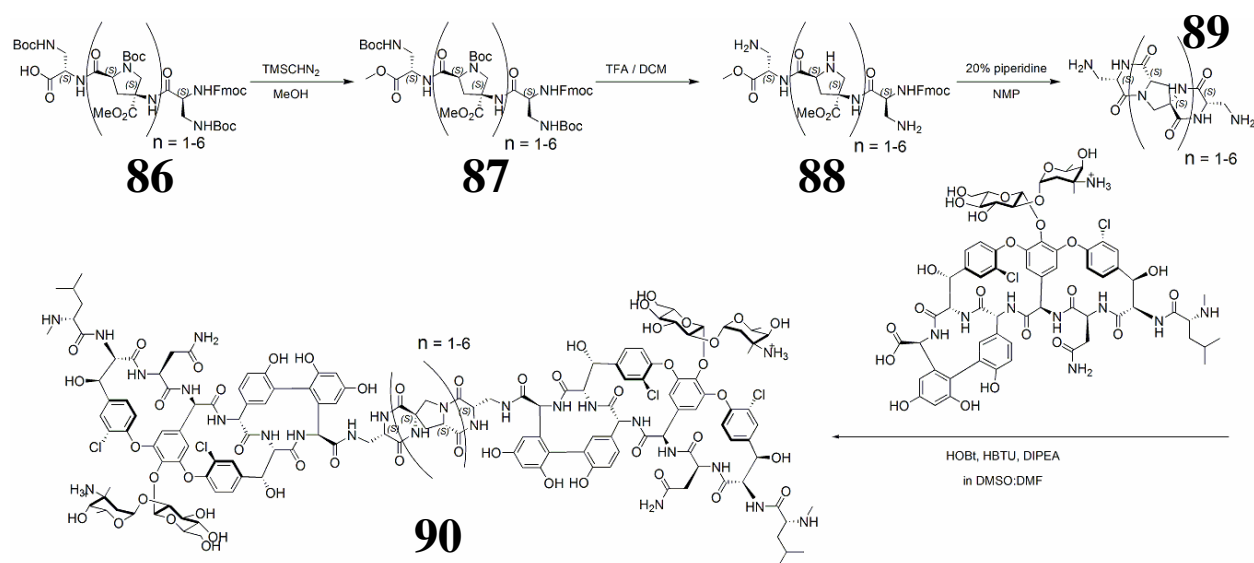


Figure 124 Synthesis of scaffold diamine and coupling of two vancomycins.

As mentioned above, the LCMS chromatogram used to confirm that all DKPs were closed also was used to quantify the amount of fullvene and corresponding diamine. Of course, this assumes complete recovery in the ether precipitation. Amounts of vancomycin, HOBt, and HBTU, which could be accurately weighed (at least 5mg), were used and the coupling reagents and vancomycin were put in separate microcentrifuge tubes, and placed in an ice bath. To each tube, $\text{DMF}:\text{DMSO}$ (1:1) was added, and sonication was used to solubilize the reagents. Vancomycin required particular attention to totally dissolve all material, although better results were seen when the DMSO was added first, followed by sonication, and then addition of the DMF . Appropriate volumes containing 5eq of each reagent were then added to the diamine pellet in an ice bath. Cycles of a few seconds of sonication followed by a minute in the ice bath served to solubilize the pellet and mix it with the reagents while keeping the temperature low. Addition of base started the coupling reaction, which was monitored by LCMS every 30min (Figure 125). Not much difference was seen between $t=30\text{min}$ and $t=60\text{min}$, so the reaction was deemed to be complete. Diamine was visible by LCMS, although it was not possible to quantitate, beyond saying it was still present. Additionally, the presence of a scaffold containing only one vancomycin was identified by MS, but not deemed to be significant by LC.

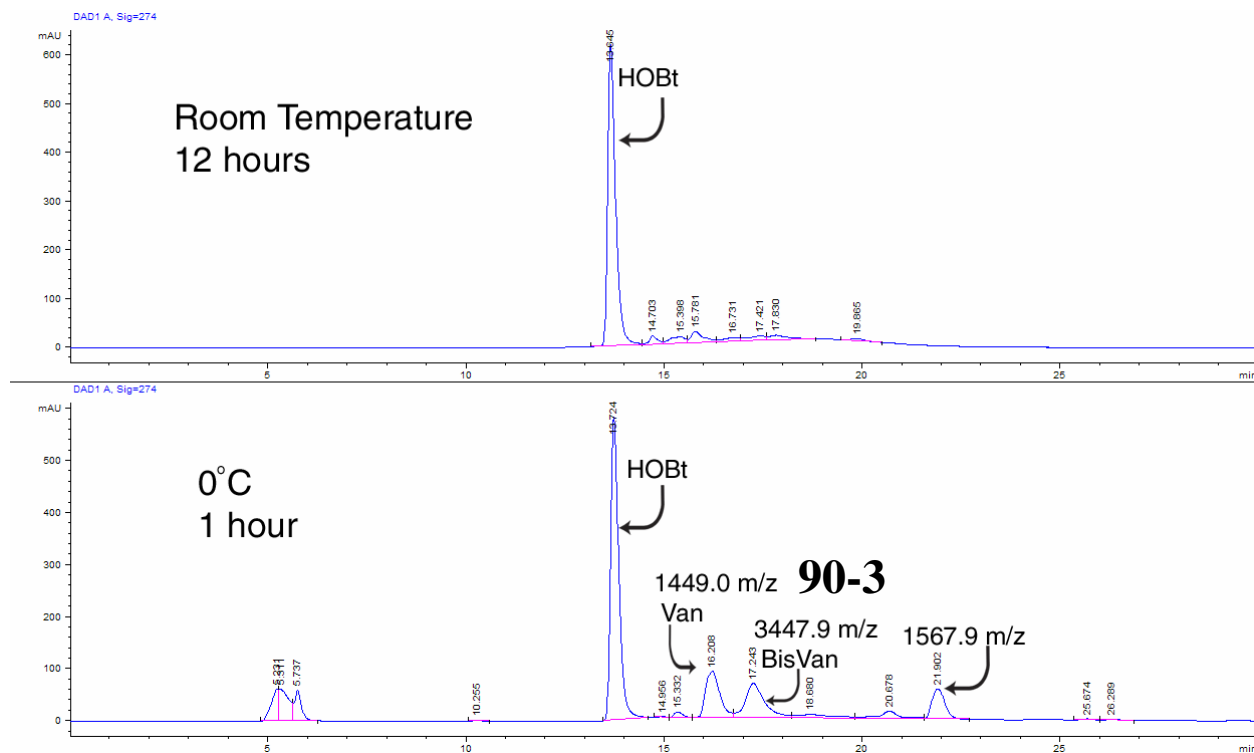


Figure 125 HPLC chromatograms illustrating the importance of time and temperature on the synthesis of a *bis*-vancomycin, 3mer.

The ease of isolation of the desired product could be improved by addition of a hydrophobic amine which would react with excess vancomycin to form a more nonpolar vancomycin amide. Initially, octadecylamine (C18) was employed, but it was found to be insoluble in DMF:DMSO. Octylamine was settled on, as it was readily soluble in DMF and, being a liquid, it was easily added to the reaction. Simply adding the amine to the reaction after one hour served to “scavenge” excess vancomycin (Figure 126/127) and convert it to a product that was much more nonpolar, and therefore, easier to isolate away from the desired product. In spite of addition of excess amine, it was not possible to remove all of the vancomycin. This was thought to be due to the partial consumption of the active ester-vancomycin adduct, by advantageous water in the solvent or, more likely, water of hydration of the vancomycin starting material. Addition of more coupling reagent when the amine was added was considered, but it was deemed to be too risky, as the desired product was present, and any further perturbation of it was thought not to be wise.

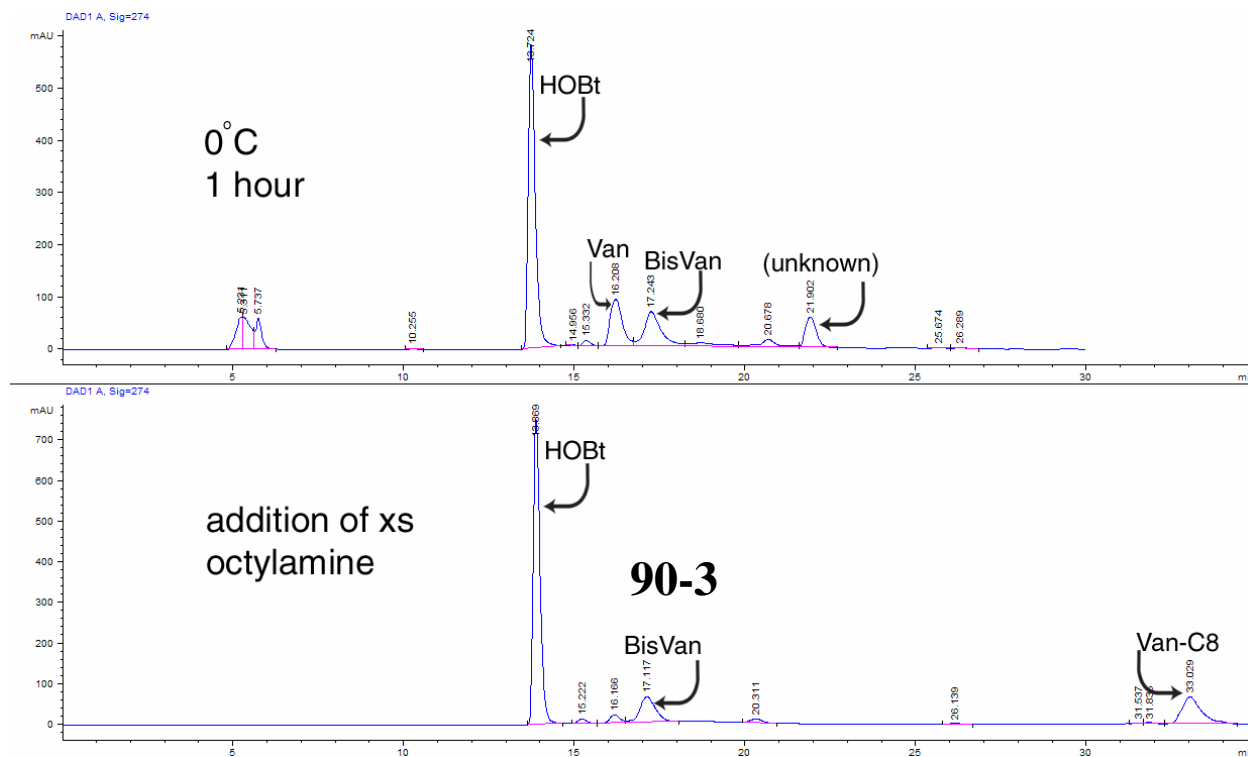


Figure 126 HPLC chromatograms illustrating the effective scavenging of excess vancomycin by octylamine.

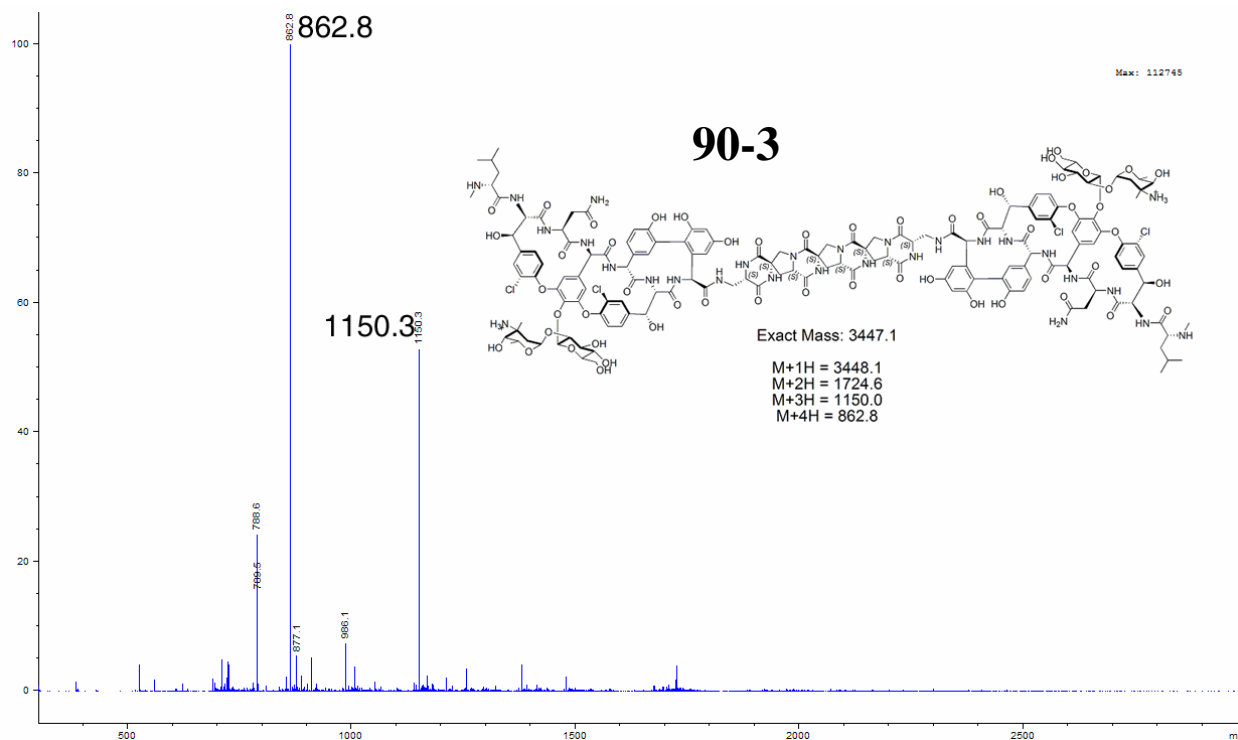


Figure 127 Mass spectrum of the bis-vancomycin, 3mer oligomer peak.

After completion of the scavenging reaction (30 additional minutes) the reaction was quenched by adding 10 volumes of water, 10% ACN, 0.5% formic acid. This was then filtered and purified as a single injection on the preparative HPLC. Again all peaks were collected, and saved until reinjection on the LCMS confirmed the identity of each. Tubes containing product were pooled and lyophilized (some representative chromatograms are listed below in Figures 128- 132). The product was then redissolved in 0.5mL water, 10% acetonitrile, and a small injection of this on the analytical HPLC facilitated quantification using a calibration curve from known amounts of vancomycin. Lyophilizing again yielded the final product, which was stored at 4°C.

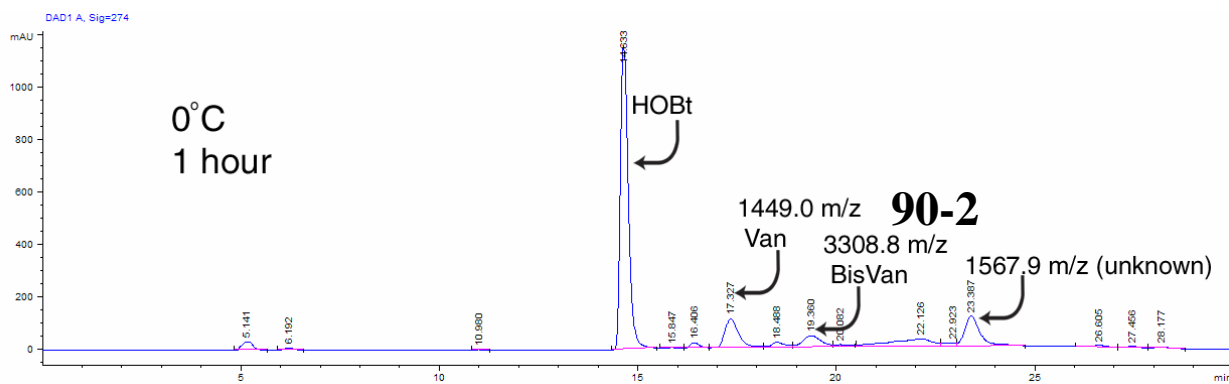


Figure 128 HPLC chromatogram of the crude *bis*-vancomycin, 2mer product (the mass listed above is the calculate M+1H, derived from the various multiply charged species).

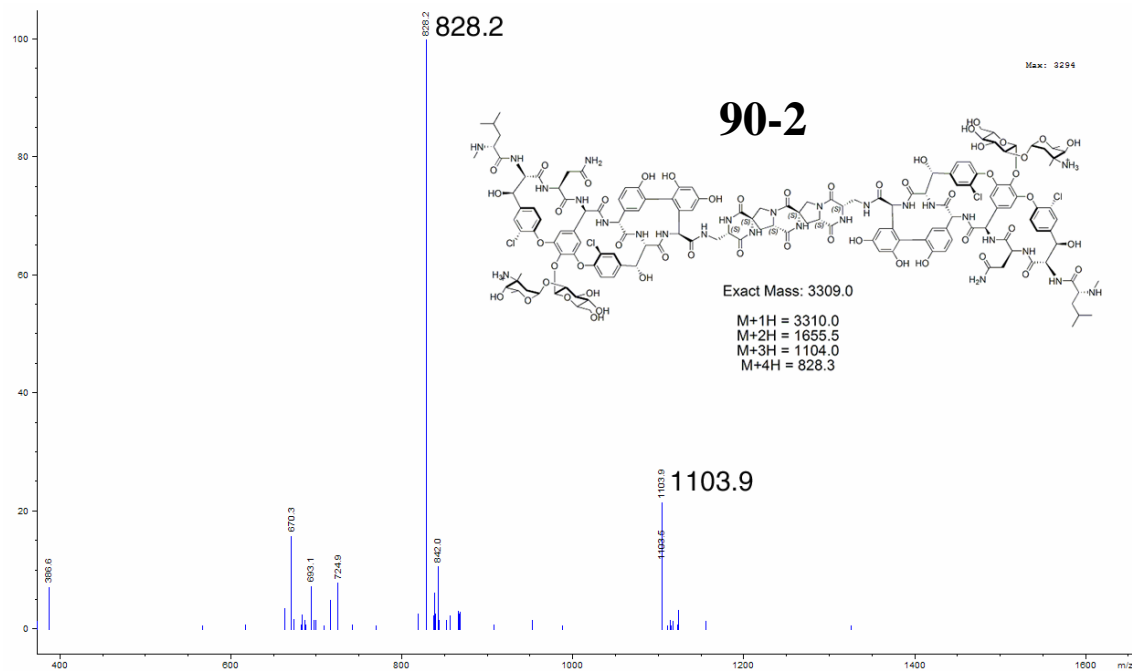


Figure 129 Mass spectrum of the *bis*-vancomycin, 2mer oligomer peak.

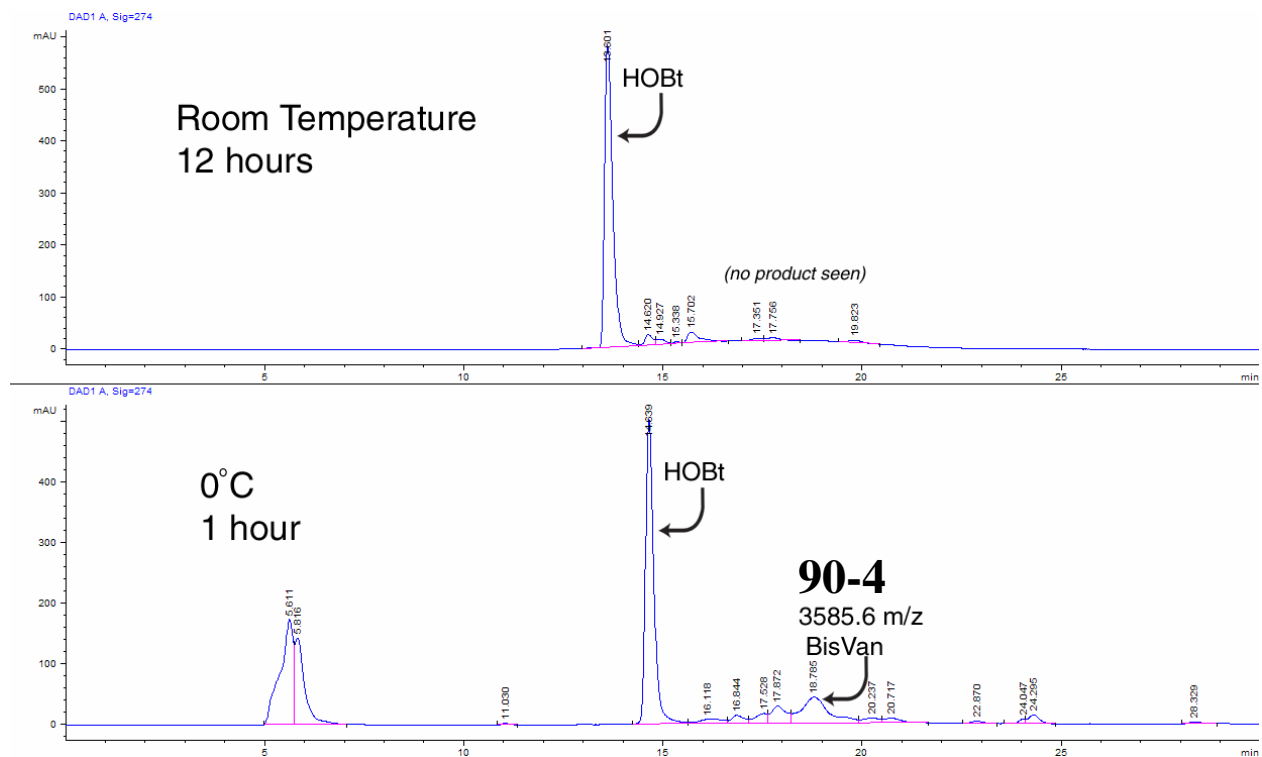


Figure 130 HPLC chromatograms illustrating the importance of time and temperature on the synthesis of a bis-vancomycin, 4mer (the mass listed above is the calculate M+1H, derived from the various multiply charged species).

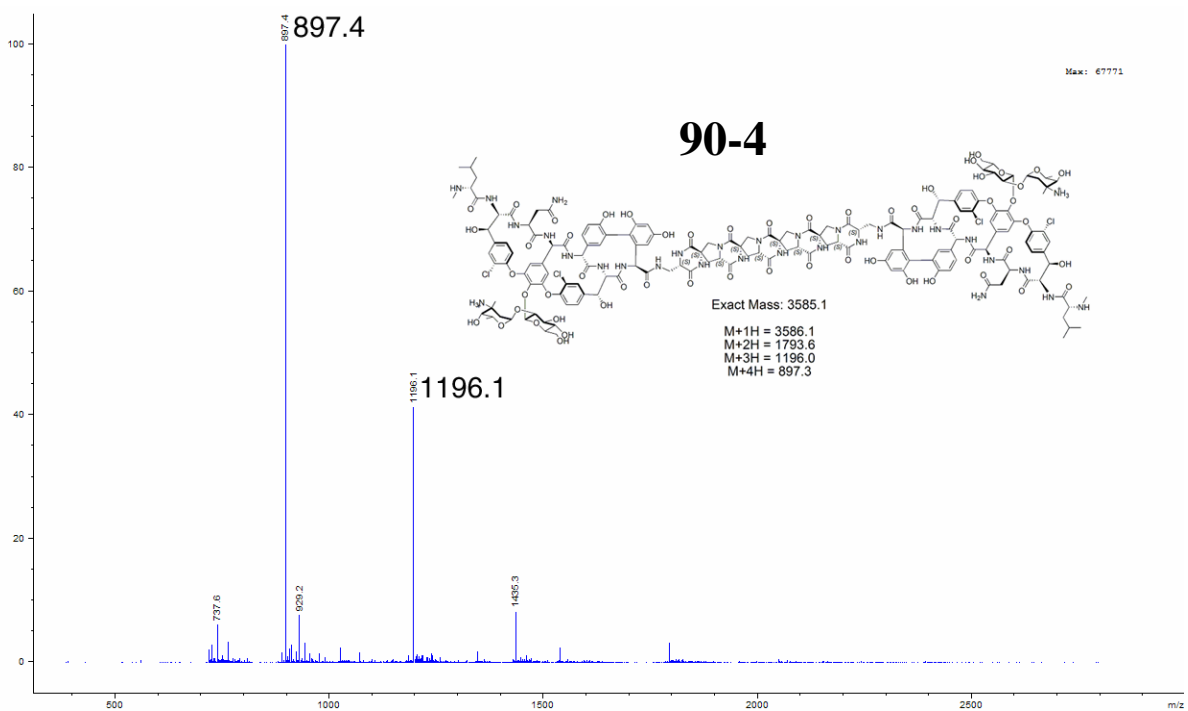


Figure 131 Mass spectrum of the bis-vancomycin, 4mer oligomer peak.

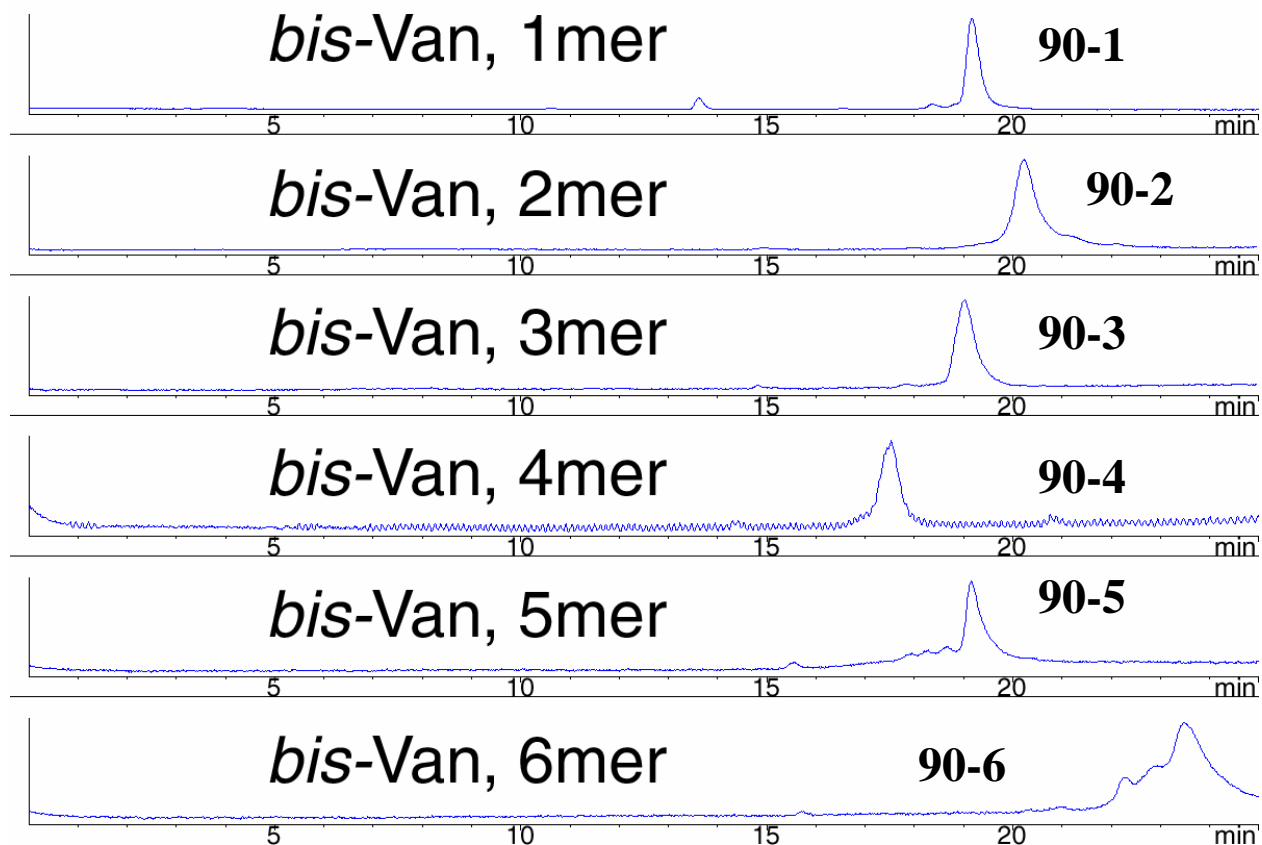


Figure 132 Final purified *bis-Van*, 1-6mer oligomers.

The synthesis and purification of the varying lengths of scaffolds, *bis-vancomycin* derivatives were, for the most part, successful, although some improvements could be made. Namely, the amount of material initially present was, frankly, miniscule. Nanomoles of scaffold were present, so trace water and minor impurities were more of any issue than would be the case if the scale were larger. Nevertheless, five of six *bis-vancomycin* scaffolds were made in acceptable yield and good to excellent purity. In these cases, the products were free of any other contaminating byproducts containing vancomycin, which was an absolute requirement for studies of bivalent molecules. In the only other sample, the desired product was the major component, however, the purity was questionable.

4.3 CONCLUSIONS

The synthesis of a series of *bis*-vancomycin and *bis*-D-Ala-D-Ala bivalent molecules has been successfully completed. Capillary electrophoresis is currently being employed to measure the binding constants of each individual *bis*-D-Ala-D-Ala with each individual *bis*-vancomycin, according to procedures and methods outlined by Whitesides.[66, 71-73] After this initial work is done, competition experiments are planned, where a single *bis*-vancomycin can choose multiple *bis*-D-Ala-D-Ala in which to bind. Finally, multiple *bis*-vancomycin and multiple *bis*-D-Ala-D-Ala pairs will be analyzed to assess the extent of selectivity as matched bivalent pairs.

4.4 EXPERIMENTAL

General: Dichloromethane was distilled from CaH₂. All other reagents were used as received, unless stated otherwise. Column chromatography was performed using ICN Silitech 32-63 D (60 Å) grade silica gel and TLC analysis was performed on EM Science Silica Gel 60 F₂₅₄ plates (250µm thickness). NMR spectroscopy was performed using a Bruker 300 MHz instruments. Chemical shifts were reported in parts per million downfield relative to trimethylsilane and categorized as br = broad, s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, q = quartet, and m = multiplet. Gradient normal phase chromatography was done on the Isco Combiflash Companion, with detection at 254nm. Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. Diisopropylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and *N*-Hydroxybenzotriazole hydrate (HOBt) were obtained from Novabiochem. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and

an inert atmosphere over the reaction. HPLC-MS analysis was performed on a Hewlett-Packard Series 1050 instrument equipped with a Waters Xterra MS C₁₈ column (3.5 μ m packing, 4.6 mm x 150 mm) and a diode-array detector, while the MSD-ESI was Series 1100. Preparative purification was done using a Varian Prostar 500 equipped with a Waters Xterra Prep MS-C₁₈ column (5 μ m packing, 10 mm x 100 mm), equipped with a dual wavelength detector.

N- α -Carbobenzyloxy-D-alanine-D-alanine-*tert*-butyl ester (75).

A solution of N- α -Carbobenzyloxy-D-alanine free acid (mmol) HOBt (mmol) and HBTU (mmol) in dry DMF was stirred for 5min in a 100mL round-bottom flask. DIPEA (mmol) was then added, producing a yellow color characteristic of an OBt active ester. The amine, alanine-*tert*-butyl ester (mmol) was then added, and the mixture was stirred for 2hrs. The reaction mixture was then poured into 20 volumes of ice water, producing a dense precipitate, which was collected using a suction filter. The precipitate was then dried *in vacuo* for 3hrs, then dissolved in chloroform and transferred to a 250mL round-bottom flask. 20g celite was then added and the solvent removed using a rotovap. The water bath was then turned to 50C, and rotovapping continued for 30min to remove residual solvent. The celite was easily transferred to an Isco loading cartridge. Automated flash chromatography using an automated Isco Companion and a custom-built steel column containing ~300g silica was then performed using 3:2 ethyl acetate:hexanes. The fully protected dipeptide was obtained in good yield (%). ¹H NMR

NH₂-D-alanine-D-alanine-*tert*-butyl ester (73).

The Cbz protected dipeptide (mmol) was dissolved in distilled EtOAc in a 100mL round bottom flask. Palladium-on-charcoal (mmol) was added, along with a stir bar. A double-balloon of hydrogen was connected to the flask using a three-way valve, and the side-arm of the valve was connected to an aspirator. At least five cycles of aspiration (until boiling) and H₂ backfill were performed to fully degas the solvent as well as charge the palladium with hydrogen. The reaction was allowed to stir overnight. Upon completion, the Pd/C was removed by filtration, followed by washing with 3 x 30mL of EtOAc. The filtrates were combined and the solvent was removed *in vacuo*. The free base was obtained in good yield (%). ¹H NMR (300MHz, 25°C, CDCl₃) δ 7.7 (br, 1H), 4.39-4.44 (m, 1H), 3.45-3.52 (q, 1H), 2.77 (s, 1H), 1.60 (br, s, 2H), 1.44 (s, 9H), 1.30-1.36 (dd, 6H)

***ortho*-phthalate-D-alanine-D-alanine-*tert*-butyl ester (74).**

The free base (mmol) was dissolved in dichloromethane, and stirred in a 50mL conical flask. The phthalic anhydride (mmol) as the limiting reagent was added and stirred for 1hr. Removal of the solvent *in vacuo* afforded an oil. The crude product was dissolved in 5mL chloroform and taken up in a syringe and injected onto an Isco column containing 40g of silica, equilibrated in chloroform. A gradient of chloroform to 10% MeOH in chloroform, performed on the Isco Companion served to purify the final product, and removal of the solvent *in vacuo* produced the final product in good yield (%). $^1\text{H NMR}$ (300MHz, 25°C, CDCl_3) δ 10.0 (br, 1H), δ 7.9 (d, 1H, $J = 7.6\text{Hz}$), δ 7.5 (m, 4H), δ 7.3 (d, 1H, 7.3Hz), δ 4.8 (m, 1H), δ 4.5 (m, 1H), δ 1.5 (d, 3H, $J = 7.0$), δ 1.4 (s, 9H), δ 1.4 (d, 3H, $J = 7.2\text{Hz}$),

Solid-phase synthesis of resin-Dab-(ivDde)-pro4-(2S4S)_n-Dab-(ivDde), end-DKP (n=1-6) (76).

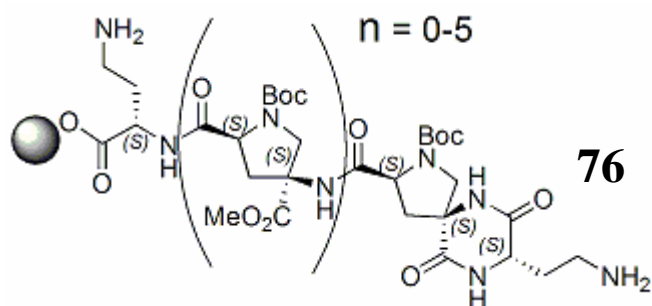


Figure 133 Synthesis of resin bound intermediate for *bis*-D-Ala-D-Ala molecules.

To a 10mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added chlorotriyl chloride Resin (Novabiochem) (30mg, 36 μmol loading). The bottom on the reactor was capped and a small stir bar was added. A solution of Fmoc-L-Dab(ivDde)-OH (Novabiochem) (99 mg, 180 μmol) in 900 μL of distilled DCM and 94 μL of DIPEA (540 μmol) was prepared in a 1.5 mL microcentrifuge tube and then added to the reactor and stirred for 2hrs. The stir bar and cap were removed, the solution drained and the beads were washed 5x with DCM, and then washed with DMF, 2x. The Fmoc-protected amine was deprotected in 2mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5 mL microcentrifuge tube, the coupling solution was made by dissolving 39.2 mg (72 μmol) of Boc-protected pro4(2S4S) building block[17] and 27.4 mg of HATU (72 μmol) in 360 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 25.1 μL (144 μmol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release. After the Fmoc deprotection, the resin was washed with DCM several times, and allowed to dry for five minutes via aspiration. The beads were now easily removed from the reactor and then weighed. An appropriate weight fraction (i.e. 1/6th removed after 1st building block was coupled, then 1/5th after second building block, etc.) was then removed, placed in a fresh 1.0 mL reactor and stored in a desiccator. The resin was then swelled in DMF for 10 min. The process of coupling/deprotection/aliquot removed was repeated five additional times, making 6 portions of equimolar amounts of 6 different lengths, 1-6 building blocks, with the final amine deblocked

In a 1.5 mL microcentrifuge tube, the final coupling solution was made by dissolving 39.3 mg (108 μmol) of Fmoc-L-Dab(ivDde)-OH (Novabiochem) and 41.1 mg of HATU (108 μmol) in 540 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 37.6 μL (216 μmol) of N,N-diisopropylethylamine (DIPEA) was added to make the active ester. After 10min activation time, the solution was added, in six equal portions to the 6 resins of varying lengths, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF, and the coupling was repeated. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DMF for 30min. The Diaminobutanoic acid side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the two side chain amines. After deprotection, the resin was washed with DMF, IPA, DMF, IPA, DCM, DCM, DCM, and allowed to dry under aspiration for 5min. The deprotected resins of varying numbers of building blocks was split into 3 equal portions, with one being stored in the desiccator and the other two being used for subsequent conversion into the two types of bivalent D-Ala-D-Ala.

Synthesis of bivalent D-Ala-D-Ala of lengths n=1-6 (80).

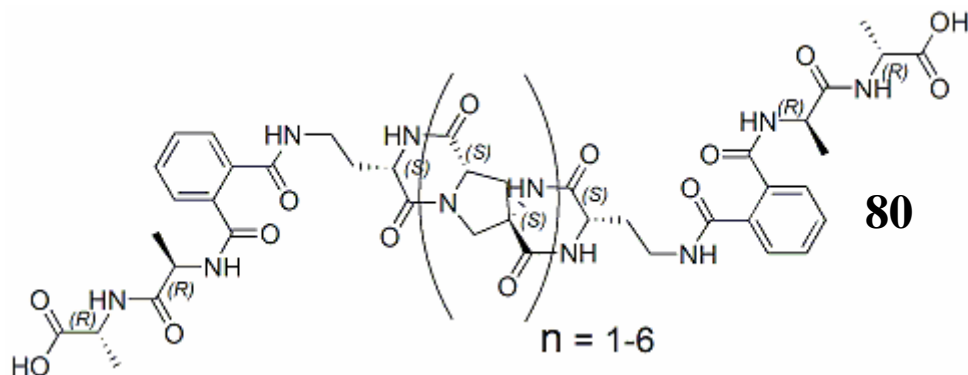


Figure 134 Bivalent D-Ala-D-Ala scaffolds.

The 6 resins of differing lengths, consisting of approximately 6 x 2 μ mol deprotected scaffold (6 x 4 μ mol free amines), were swelled in DMF for 30min. During which, *ortho*-phthalate-D-alanine-D-alanine-*tert*-butyl ester (26.2 mg, 72 μ mol) and HATU (27.4 mg, 72 μ mol) was dissolved in 720 μ L 20% DCM/DMF, and then, after addition of 25.1 μ L of DIPEA (144 μ mol), the mixture was allowed to sit for ten minutes. The preactivated substrate was then added to the resins in 6 equal portions, and allowed to react with argon bubbling for 30min. The beads were then washed with DMF, 3x, and the coupling reaction was repeated again, followed by an extensive washing with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then overnight drying, *in vacuo*.

The 6 reactors were then cleaved in 1mL of 20% AcOH, 20% trifluoroethanol, in DCM, with stirring for 2hrs. This was then drained and 3 x 1mL additional cleavage cocktail with stirring for 3 x 15min served to remove any residual product. The pooled cleavage solutions were transferred into a 13mm glass tube, and the solvent was removed in a SpeedVac. An additional ~4mL of DCM and ~2mL hexanes served to azeotropically distill off residual acetic acid, and was repeated an additional three times, resulting in a dry white powder.

The white powder was dissolved in 1mL of dry MeOH, a stir bar was added and ~25eq trimethylsilyldiazomethane (TMSCHN₂), as a 2M solution in ether was added dropwise. Slight bubbling and yellow color resulted. The reaction was stopped after 10min by addition of an identical volume of trifluoroacetic acid, also dropwise. More bubbling was apparent and the color disappeared. The solvent was again removed in the SpeedVac. A stir bar was added and the residue was dissolved in 4mL TFA/DCM (1:1) and stirred for 2hrs. The solvent was

removed in the SpeedVac, resulting in an oily residue. 200 μL of 20% piperidine in NMP was added to the residue. Incubation at room temperature for 36hrs served to close the diketopiperazines. After this, the product was precipitated into 1.5 mL of ether, in a 2mL microcentrifuge tube. The final products of varying lengths ($n=1-6$ building blocks) were purified on a preparative HPLC column, as a single injection. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. The tubes containing the desired product were lyophilized, redissolved in 0.5mL of 25% ACN in water. Using an HPLC calibration curve derived from the glycine methyl ester diamide of phthalic acid, a 5 μL injection allowed for quantitation of the amount of bivalent D-Ala-D-Ala. The remaining samples were again lyophilized and the tubes were then stored at 4°C.

Synthesis of bivalent β -Ala-D-Ala-D-Ala of lengths $n=1-6$ (84).

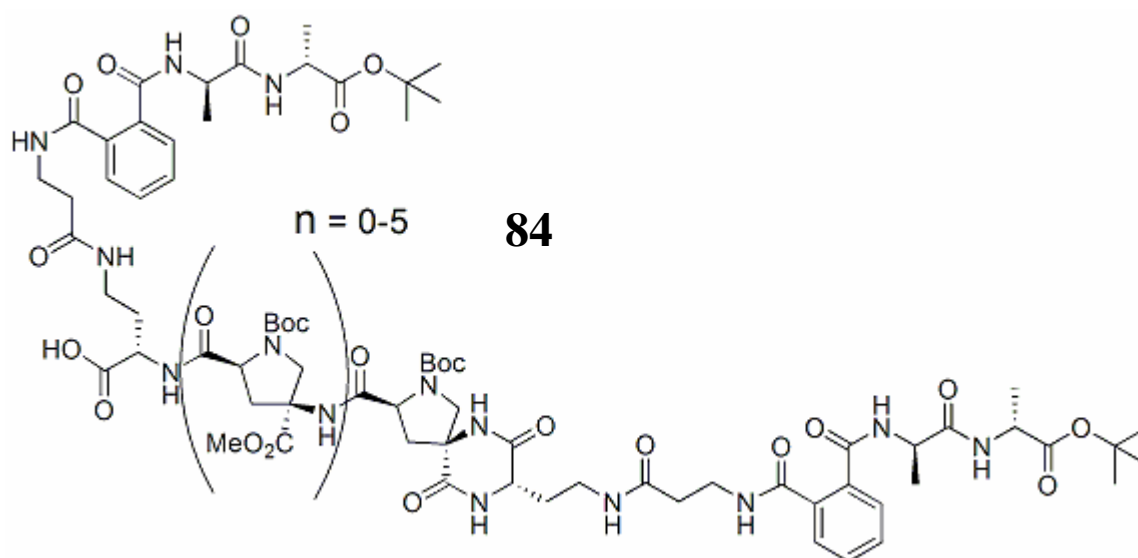


Figure 135 Bivalent β -Ala-D-Ala-D-Ala scaffolds.

The 6 resins of differing lengths, consisting of approximately 6 x 2 μmol deprotected scaffold (6 x 4 μmol free amines), were swelled in DMF for 30min. During which, Fmoc- β -Ala (37.3 mg, 120 μmol) and HATU (45.6 mg, 120 μmol) was dissolved in 600 μL 20% DCM/DMF, and then, after addition of 41.8 μL of DIPEA (240 μmol), the mixture was allowed to sit for ten minutes. The preactivated substrate was then added to the resins in 6 equal portions, and allowed to react with argon bubbling for 30min. The beads were then washed with DMF, 3x, and the coupling reaction was repeated again, followed by Fmoc deprotection for 30min in 0.5mL of

20% piperidine/DMF. The Abs at 301nm of a 1/100th dilution was measured to quantify the fullvalene released. The resins were then washed with DMF, IPA, DMF, IPA, DMF, and swelled in DMF for 10min. This was followed by double coupling of the *ortho*-phthalate-D-alanine-D-alanine-*tert*-butyl ester followed by drying *in vacuo* and cleavage as outlined above.

Removal of the solvent from the cleavage solution followed by methyl esterification using TMSCHN₂, removal of the Boc groups and *tert*-butyl ester using TFA, and diketopiperazine closure in piperidine/NMP was done identically as outlined above. Purification and quantifying the final purified product resulted in bivalent β-Ala-D-Ala-D-Ala, varying in length of the spacer from 1-6 building blocks.

Solid-phase synthesis of resin-Dpr-(Boc)-pro4-(2S4S)_n-Dpr-(Boc)-Fmoc (n=1-6) (88).

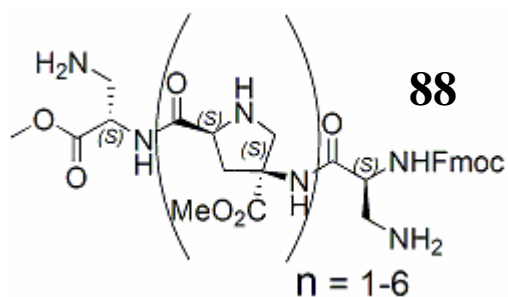


Figure 136 Diamine scaffolds, before DKP closure.

To a 10mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added chlorotriyl chloride Resin (Novabiochem) (30mg, 36μmol loading). The bottom on the reactor was capped and a small stir bar was added. A solution of Fmoc-L-Dpr(Boc)-OH (Novabiochem) (76.7 mg, 180 μmol) in 900 μL of distilled DCM and 94.1 μL of DIPEA (540 μmol) was prepared in a 1.5 mL microcentrifuge tube and then added to the reactor and stirred for 2hrs. The stir bar and cap were removed, the solution drained and the beads were washed 5x with DCM, and then washed with DMF, 2x. The Fmoc-protected amine was deprotected in 2mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 2mL microcentrifuge tube, the coupling solution was made by dissolving 39.2 mg (72 μmol) of Boc-protected pro4(2S4S) building block[20] and 27.4 mg of HATU (72 μmol) in

360 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 25.1 μL (144 μmol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release. After the Fmoc deprotection, the resin was washed with DCM several times, and allowed to dry for five minutes via aspiration. The beads were now easily removed from the reactor and then weighed. An appropriate weight fraction (i.e. 1/6th removed after 1st building block was coupled, then 1/5th after second building block, etc.) was then removed, placed in a fresh 1.0 mL reactor and stored in a desiccator. The resin was then swelled in DMF for 10 min. The process of coupling/deprotection/aliquot removed was repeated five additional times, making 6 portions of equimolar amounts of 6 different lengths, 1-6 building blocks, with the final amine deblocked

In a 1.5 mL microcentrifuge tube, the final coupling solution was made by dissolving 46.0 mg (108 μmol) of Fmoc-L-Dpr(Boc)-OH (Novabiochem) and 41.1 mg of HATU (108 μmol) in 540 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 37.6 μL (216 μmol) of N,N-diisopropylethylamine (DIPEA) was added to make the active ester. After 10min activation time, the solution was added, in six equal portions to the 6 resins of varying lengths, and allowed to react by argon mixing for 30min. The resin was then washed 3 x 2min with DMF, and the coupling was repeated. The last Fmoc groups MUST be left on to ensure the success of the subsequent steps. The resin was washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then overnight drying, *in vacuo*.

The 6 reactors were then cleaved in 1mL of 20% AcOH, 20% trifluoroethanol, in DCM, with stirring for 2hrs. This was then drained and 3 x 1mL additional cleavage cocktail with stirring for 3 x 15min served to remove any residual product. The pooled cleavage solutions were transferred into a 13mm glass tube, and the solvent was removed in a SpeedVac. An additional ~4mL of DCM and ~2mL hexanes served to azeotropically distill off residual acetic acid, and was repeated an additional three times, resulting in a dry white powder.

The white powder was dissolved in 1mL of dry MeOH, a stir bar was added and ~25eq trimethylsilyldiazomethane (TMSCHN₂), as a 2M solution in ether was added dropwise. Slight bubbling and yellow color resulted. The reaction was stopped after 10min by addition of an identical volume of trifluoroacetic acid, also dropwise. More bubbling was apparent and the

color disappeared. The solvent was again removed in the SpeedVac. A stir bar was added and the residue was dissolved in 4mL TFA/DCM (1:1) and stirred for 2hrs. Each of the six reactions was split into 4 equal volumes for optimization of subsequent steps. The solvent was removed in the SpeedVac, resulting in an oily residue. The tubes were stored in a desiccator until needed.

Solid-phase synthesis of resin-Dpr-(Boc)-pro4-(2S4S)_n-Dpr-(Boc)-Fmoc (n=1-6) (90).

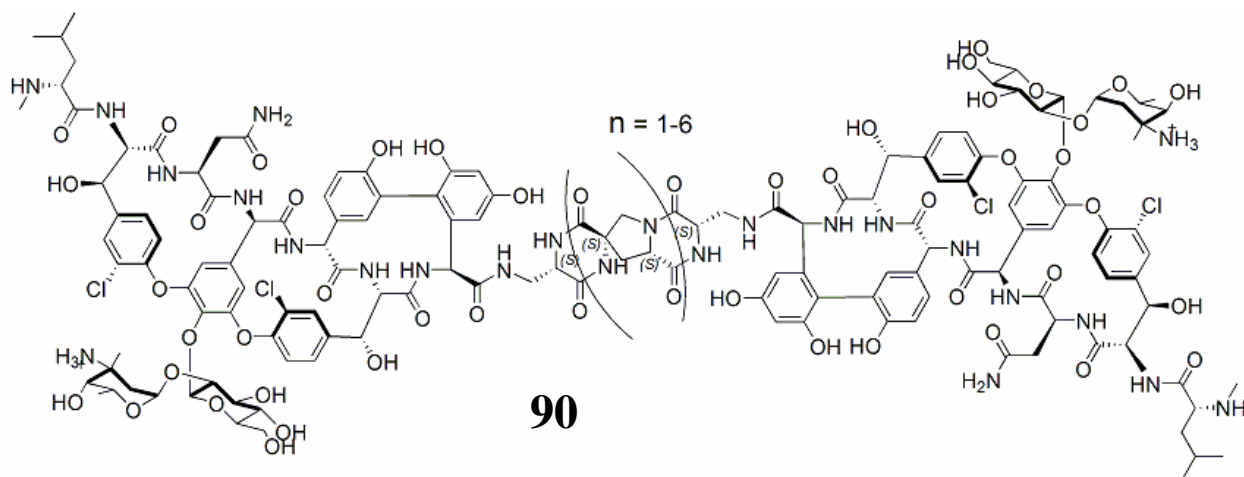


Figure 137 Bivalent vancomycin scaffolds.

200 μ L of 20% piperidine in NMP was added to the residue. Incubation at room temperature for 36hrs served to close the diketopiperazines. After this time, a 5 μ L injection was done on the LCMS and the product was precipitated into 1.5 mL of ether, in a 2mL microcentrifuge tube. Using an HPLC calibration curve, made by dissolving a known amount of Fmoc-glycine in 20% piperidine/DMF, the number of moles of diamine substrate was found. For every 1 equivalent of diamine, 5eq of HOBt, HBTU, and Vancomycin hydrochloride hydrate were used, along with 17.5eq of DIPEA at a concentration in DMF:DMSO (1:1) of 20mM. The time, temperature and order of addition were critical variables, as the best conditions were found to consist of 1hr at 0°C, while first dissolving the HBTU/HOBt in 0.2vol DMF:DMSO and sonicating, then separately dissolving vancomycin in 0.8vol of DMF:DMSO and sonicating. These two tubes, along with the ether precipitated pellet of the diamine in another tube were all incubated in an ice bath. The vancomycin was mixed with the coupling reagents first, and then added to the diamine, with 3 cycles of sonication followed by cooling in the ice bath, as

sonication tended to heat the tube. Finally, base was added, and the reaction was incubated on ice for 1hr. LCMS analysis confirmed successful synthesis of the *bis*-vancomycin. In some cases, if the vancomycin starting material was present in large amounts or if its retention time was too close to the desired product, the vancomycin active ester could be scavenged by addition of a long chain amine, usually octylamine, added as a neat liquid in excess molar amounts. The coupling reaction was stopped by addition of 20 volumes of 10% ACN in water with 0.5% formic acid. The crude product was purified on a preparative HPLC column, as a single injection. By observing at 278nm and 310nm, vancomycin-containing products were easily distinguished from HOBt derivatives. All peaks were collected and re-injected on the LCMS to obtain the identity of the collected peak as well as the purity. The tubes containing the desired product were lyophilized, redissolved in 0.5mL of 25% ACN in water. Using an HPLC calibration curve derived from vancomycin, a 5 μ L injection allowed for quantitation of the amount of bivalent vancomycin. The remaining sample was again lyophilized and the tubes were then stored at 4°C.

5. ELECTRON TRANSFER THROUGH CHIRAL SCAFFOLDS

5.1 INTRODUCTION

The process of electron transfer underlies many chemical and biological reactions and is of primary importance in many technologies. Consequently, the nature of electron transfer has been under experimental and theoretical study for many years.[74, 75] Despite these efforts, little attention has focused on the influence of molecular chirality on electron transfer.

On a fundamental level, spin-polarized electrons have been used to perform chemistry and are implicated in the origin of chiral selectivity in biology.[74] On a technological level, molecular chirality could be used to introduce a new control parameter for spin-sensitive devices.

Naaman reported the first investigation of spin dependent electron transmission through thin chiral films of stearyllysine[74] and more recently observed an asymmetry for electron transmission through monolayers of L (or D) polyalanine films.[74] The magnitude of the effect is 10^3 to 10^4 times larger than the chiral selectivity found for the interaction of polarized electrons with molecules that are not organized into two-dimensional arrays.[74-85]

In photoemission through an organic monolayer film, the electron wavefunction can be delocalized among many chiral molecules in the film, whereas tunneling electrons are more localized. Hence, it is interesting to ask if such large effects are possible for electron tunneling. Spin polarized tunneling has been observed in Metal-Oxide-GaAs (MOS) structures with an asymmetry of the order of 1%.[74] In those studies the polarized distribution of carriers is generated in the GaAs by circularly polarized light and tunneling occurs through a thin Al_2O_3 (2 to 20 nm) on Al. In recent work spin polarized electrons were selectively transmitted between two quantum dots through organic molecules.[74] Those findings show that it is possible to create the polarized distribution of charge carriers and observe asymmetry in electron tunneling.

This study investigates the photocurrent, induced by circular polarized light, through organic monolayer films on Au electrodes that are immersed in an electrochemical cell. The films are composed of a chiral scaffold molecule, which is linked to the Au by a cysteine amino acid at one end and presents a porphyrin chromophore at the other end. Although related systems have been studied previously (*e.g.*, Morita *et al.*[74] placed helical peptides containing a carbazoyl chromophore on gold electrodes), the effects of molecular chirality and light polarization were not explored. Under photoexcitation of the porphyrin, an electron is transferred to an acceptor (*e.g.*, methylviologen in Figure 138), and the resulting cation of the porphyrin is reduced by the gold electrode (Figure 139). By measuring the dependence of the photocurrent on the polarization of the light field and correlating it with the scaffold's chirality, a preference for electron tunneling of one handedness may be determined.

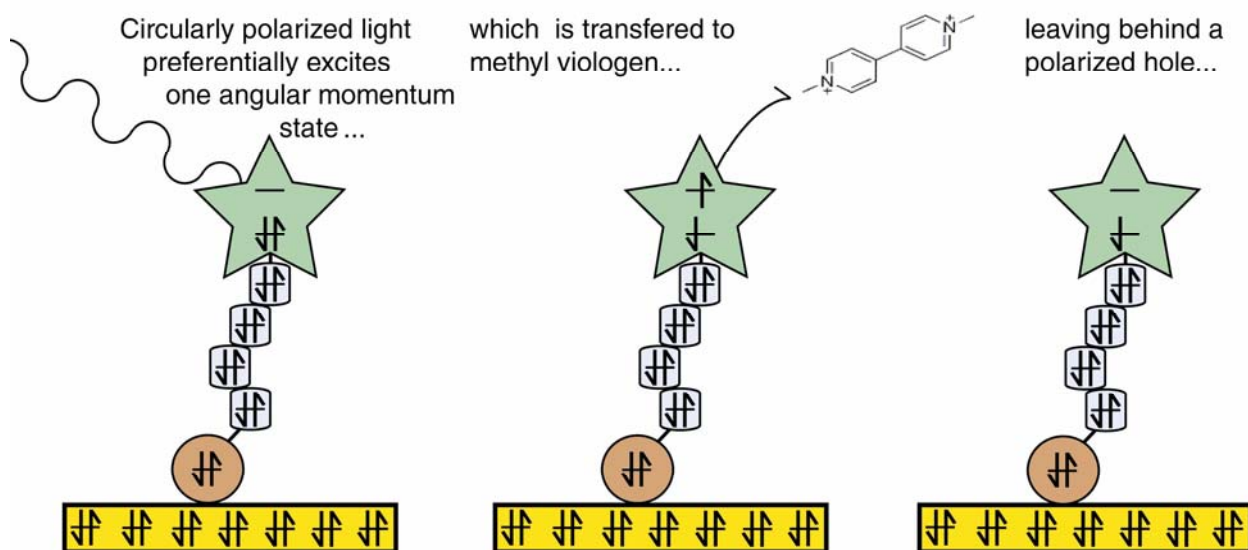


Figure 138 Cartoon depiction of the origin of the photocurrent.

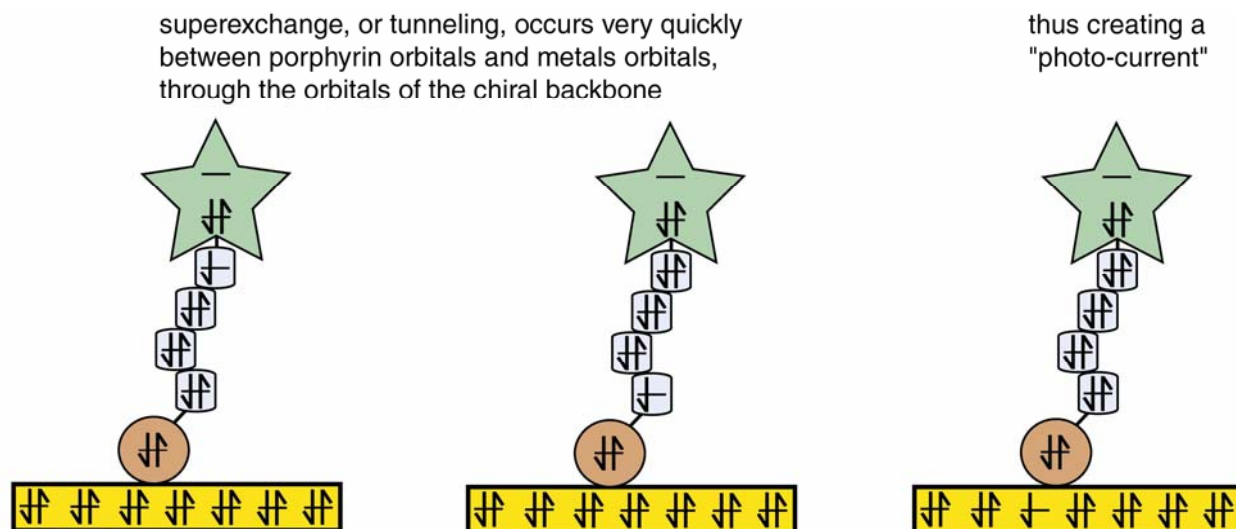


Figure 139 The process of "hole transport."

5.2 RESULTS AND DISCUSSION

(NOTE: some of the following figures were created by Jianjun Wei or Amit Paul in Prof. Waldeck's lab, and for their work I am very grateful)

The synthesis of these porphyrin containing scaffolds (Figure 140) was done on the Rink Amide resin. Incorporation of trityl-protected cysteines, either L or D occurred first. Then a series of four Boc-protected pro4 building blocks, either SS or RR (naming system stereochemistry based: D or L for cysteine, then SS or RR for building block; DRR or LSS), completed the scaffold sequence. Incorporation of the carboxy-tetraphenyl porphyrin proceeded cleanly to yield the desired product, having a dark purple color. Cleavage of the product in 95% TFA gave the crude product with a vivid green color. Closure of the diketopiperazines (DKPs) in 20% piperidine/NMP gave the final product, having a red-purple color.

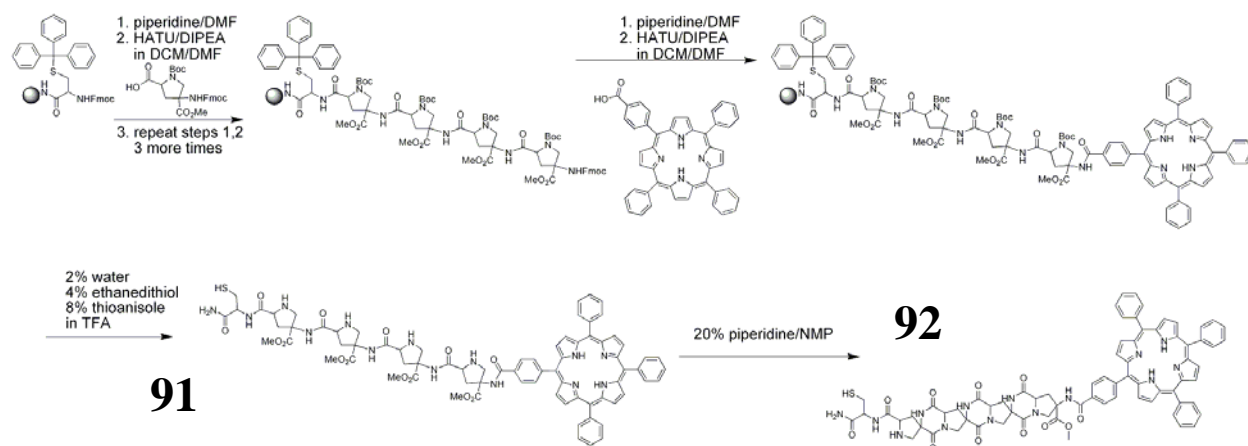


Figure 140 Solid-phase synthesis of the porphyrin-containing scaffolds for photocurrent measurements.

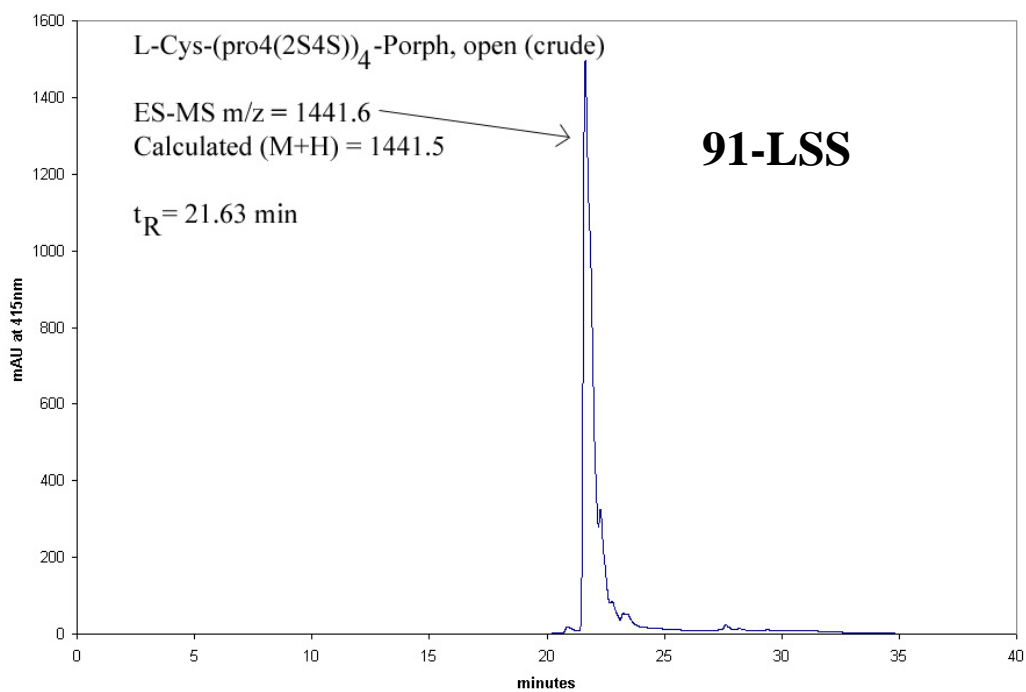


Figure 141 L-Cys-SS₄-Porph cleavage product

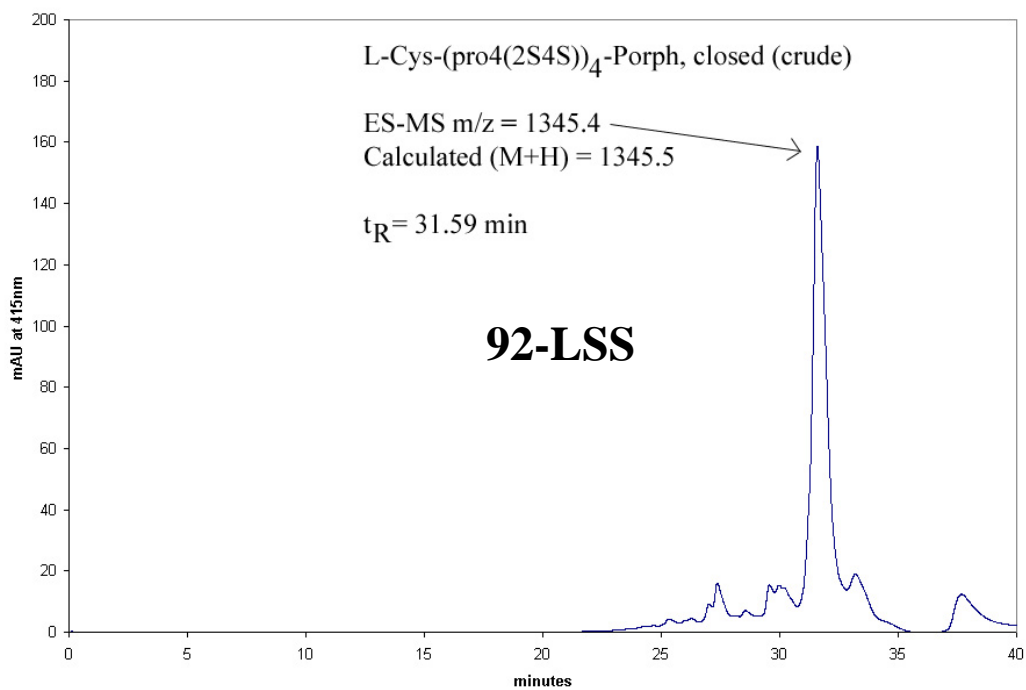


Figure 142 L-Cys-SS₄-Porph crude DKP product.

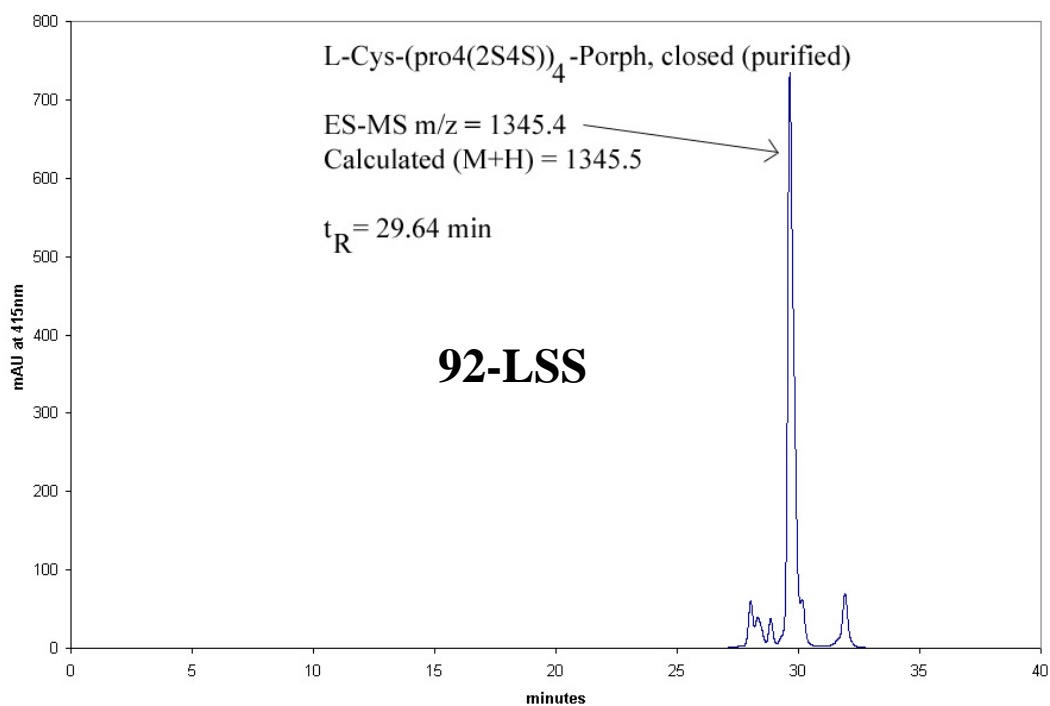


Figure 143 L-Cys-SS₄-Porph purified final product.

Several small breakthroughs were made in this project. First, it was found that closing the DKPs in NMP instead of DMF gave cleaner product in this case. This observation was subsequently validated by others, and from that point on, NMP has been universally adopted as the solvent of choice for DKP formation. In some instances before NMP was used, DKP closure in DMF produced significant impurity (>50%) closely corresponding to incorporation of iron, by LCMS. This iron could potentially have been incorporated into the porphyrin ring, as in heme, but this coordination was believed to require higher temperature. Also, it was thought that incorporation of iron would change the UV-Vis spectrum of the porphyrin, which was not seen. As the only evidence of heme formation was a close match of the expected mass to the observed mass, this conclusion of an iron-containing impurity may be suspect. Regardless, utilization of NMP suppressed this impurity to below 5%.

A second breakthrough involved the initial attempts to remove this unknown impurity. This impurity was only observed when formic acid was used in the HPLC separation (with a Waters Xterra column) and not when TFA was used. Therefore, these formic acid conditions were transferred to the preparative column which was a Varian Microsorb C18 column. It was found that the compound, which normally eluted near 80% ACN would not elute even at 100% ACN for extended times. It was at this time that a greater understanding of the features of a C18-silica particle was attained. The Microsorb particle is an older-type particle, and is alkylated with C18 to less than 100% completion. The un-alkylated silinol is acidic and is much less likely to be protonated by formic acid than by TFA. As a consequence, the deprotonated silinol will interact ionically with the free amine in the scaffold. This observation was seen in other compounds as well. As we were moving more towards a formic acid system for the benefit of MS analysis, the obsolete Varian columns in the lab were universally replaced with the Waters Xterra columns, in which the un-alkylated silinols are then capped to enable performance in low acid, or even acid-free, conditions. This column and formic acid conditions have also been adopted by all in our group, with great success.

The third breakthrough came at the end of the project. Throughout the project, the purification of the free-thiol containing product had been a real nuisance. Closing the DKP in piperidine formed exclusively the disulfide product. In the process of purification, obviously the disulfide, impurity-product mixed disulfide and free thiol products would all have different retention times, so the disulfide needed to be reduced. This required large amounts of the

phosphine-reducing agent, *tris*-carboxyethyl phosphine (TCEP). Also, it was learned that the final product HPLC fractions could not be lyophilized or else numerous impurities would appear. This was the main drawback of using unprotected cysteines, as only the most concentrated, most pure fractions could be used for subsequent photocurrent measurements. This led to a great waste of usable material, as slightly impure fractions could not be dried-down and repurified. This free-thiol nuisance was creatively remedied by employing a different cysteine protecting group: the acetamido protecting group (Acm = -CH₂NH-Acetyl). Transition metals like mercury, silver and thallium are known to cleave this group. To our enjoyment, so did the gold of the electrode. The gold removed the protecting group and then complexed the liberated thiol, to form monolayers with exactly the same properties as those prepared with free thiols.

The following contains some results of the characterization of the scaffold-porphyrin monolayers, as well as the photocurrent measurements and analysis. For more details on the measurements by Wei and Amit, as well as the mechanism, the reader is referred to the publication.[86] A brief summary of the highlights follows.

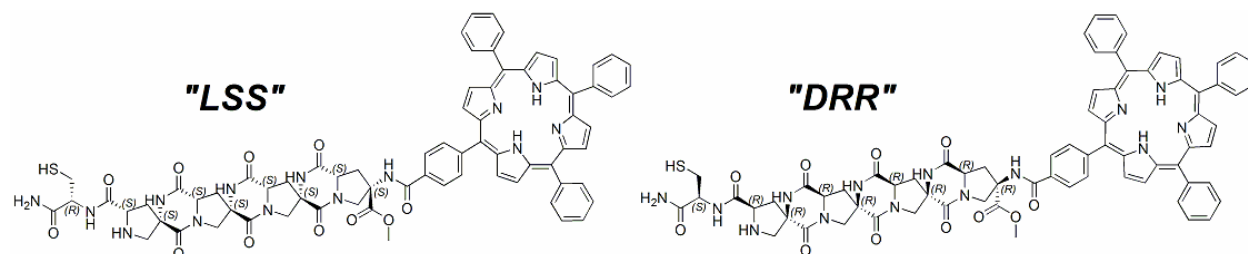


Figure 144 The two enantiomeric porphyrin-containing scaffolds used in this study.

The figure below shows the absorption spectra of the two enantiomeric compounds in 80%ACN/20%H₂O/0.1%TFA acid solution. No significant peak shift is found in the Soret bands ($\lambda_{\text{max}}=435$ nm) and Q bands (649 nm for original porphyrin and 650 nm for scaffold porphyrins) in comparison to the free porphyrin (H₂TPP). This result suggests no significant change of the porphyrin electronic structure in the two compounds. The figure below also shows the CD spectra of the two porphyrin scaffolds in the far UV region (180-260 nm). It is well known that the transitions in a polypeptide involve the nonbonding electrons on the oxygen of the carbonyl group and the nearest nitrogen atoms. The complimentary signals of the two scaffolds reflect the different chirality of these molecules. This CD spectrum was obtained in unideal conditions. The presence of the TFA in the sample likely absorbs a large amount of low UV light used for

the measurement. Because of this, the preference of this molecule for absorbance of one ellipticity over the other is less accurate, as this would be a situation of a small difference of large numbers. The measurement would be more accurate if it was done in pure water or pure methanol, as they both absorb very little around 210nm.

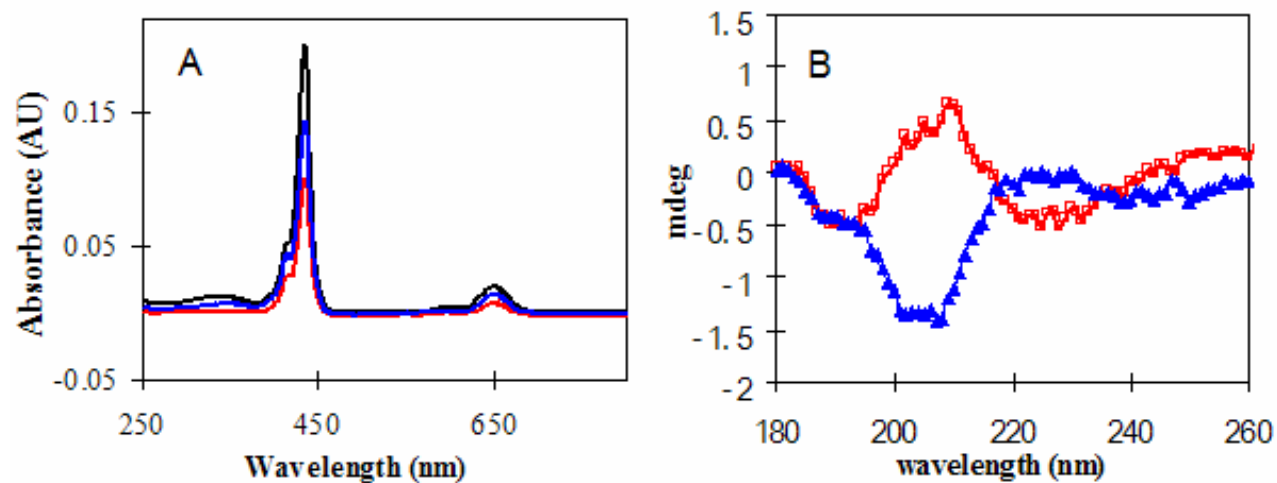


Figure 145 Panel A shows the absorption spectra of porphyrin only (black), **DRR** (red), and **LSS** (blue) scaffold with porphyrins attached in 80%ACN/20%H₂O/0.1%TFA acid solvent. Panel B shows the CD spectra of chiral scaffold molecules, a) red (**LSS**) and b) blue (**DRR**).

The action spectrum of scaffold porphyrins at gold electrodes was obtained by measuring the cathodic photocurrent under irradiation with light, whose wavelength was selected with bandpass filters. The figure below shows the photocurrent action spectrum of a **DRR** SAM, and the inset shows its absorbance spectra under different conditions. The greatest photocurrent is observed in the wavelength range of 400 to 450 nm, the Soret band region. The films display a broadened Soret band (compared to the solution porphyrin). The photocurrent action spectrum and the absorbance spectrum of scaffold porphyrins at the gold surface demonstrate that the porphyrin is the photoactive species responsible for the photocurrent generation.

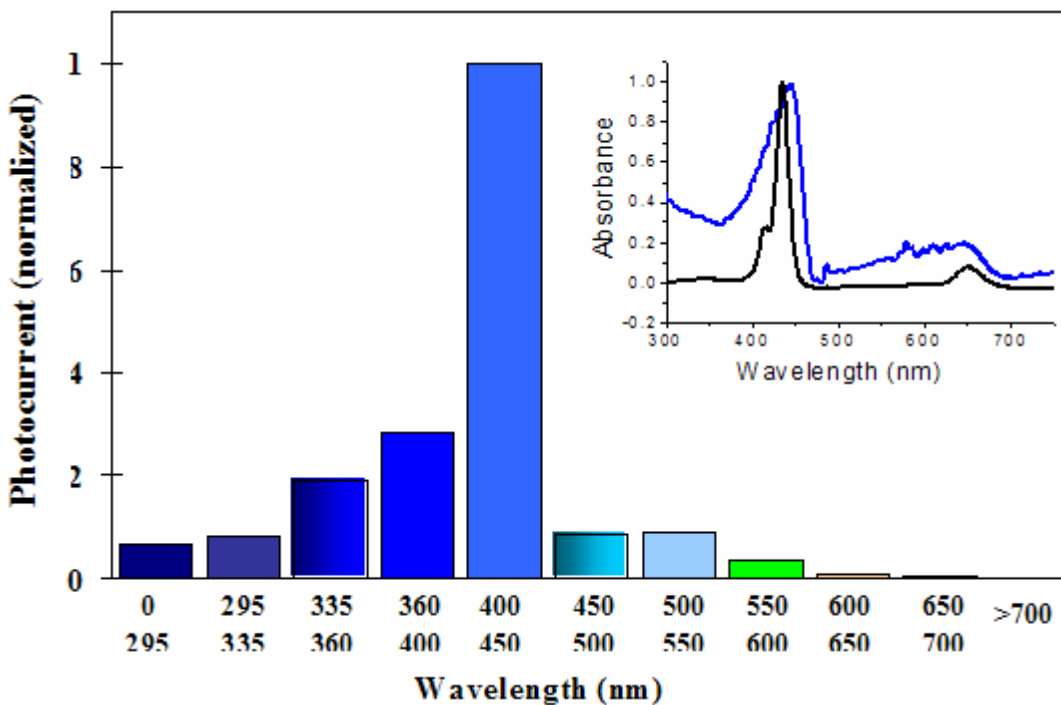


Figure 146 The photocurrent action spectrum. The inserted graphic is the UV-visible spectra of a scaffold porphyrin (DRR) in solution (black curve), the scaffold assembled at a gold coated transparent (blue curve).

Photoelectrochemical measurements were performed in a 0.1 M Na₂SO₄ aqueous electrolyte solution containing 10 mM methyl viologen (MV⁺) and saturated oxygen as electron acceptors. A cathodic photocurrent from the porphyrin modified gold electrode was observed immediately upon irradiation by a 435 nm laser beam with a power of 1.35 mW at an applied voltage bias of 0.0 V versus Ag/AgCl (3.0 M KCl) reference electrode. A time profile of the raw photocurrent for the LSS SAM is shown in the figure below. The dark current in cathodic photocurrent measurements changes positively with the voltage bias change from 0 to 0.6 V, indicating that the LSS SAM is not so compact. Nevertheless, the magnitude of photocurrent was stable, and reproducible. The photocurrent was linear in light intensity for laser powers <3.0 mW. The figure below shows the voltage dependence of the photocurrent, which decreases monotonically with increasing positive bias. These results demonstrate that the electron flows from the gold electrode to the electrolyte through the scaffold porphyrin SAMs.

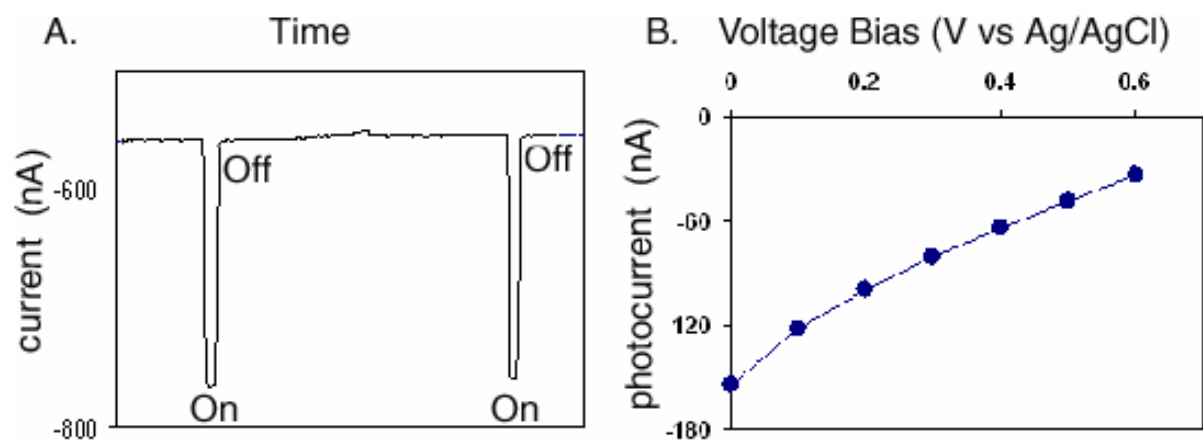


Figure 147 Representative photoelectrochemical responses from the LSS scaffold porphyrin SAM modified Au electrode at an applied voltage bias of 0.0 V in a three-electrode cell (counter: Pt; reference: Ag/AgCl); the dark interval between photocurrent peaks is 60 s duration. B) The voltage bias dependent photocurrents for the Au-Porphyrin/MV²⁺/Pt system. The excitation wavelength is 435 nm and the average power is 1.35 mW. The photocurrent in panel B is defined as $I_{\text{photo}} = I_{\text{on}} - I_{\text{off}}$.

Similar wavelength responses and voltage dependencies were found for the **DRR** and **LSS** SAMs at gold electrodes. In addition, no photocurrent was observed from the bare gold electrodes under the irradiation. The photoelectrochemical characterization confirms that excitation of the porphyrin is responsible for photocurrent generation.

To study the effect of molecular chirality and electron helicity on the electron transfer, photocurrent generated under irradiation with circularly polarized light (either right circularly polarized light, RCP, or left circularly polarized light, LCP) was examined for both **LSS** and **DRR** SAMs. The figure below shows representative photocurrents generated under illumination with circular polarized light for the two chiral scaffold porphyrins at gold electrodes. The RCP or LCP polarizations were obtained by rotating a $\lambda/4$ wave plate at a specific tilt angle (see experimental section for details). The incident light intensity was measured for every illumination. For the **LSS** scaffold porphyrins, the magnitude of photocurrent under LCP irradiation is slightly larger than that under RCP irradiation as shown in the figure below. In contrast, the **DRR** scaffold porphyrin film has a larger photocurrent under RCP irradiation than that under LCP irradiation. Although the preference is small, less than 1%, it was highly reproducible for a given sample and stable over a period of many hours. In some cases,

measurements were performed over more than one day on the same electrode and found to be reproducible.

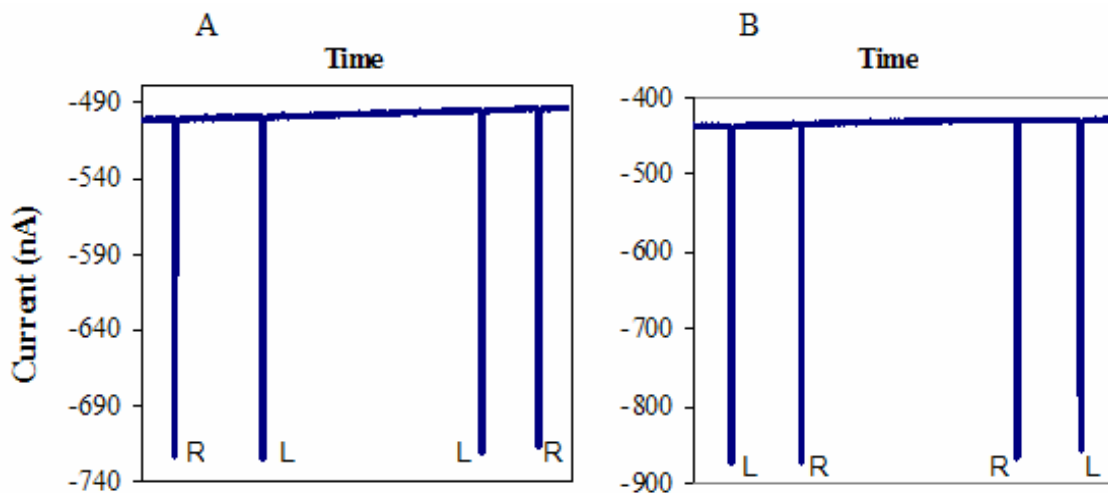


Figure 148 Representative photocurrent signals generated under circular polarized light for A) **LSS** and B) **DRR** scaffold porphyrins at gold electrodes.

About ten electrodes for each sample type (**DRR** and **LSS**) were studied under the same conditions and the propensities of the asymmetry in photocurrents were measured. Control experiments, using a linearly polarized laser beam, showed no asymmetry.

An asymmetry factor A for the photocurrent was defined as:

$$A = \frac{j(\sigma+) - j(\sigma-)}{j(\sigma+) + j(\sigma-)}$$

in which $j(\sigma+)$ and $j(\sigma-)$ are photocurrents (normalized to light power) for RCP and LCP illumination at the same electrode, respectively. The asymmetry factor was calculated for each RCP and LCP irradiation pair. The **LSS** scaffold (4-mer) gave an average asymmetry factor of -0.0048, and the **DRR** scaffold (4-mer) gave an average asymmetry factor of +0.0054.

The figure below plots the asymmetry factor obtained for all of the experiments. Panels a) and b) show the distribution (a descending sort) of asymmetry factors for **DRR** and **LSS** films, respectively. The asymmetry factors of the **DRR** scaffold range from -0.017 to 0.034 and most of them are positive values, whereas the asymmetry factor of the **LSS** scaffold range from -0.033 to 0.012 and most of them are negative values. Panels c) and d) show a histogram (bin size of 0.001) for the asymmetry factors. A Gaussian function (solid curve in c and d) is fit to the distribution. This fit yields an average value of 0.004 and a standard deviation of 0.006 for the

asymmetry factor of the **DRR** scaffold, and it yields an average value of -0.005 and a standard deviation of 0.004 for the **LSS** scaffold. This analysis gives an average asymmetry factor of 0.004 ± 0.002 for the **DRR** scaffold and -0.005 ± 0.001 for the **LSS** scaffold.

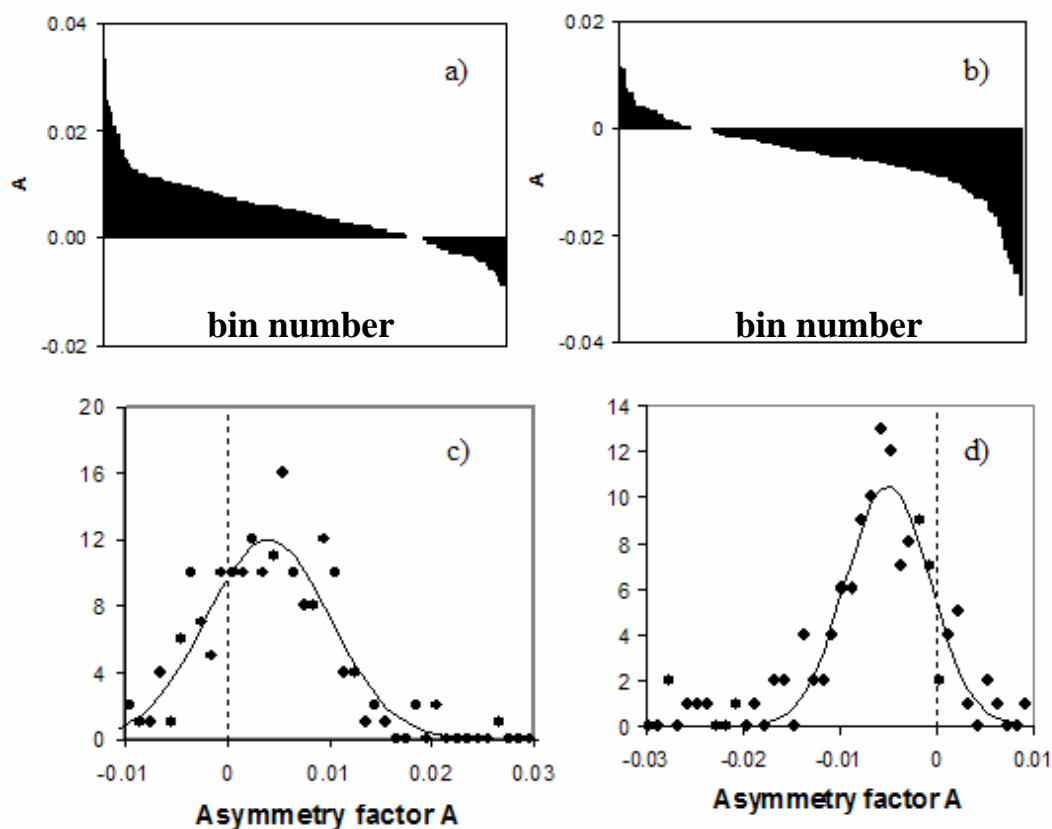


Figure 149 Distributions of asymmetry factors and statistical analysis of the helicities. a) and b) respectively present the distributions of the asymmetry factors in a descending sort for **DRR** and **LSS** scaffold porphyrin electrodes, and c) and d) are the histograms of the number of observations vs. the observed ranges of asymmetry factors, corresponding to a) and b) respectively.

5.3 CONCLUSIONS

Porphyrin-containing *bis*-amino acid oligomers have been synthesized, purified and organized into a monolayer on a gold electrode. The effect of the chirality of the scaffold backbone and the helical polarization of the excitation light on the photocurrent generation has been studied, and an asymmetry was found. The average asymmetry factor obtained for a right-handed monolayer

is 0.004 ± 0.002 and for a left-handed monolayer is -0.005 ± 0.001 , with confidence limits of 95%. Further studies are ongoing to elaborate and improve this asymmetry factor.

5.4 EXPERIMENTAL

General: Dichloromethane was distilled from CaH_2 . All other reagents were used as received, unless stated otherwise. Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. *N,N*-diisopropylethylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and an inert atmosphere over the reaction. HPLC-MS analysis was performed on a Hewlett-Packard Series 1050 instrument equipped with a Waters Xterra MS C_{18} column (3.5 μm packing, 4.6 mm x 150 mm) and a diode-array detector, while the MSD-ESI was Series 1100. Preparative purification was done using a Varian Prostar 500 equipped with a Waters Xterra Prep MS- C_{18} column (5 μm packing, 10 mm x 100 mm), equipped with a dual wavelength detector.

Synthesis of Porphyrin Scaffold L-Cys-(pro4(2S4S))₄-Porph (91-LSS).

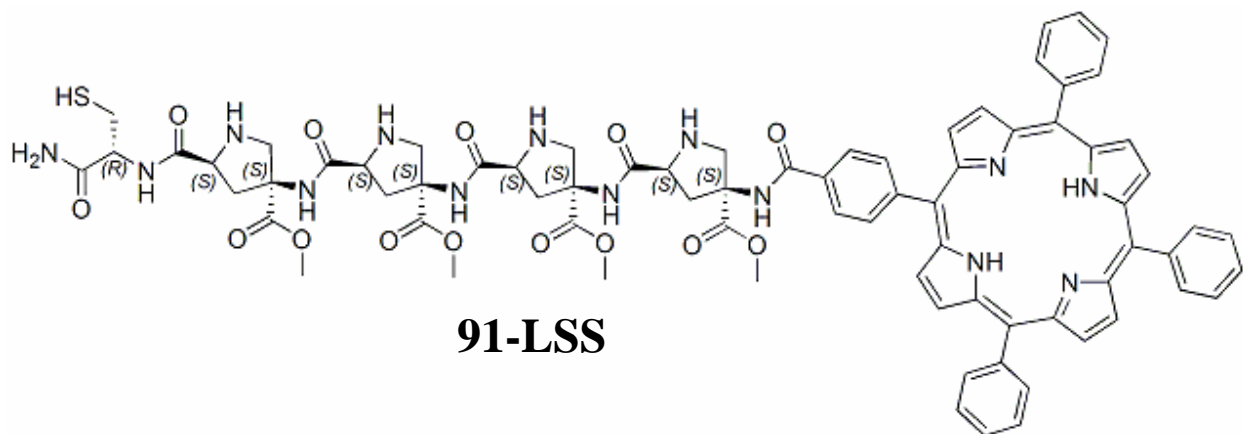


Figure 150 L-Cys-(pro4(2S4S))₄-Porph, open form, cleavage product

To a 1.5mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (20mg, 12.6 μ mol loading). The resin was swollen for 1hr in dimethylformamide (DMF). The terminal Fmoc-protected amine was deprotected in 0.5mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 36.9mg (63.0 μ mol) of Fmoc-L-Cys(Trt)-OH (Novabiochem) and 24.0mg of HATU (63.0 μ mol) in 315 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 21.9 μ L of N,N-diisopropylethylamine (DIPEA) was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react for 30min. The resin was then washed 3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 13.7mg (25.2 μ mol) of Boc-protected pro4(2S4S) building block[17, 20] and 9.6mg of HATU (25.2 μ mol) in 126 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 8.8 μ L of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react for 30min. The resin was then washed 3 x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release. This process of coupling/deprotection was repeated three additional times.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 12.5mg (18.9 μ mol) of 5-(4-Carboxyphenyl)-10,15,20-triphenyl-21,23H-porphyrin (Porphyrin Systems) and 7.2mg of HATU (25.2 μ mol) in 126 μ L of DMF. This solution was mixed using a micropipettor, after which 6.6 μ L of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react for 30min. The resin was then washed 3 x 2min with DMF. Double couplings produced the final product, after which the resin was prepped for cleavage by washing with DMF, isopropanol, DMF, isopropanol, DCM, methanol, DCM, methanol, DCM, for 2 min each. The reactor was then put in vacuum tube, and dried, *in vacuo*, overnight.

The resin-bound product was cleaved in 1mL of 2% water, 4% ethanedithiol, 8% thioanisole in trifluoroacetic acid (TFA), with stirring for 2 hours. The green solution was filtered away from the resin beads, and one additional mL was used to wash the beads. The green solutions were pooled and the solvent was removed under a stream of dry nitrogen. Residual solvent was removed, *in vacuo*, for one hour, yielding a very dark green residue.

Closed form of Porphyrin Scaffold L-Cys-(pro4(2S4S))₄-Porph (92-LSS).

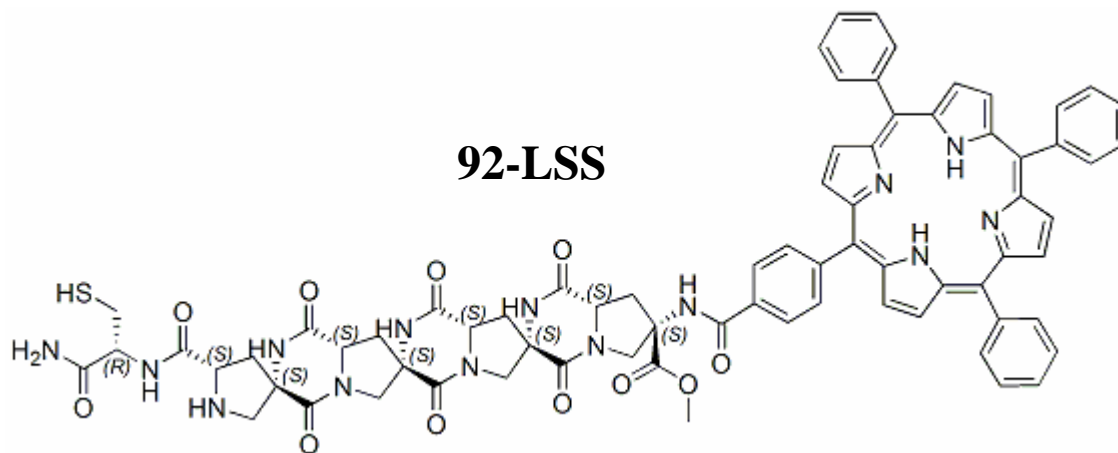


Figure 151 L-Cys-(pro4(2S4S))₄-Porph, DKP closed, final product

The cleaved product was dissolved in 1mL of 20% piperidine/ N-methylpyrrolidinone (NMP), changing the color to a dark red. After 48hrs at room temperature, the product was precipitated by dripping into 40 mL of ether in a 50mL polypropylene tube. Some precipitate was evident, but more was obtained by a 1hr incubation at -20°C. The precipitate was collected by centrifugation at 3000 x g, 4°C, for 30min. The pellet was washed with 40mL of fresh ether,

vortexed, and the centrifugation was repeated. The ether was removed and the pellet allowed to dry. The pellet was dissolved in 2 mL of 1:3 water: acetonitrile, with 0.5% TFA, and mixed by pipetting. Tris-(2-carboxyethyl)phosphine (TCEP) was added to a concentration of 10mg/mL, and allowed to reduce the disulfide bonds for 2 hrs, before analysis or purification.

The reduced product was purified on a Varian ProStar instrument with dual wavelength detection using Varian Chrompack Microsorb 100 C₁₈ column 8 μ m packing, 21.4m x 250mm (mobile phase, acetonitrile (0.05% TFA) / water (0.1% TFA), 5% to 100% acetonitrile over 31.67 min, then 100% acetonitrile until 40min; flow rate, 15 mL/min; UV detection at 415 nm). It was found that when a purified fraction containing a single peak was lyophilized and resuspended, multiple peaks were found, even with added TCEP. Fractions of sufficient concentration (100 μ M) were obtained by making a single large injection and collecting the eluting peak in numerous 3-5 mL fractions. LC/MS was then used to confirm the purity. Then the most pure and most concentrated fractions were pooled to give the purified product **1**. The final concentration was found using a Varian Cary 50 UV/Vis spectrometer using $\epsilon = 232000 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 435\text{nm}$.

Synthesis of Porphyrin Scaffold D-Cys-(pro4(2R4R))₄-Porph (**91-DRR**).

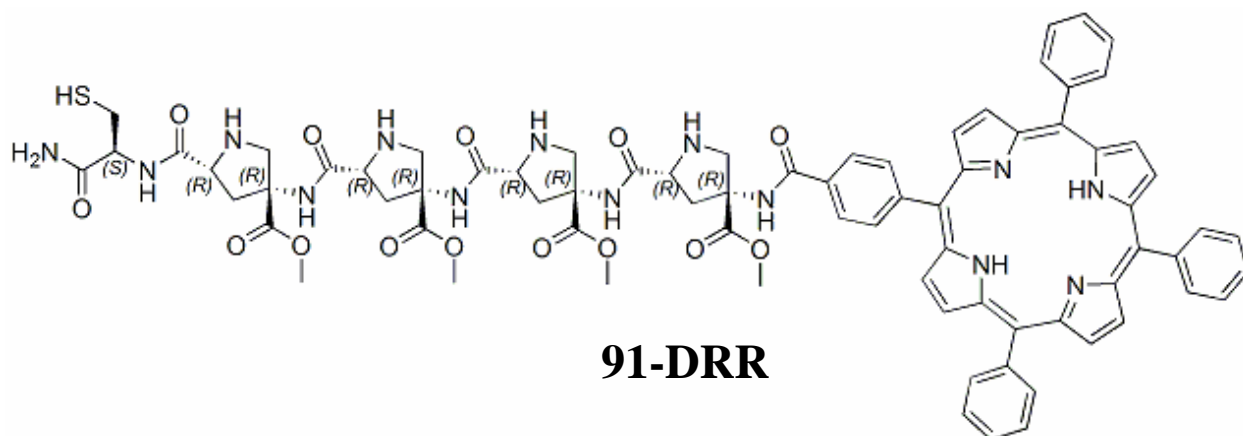


Figure 152 D-Cys-(pro4(2R4R))₄-Porph, open form, cleavage product

The enantiomeric compounds were prepared in an identical manner to what was described for above, except with the following obvious changes: used Fmoc-D-Cys(Trt)-OH (Novabiochem) and Boc-protected pro4(2R4R).[17]

Closed form of Porphyrin Scaffold D-Cys-(pro4(2R4R))₄-Porph (92-DRR).

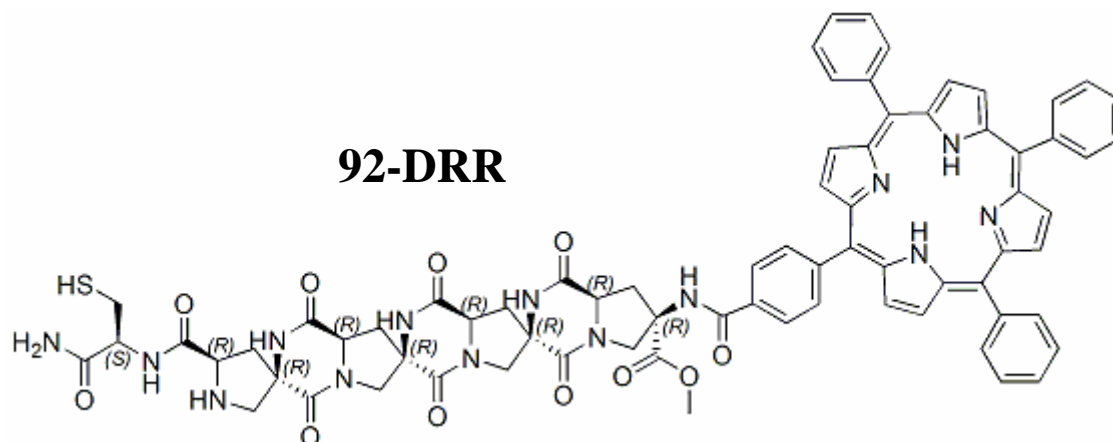


Figure 153 D-Cys-(pro4(2R4R))₄-Porph, DKP closed, final product

For a detailed description of the monolayer preparation and characterization, and the measurements of the photocurrent, the reader is referred to the original publication.[86]

6. MEASURING DISTANCE THE BETWEEN TWO ENDS OF A SCAFFOLD USING ESR

6.1 INTRODUCTION

The following is a quote about the properties of a molecular rod: “In contrast to freshman students' expectations, molecular "rigid" rods are not rigid at all but are highly flexible, even though their equilibrium structure may be linear. At room temperature, even quite short rods bend and flex vigorously and should be thought of as rubber sticks rather than steel rods. With the possible exception of nanotubes, truly long rods many nanometers in length should be thought of as boiled rather than raw spaghetti... The rigidity issue is likely to complicate the construction of structures that use rods longer than 2-3 nm.”[87]

Using the unique technology being developed in this lab, we hope to study the feasibility of creating a molecular rod that can hold a designed shape. This study will utilize 2-D ESR techniques, which are capable of measuring distances between two unpaired electrons, to first study the control of distance and then the control of shape that are inherent to the *bis*-amino acid ladder oligomers being constructed in this lab.

The flexibility of the molecular rods can be characterized by the standard deviation, σ , of the distribution function. The sensitivity of Double Electron-Electron Resonance (DEER)-ESR experiment to the measurement of interspin distances in the ~ 15 -80 Å length range[88, 89] has been used to determine global folding patterns in proteins,[90-93] nucleic acids,[94] ionic polymers,[95] and conformational and aggregation states of polypeptides.[96]

The key advantage of the use of ESR is twofold. First, large distance constraints can be measured from which the overall shape of the conformationally restricted material can be rapidly inferred. NMR has been used to determine short-range distances in bis-peptides[17, 20] but the rod-like nature of these materials precludes the measurement of distance between residues far

apart in the linear sequence. This can lead to a substantial uncertainty in the modeling of the overall structure. In principle, energy transfer in fluorescence resonance energy transfer (FRET)[17, 97, 98] is sensitive to distances in these length scales. However, the correct interpretation of the energy transfer in terms of distances requires assumptions about the rotational averaging of the donor and acceptor groups and a careful accounting of molecular dynamics. The second advantage is that ESR measures the full distance distribution function, from which the flexibility of the nanostructured materials can be directly assayed. The shape and flexibility are both important criteria for the design of nanostructured materials with targeted functions.

6.2 RESULTS AND DISCUSSION

(NOTE: some of the following figures were created by Soraya Pornsuwan in Prof. Saxena's lab, and for their work I am very grateful)

The synthesis of *bis*-spin labeled scaffolds, consisting of 4-8 pro4(2S4S) building blocks utilized the Rink amide AM resin (Figure 154). A single batch of resin was used, up until the Fmoc of the fourth building block was removed. After this, the resin was split into 4/5th and 1/5th batches, with an additional building block being added to the larger 4/5th portion of the resin. This Fmoc was removed, and again portioned into 3/4th and 1/4th. The process was repeated until 5 lengths were produced, all with equimolar amounts of material attached to the resin. The final deprotected amine was acylated with an excess of the pyrrolidine-based carboxylic acid nitroxide. The scaffold was cleaved with TFA, the DKPs were closed, and a second nitroxide was attached to the remaining free amine. An excess of the spin label, activated with HATU ensured complete coupling in less than 30min. After this time, the coupling reaction of about ~75 μ L was dripped into 2 mL of ether, in which the excess unreacted nitroxide was conveniently soluble. The resulting precipitate was centrifuged, redissolved in ~50 μ L of methanol and purified on an analytical C18 HPLC column to give products **93-(4-8)**. The nitroxide/ α - β -unsaturated system had a modest absorbance at ~254 nm, although a distinct peak

was absent. Using a HPLC calibration curve derived from a known amount of the carboxamide nitroxide, the concentration could be estimated (Figure 155). This was used to make the 0.2 mM solution for subsequent distance measurements.

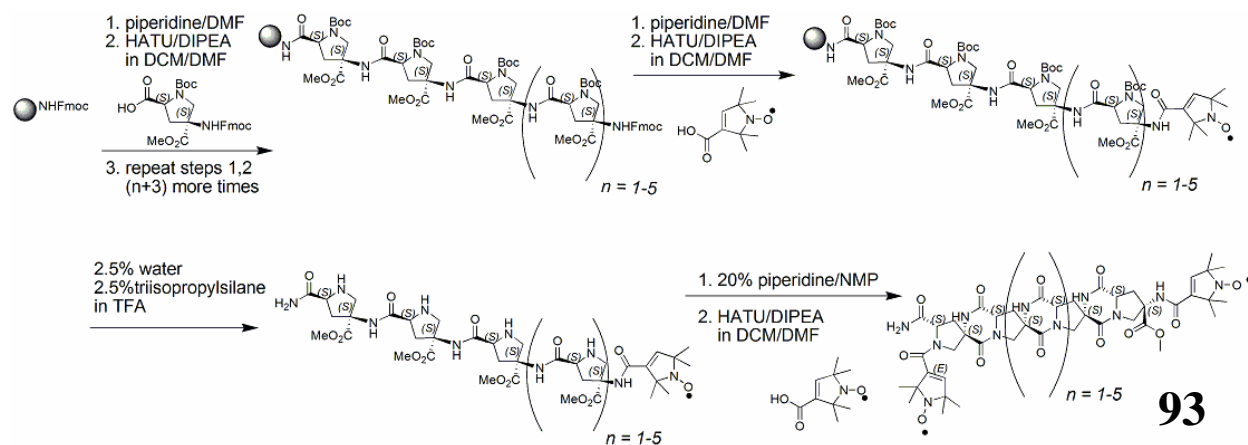


Figure 154 Synthesis of bis-spin labeled scaffolds of different lengths.

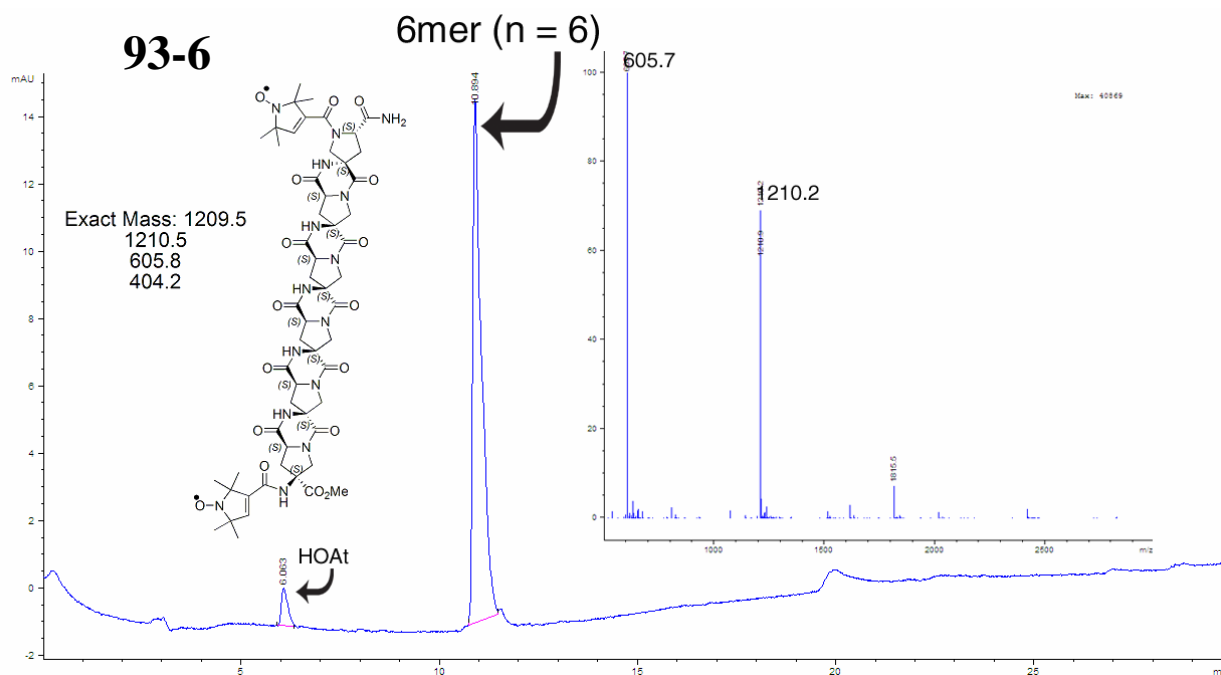


Figure 155 Representative chromatogram of the final purified product, 6mer.

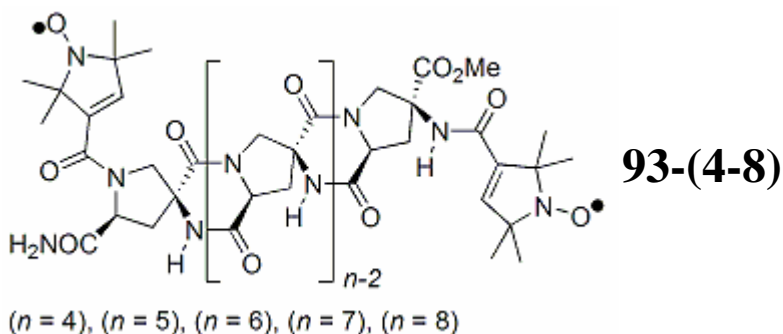


Figure 156 Representative structure of the different lengths studied (4mer, 5mer, 6mer, 7mer, and 8mer).

The figures on this page and the following pages (Figures 157-161) show the distance distribution functions obtained from molecular dynamics and from ESR. For compounds $n = 4, 5$ molecular dynamics predicts a significant population of conformers with interspin distances shorter than 2 nm. These conformers are unlikely to be sampled by the DEER experiments due to the use of pump pulses of 48 ns. Limitations in the excitation bandwidth of this pulse are expected to suppress the distance distribution function below 2 nm,[99] which possibly accounts for the discrepancy between experiment and dynamics in the lower wings of the distribution function.

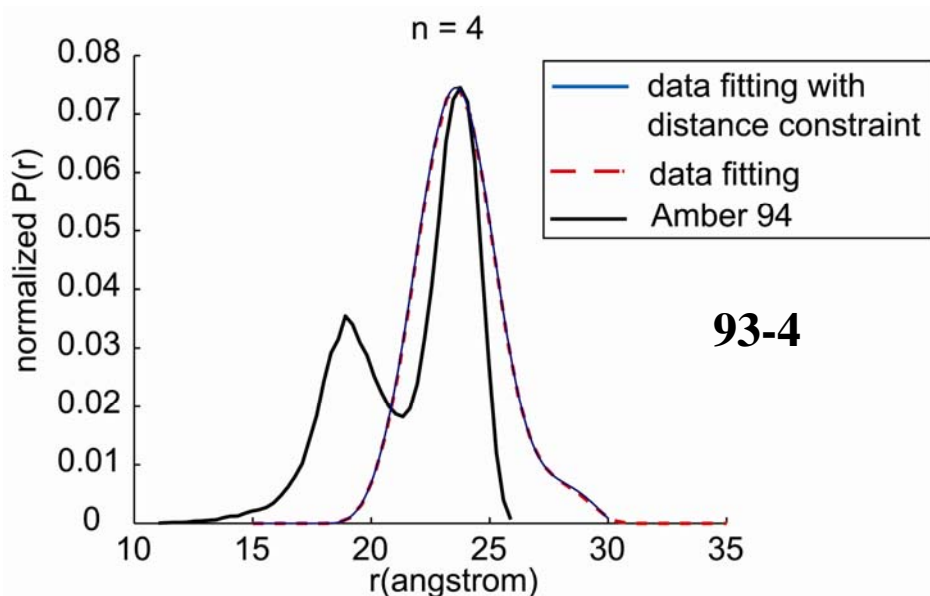


Figure 157 Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the $n = 4$ labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.

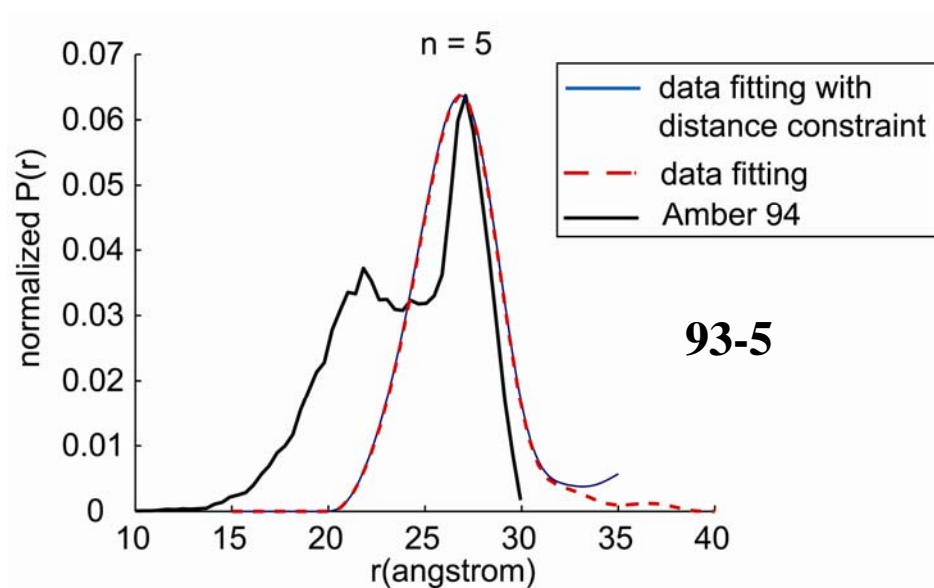


Figure 158 Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the $n = 5$ labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.

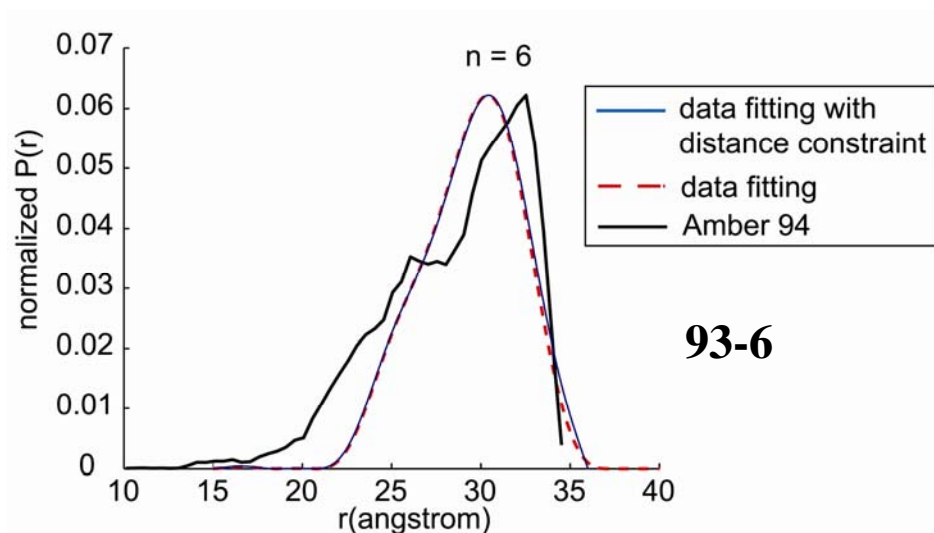
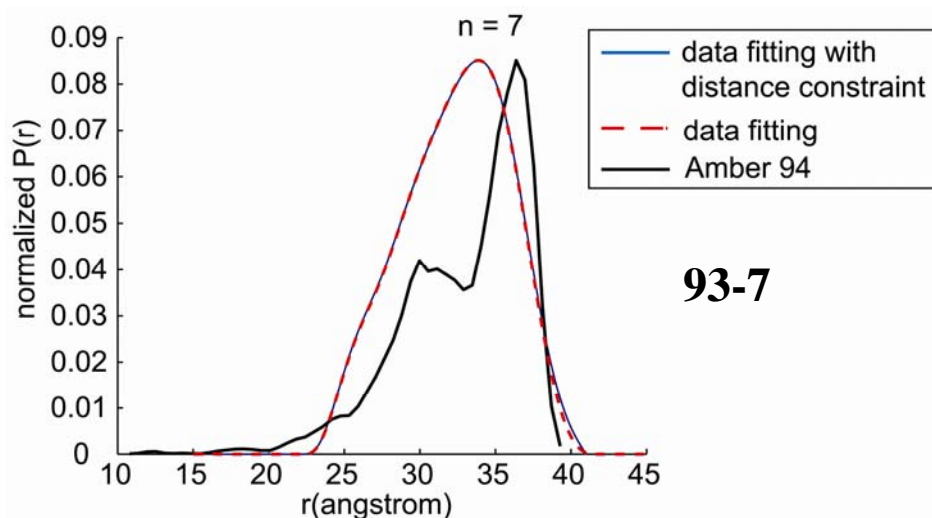
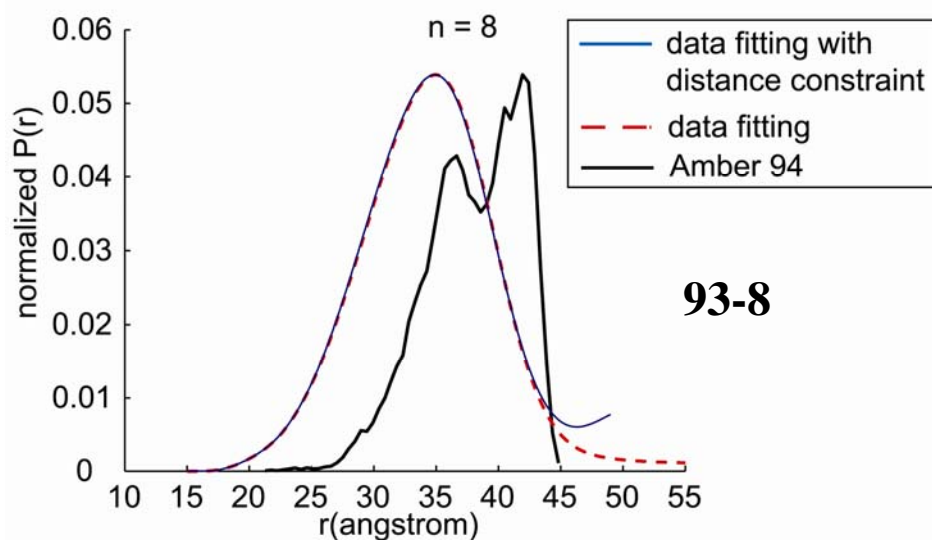


Figure 159 Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the $n = 6$ labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.



93-7

Figure 160 Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the $n = 7$ labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.



93-8

Figure 161 Overlay of the distance between nitroxide nitrogens calculated from a 5 nanosecond Amber molecular dynamics simulation at 300 K of the $n = 8$ labeled scaffold (black) on the distance distribution determined from ESR for the same molecule.

The mean distance and standard deviation (σ) were calculated from the distribution functions using first and second moment as defined by Equations [1] and [2]

$$\langle r \rangle = \frac{\sum_{r_1}^{r_2} r \cdot P(r)}{\sum_{r_1}^{r_2} P(r)} \quad [1]$$

$$\sigma = \sqrt{\frac{\sum_{r_1}^{r_2} r^2 \cdot P(r)}{\sum_{r_1}^{r_2} P(r)} - \langle r \rangle^2} \quad [2]$$

where $P(r)$ is distance distribution function, r is distance, r_1 and r_2 is the lower and upper range in the distribution function and σ is the standard deviation. The results are summarized in Figure 162 below.

n	Experimental		Molecular dynamics	
	$\langle r \rangle$ (Å)	σ (Å)	$\langle r \rangle$ (Å)	σ (Å)
4	23.7 ± 1.0	1.8	21.7	2.6
5	26.7 ± 1.7	2.2	24.0	3.4
6	29.4 ± 1.0	2.7	28.5	3.9
7	32.3 ± 1.4	3.5	32.9	4.1
8	34.5 ± 1.1	5.8	38.0	3.8

Figure 162 Table comparing distances and standard deviations.

Within experimental resolution, the estimates of the distances from dynamics are in reasonable agreement with the experiments for $n = 4-7$. Note, that for $n = 4$ and $n = 5$ the experimental value of the average inter nitroxide distance is shifted to higher values possibly because the conformer with r less than 20 Å are inadequately sampled by the DEER experiment (see above). However, molecular dynamics overestimates the mean distance for $n = 4$ by ~3.5 Å. Also, the molecular dynamics distributions overestimate the inter-nitroxide distance for long scaffolds (by as much as ~7 Å for $n = 8$).

In Figure 163 below, we compare the experimental data for $n = 8$ with simulated DEER data based on the probability distribution predicted by molecular dynamics. It is evident that the experimentally derived distribution function yields a better fit compared to the simulated data based on molecular dynamics results.

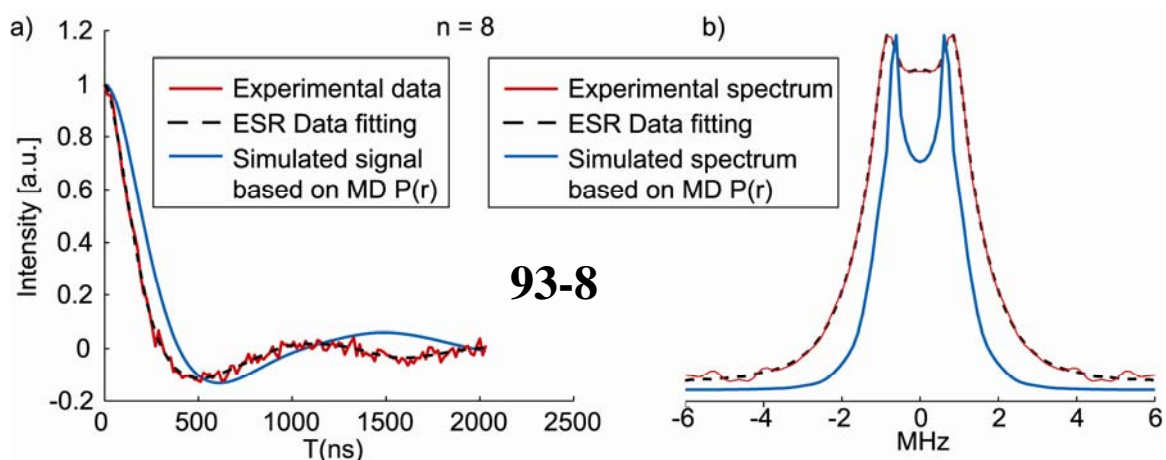


Figure 163 Overlay of the DEER time traces (a) and frequency spectra (b) of experimental data (red), ESR data fit (dashed black), and simulated DEER signal using the $P(r)$ derived from molecular dynamics (blue) for $n = 8$ scaffold.

Snapshots from the dynamics simulations for $n = 4$ and $n = 8$ shown in the Figures 164-165 on the following pages are presented to illustrate the flexibility of these molecules according to the dynamics simulations. We are currently investigating the addition of explicit solvent and parameterization of the force field for these molecules.

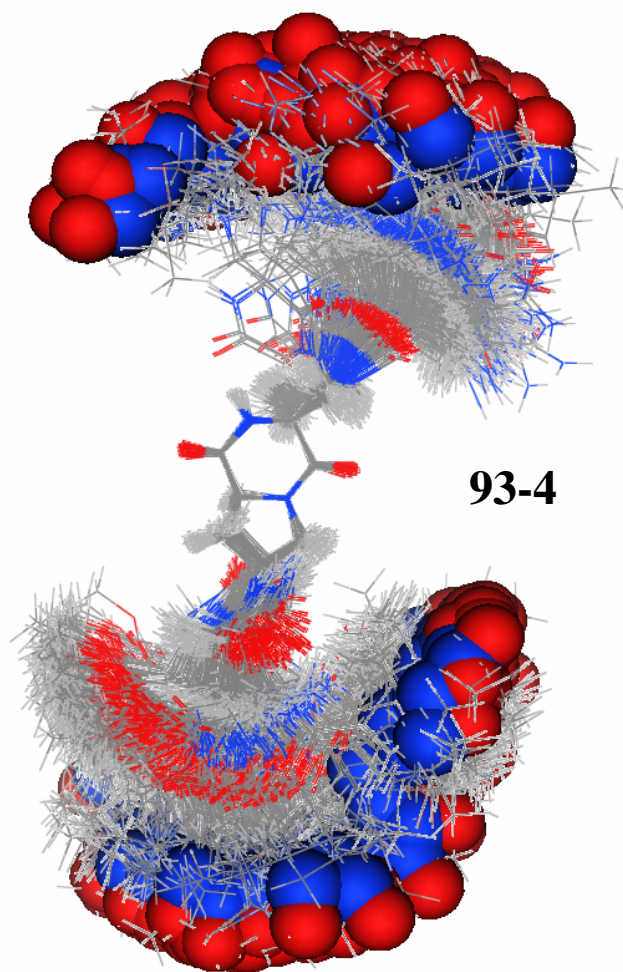


Figure 164 The superposition of the last 500 picoseconds of the 5 nanosecond molecular dynamics simulation for the $n = 4$. The central diketopiperazine ring was aligned for each structure and the nitroxide N-O atoms are rendered as solid spheres.

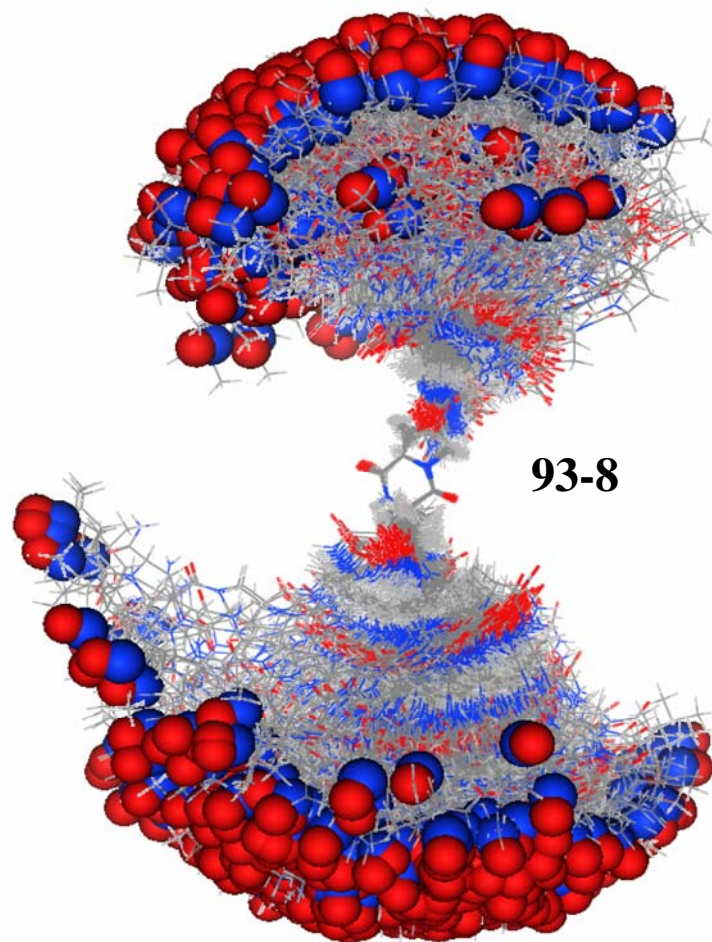


Figure 165 The superposition of the last 500 picoseconds of the 5 nanosecond molecular dynamics simulation for the $n = 8$. The central diketopiperazine ring was aligned for each structure and the nitroxide N-O atoms are rendered as solid spheres.

The mean distance and the standard deviation for these molecules, calculated using a moment analysis (see supporting information of [100]), are shown in Figure 166 below. The error is estimated by the spectral resolution ($\sim 1.0\text{-}1.7 \text{ \AA}$). The “linear” rod-like shape of these materials is readily interpreted from the plot of in (D) below. The linear fit to the data indicates that each building block adds 2.7 \AA to the average distance between the spin probes. Five-nanosecond molecular dynamics simulations were carried out in vacuo at 300 K on each oligomer. The DEER experiment inadequately samples conformers with r less than 20 \AA (see previous pages). Within this experimental limitation, the estimates of the average distance from dynamics are in reasonable agreement with the experiments for $n = 4\text{-}8$. However, molecular dynamics overestimates the mean distance for $n = 8$ by $\sim 3.5 \text{ \AA}$ (see previous pages). Distributions from molecular dynamics also progressively overestimate the most probable internitroxide distance for long scaffolds (by $\sim 2, 2.5,$ and 7 \AA for $n = 4, 5,$ and 6). The flexibility of the molecular rods can be characterized by the standard deviation of the distribution function, (E) below. The standard deviation increases from 1.8 \AA for $n = 4$ to 5.8 \AA for $n = 8$.

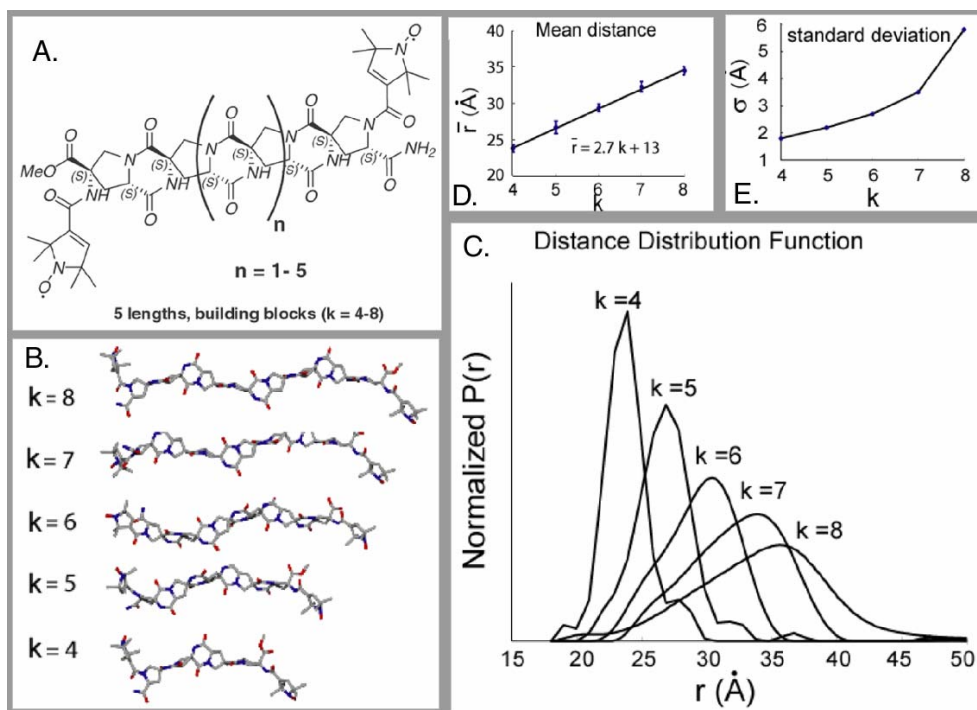


Figure 166 Compilation of length measurements: (A) general scaffold structure (B) modeled scaffold structures (C) distance distribution functions (D) plot of average distance vs. number of building blocks (E) standard deviation vs. number of building blocks.

With the ability to control distance between two ends having been demonstrated, the next question to ask was whether we can control shape. From NMR solution structures, it is known that bends and kinks are formed when the stereochemistry of adjacent monomers is different.[17, 20] So, a series of ten sequences were identified, which result in curved structures spanning a broad distance range of 20-40 nm, as estimated by modeling. The synthesis (Figure 167) was identical to what was described above. All ten sequences (Figure 168) were successfully synthesized, based on crude cleavage LCMS trace (**94-(1-10)**). However, three of the ten sequences “disappeared” during the DKP closure. Only trace amounts of product containing a few DKPs open could be seen in these cases. The other seven closed with issue and 3 of these seven were purified and their distances measured.

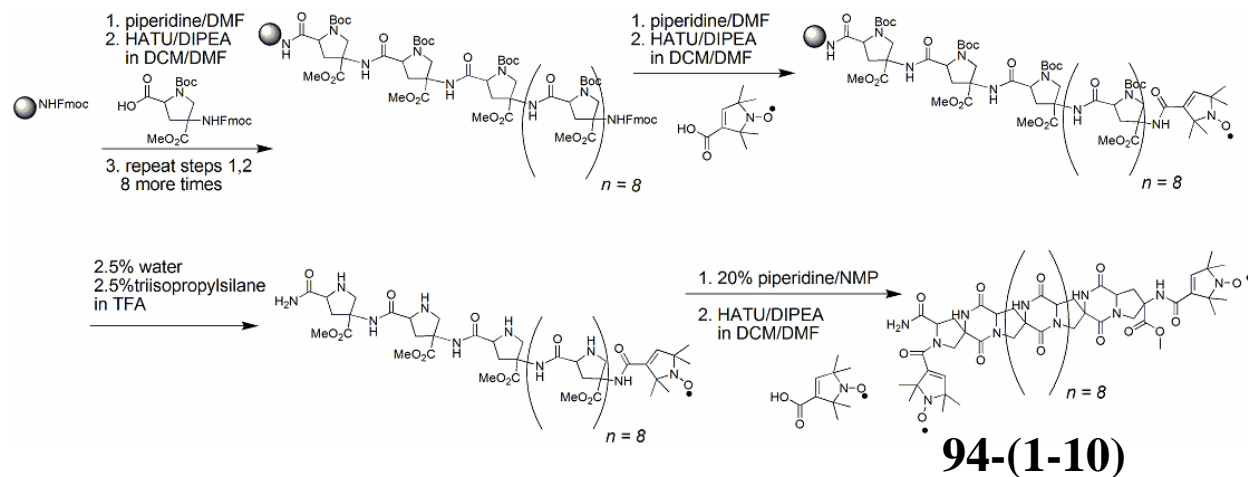


Figure 167 Synthesis of “curved” bis-spin labeled 8mers.

1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
RS	RR	RR	RS	SR	RS	SS	SR	SS	RS
SR	RR	RR	SS	RR	SS	SS	RR	RS	RS
SS	RR	SS	SS	SS	RR	RR	SS	RS	SR
RR	SS	RR	RR	RR	SS	SS	RR	SS	RS
SR	RR	RS	RS	SS	RR	RR	SS	RR	SR
SS	RS	RR	RR	SR	RS	SS	RR	RR	RS
SS	RR	SS	SS	SS	RR	RR	SS	RS	SR
RS	SS	RR	RS	RR	SS	SR	RR	SR	SS

Figure 168 Table of 1st set of “curved” scaffold sequences which were synthesized. Red = Distance measured, Green = DKPs wouldn't close and material was lost.

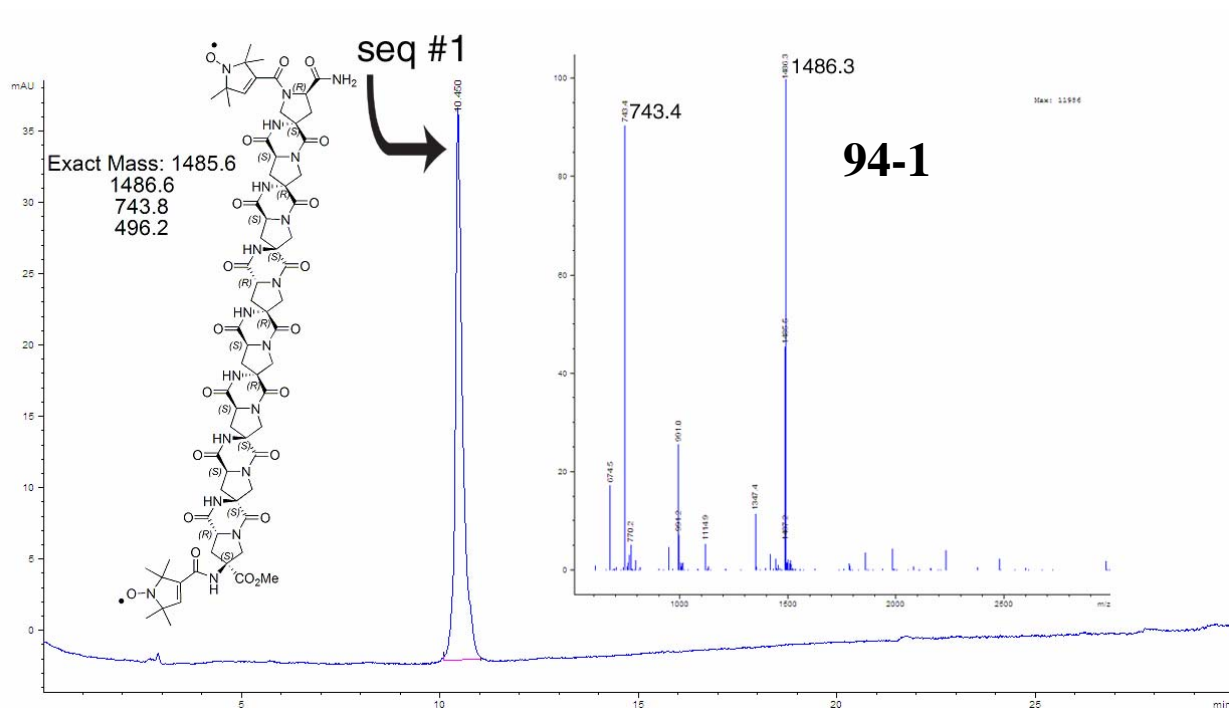


Figure 169 Representative chromatogram of the final purified product, “seq#1”.

One thing that the three sequences which failed to fully close had in common was that they all had an alternating sequence motif of SS-RR repeated three times (or RR-SS or RS-SR). Issues with these motifs failing to close their diketopiperazines had been seen several times within the lab, but now we were aware of possible problems with this repeating motif (a hypothesis for this issue was discussed in the Phage Display chapter).

Modeling suggested that the distances for the three measured sequences should have been 32 nm, 29 nm, and 22 nm. The experimentally measured distances were not consistent with the predicted measurements and no pattern was apparent.

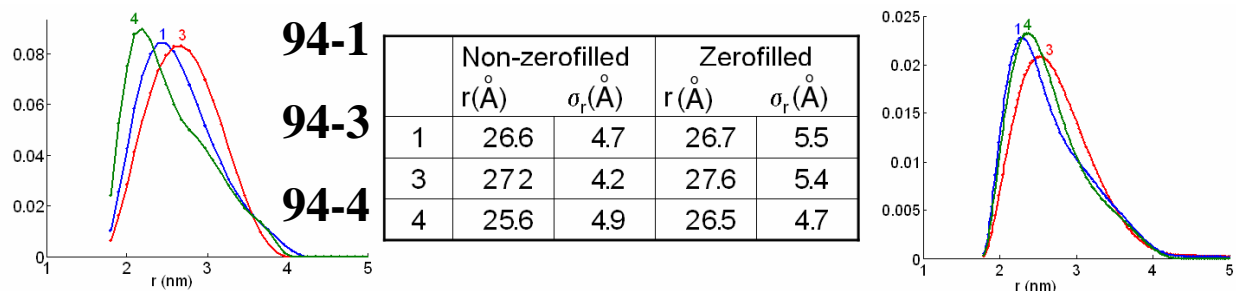


Figure 170 Distances measured for curved 1, 3, and 4. Plot on left is Non-zero-filled, right is zero-filled.

These first curved scaffolds were designed based on molecular mechanics predictions of the shapes of oligomers containing sequences of all four pro4 monomers. Most of the scaffolds incorporated SR and RS building blocks. The understanding within the lab of the conformational preferences of SR and RS building blocks is limited and based purely on molecular mechanics predictions. Our knowledge of the conformational preferences of SS and RR building blocks is more advanced and based on several NMR structures of oligomers containing different sequences of SS and RR. So I proposed that we create curved sequences in which only SS and RR building blocks would be used. Also, there would be very specific points of diversion between sequences (Figure 171). RR “mutations” would be specifically incorporated into only three positions, illustrated below.

c1	c2	c3	c4	c5	c6
SS	SS	SS	SS	SS	SS
SS	SS	SS	SS	SS	SS
RR	RR	RR	RR	SS	SS
SS	SS	SS	SS	SS	SS
SS	RR	RR	SS	RR	RR
SS	SS	SS	SS	SS	SS
SS	SS	RR	RR	RR	SS
SS	SS	SS	SS	SS	SS

95-(1-6)

Figure 171 Table of 2nd set of “curved” scaffold sequences which were synthesized. Plum = positions of variance.

Again, these molecules were made as described previously (Figure 172). And as before, in one sequence the DKPs failed to fully close. In C3, the problematic motif again resulted in a loss of material and stalling of the DKP closure. The other 5 curved sequences had their distances measured by EPR (Figure 173). Attempts to carry out *in vacuo* dynamics simulations on these sequences were foiled by the two ends coming in contact with each other early in the simulation, and remaining stuck throughout. A solution to this problem is to simulate in explicit water solvent, instead of the gas phase, but this is computationally intensive and will be done a later date.

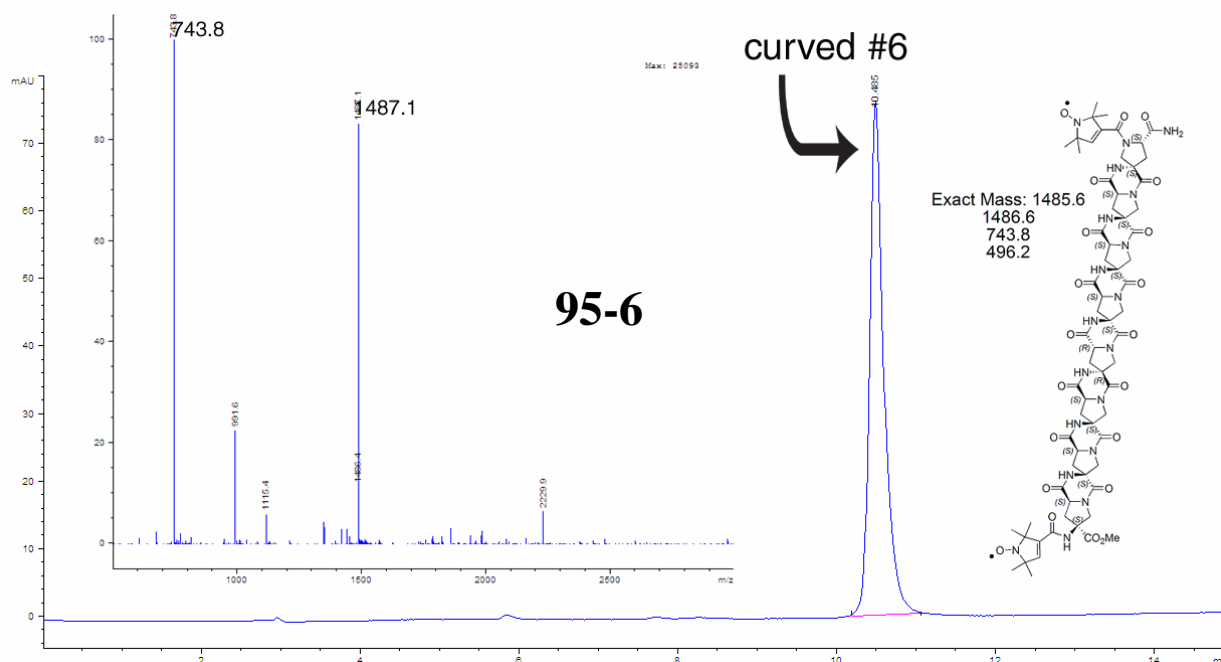


Figure 172 Representative chromatogram of the final purified product, "curved-6".

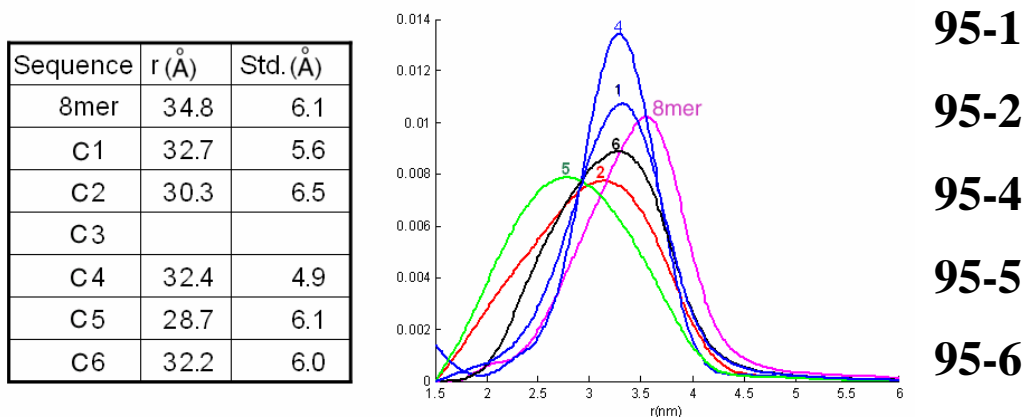


Figure 173 Distances measured for curved sequences, except C3, along with the all-SS 8mer for comparison.

Upon first glance, these results appear unremarkable, however some trends are present. C1 and C6 are among the longest of the sequences, and both of them contain one RR monomer. The sequence SS-RR-SS produced a single bend in 3-D structure, as seen with modeling. C4 contains two RR monomers, which results in two curved SS-RR-SS sequences and a 3-D structure with a zig-zag or "Z" shape. In this sequences the two bends curve in opposite directions and negate each other, resulting in a distance similar to C1 and C6. The C2 and C5 also have two RR monomers and each contain the strongly curved SS-RR-SS-RR-SS sequence and the 3-D structure resembles a "C" shape, which is consistent with the shorter distances. The

key to these observations would be the C3 sequence, which, if it is indeed the shortest, would greatly substantiate these somewhat tentative trends and conclusions.

New methods toward the synthesis of this SS-RR-SS-RR-SS-RR-SS containing motif needed to be developed. As described in a previous chapter, this sequence has repeatedly failed to close all of its DKPs and our hypothesis is that an ordered hydrogen bonding network forms and intermolecular hydrogen bonding causes stalling of the DKP closure and precipitation and loss of the material. I reasoned that perhaps by closing the DKP on the resin, these interactions could be thwarted. Additionally, the hydrogen bonding network could be suppressed by including denaturant-like molecules in the DKP closure solution.

These hypotheses were tested by first synthesizing two sequences containing the problematic motif (Figure 174), consisting of the desired SS-SS-(RR-SS)₃-NH₂ (**96-1** = **95-3**) and the “frame shifted” version SS-(RR-SS)₃-SS-NH₂ (**96-2**) sequence. By employing the regular Rink amide resin, the Boc groups could be removed without cleaving the oligomer from the resin using the previously described TMS-triflate/lutidine conditions. It was assumed that the highly reactive oxidizing environment that accompanies triflic acid cleavage would likely result in reduction and protonation of the nitroxide, thus ruling out TFA deprotection on an HF-cleavable resin. Nonetheless, repeated successes with these TMSOTf / lutidine Boc deprotection conditions provided confidence in the chosen approach, illustrated below.

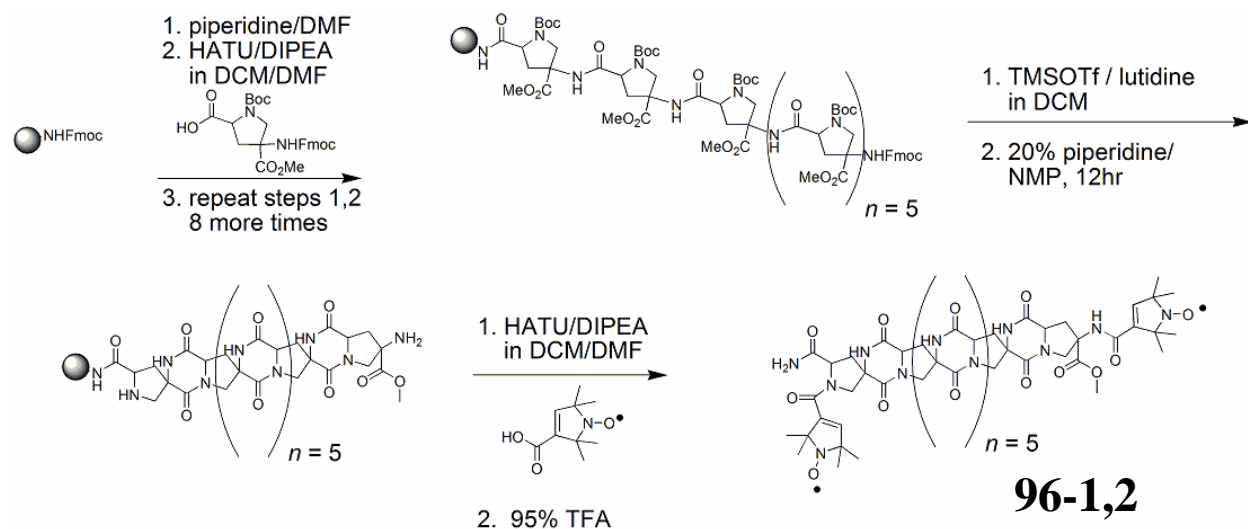


Figure 174 Synthesis of *bis*-spin labeled scaffold with alternating -(RR-SS)₃-SS- configuration.

Both of the sequences were successfully synthesized, so conditions to close the DKPs on the resin were investigated. First, the time was 20 hr, longer than the normal 10-12 hr known to close a normal series of DKPs and the temperature was 37°C-40°C, as usual. Three different conditions were tested on each of the two sequences. The first was the traditional 20% piperidine in NMP. The second had the previous conditions supplemented with 0.5M *ortho*-hydroxypyridine (HO-pyr), capable of simultaneously donating and accepting H-bonds. The final conditions were identical to buffered conditions that I discovered years before that accelerated DKP closure by a factor of >5 (phenol-piperidine, both 0.5M in NMP). After the 20hr incubation, the resins were cleaved and analyzed by LCMS. A preliminary conclusion was that the frame shifted (**96-2**) sequence totally closed, indifferent to the presence of additives, so the traditional conditions were subsequently used. The other sequence (**96-1 = 95-3**) seemed to only fully close with the addition of additive, with a slight preference for HO-pyr. These were significant breakthroughs, and were repeated on a larger scale on both sequences, followed by coupling of the spin label to both amines and cleavage with TFA. Follow-up elaboration and confirmation of the observed preferences for closure conditions are required, as attachment of a more vivid chromophore would greatly substantiate the initial conclusions. Nonetheless, material containing product was isolated (Figure 175), although clean and efficient are not synonymous with this process.

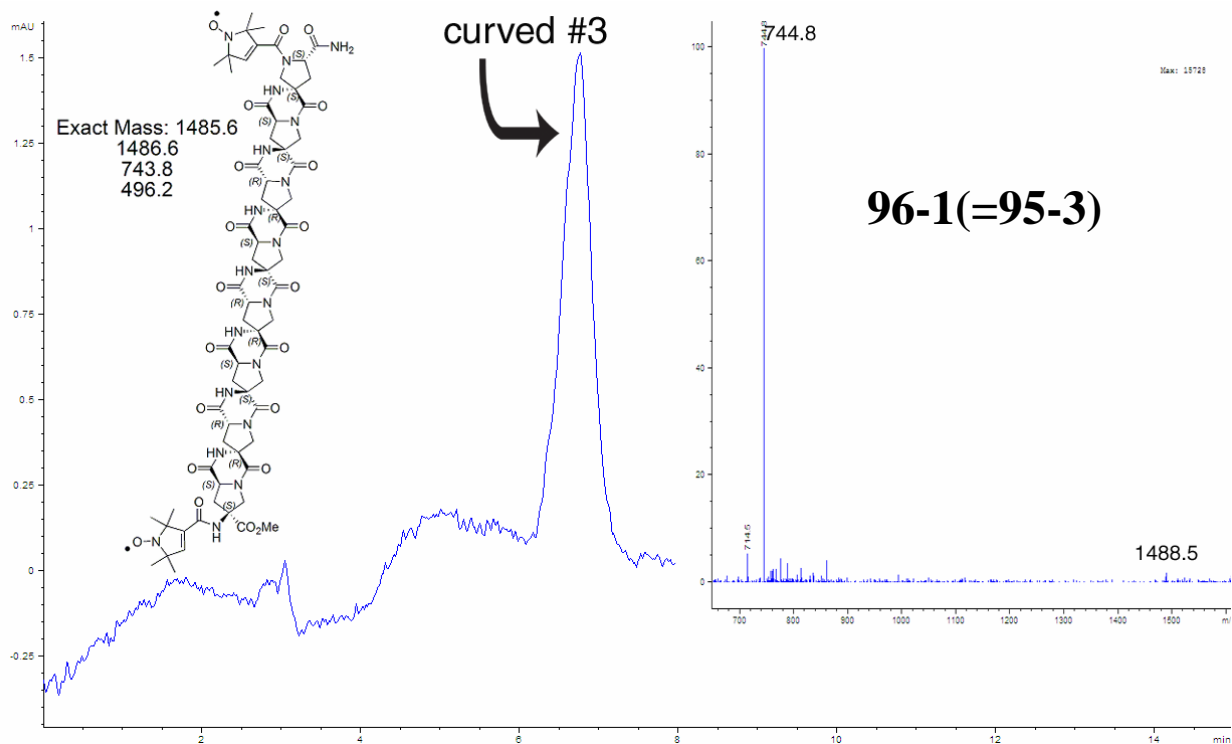


Figure 175 Final purified product, “curved-3”.

We were ecstatic with the results of this measurement (overlaid with all other sequence **95**, in Figure 176). This sequence was perfectly in line with our predictions: adding 3-RR “mutations” into the SS-sequence produces three bends, which cause a **95-3** to curve sharply.

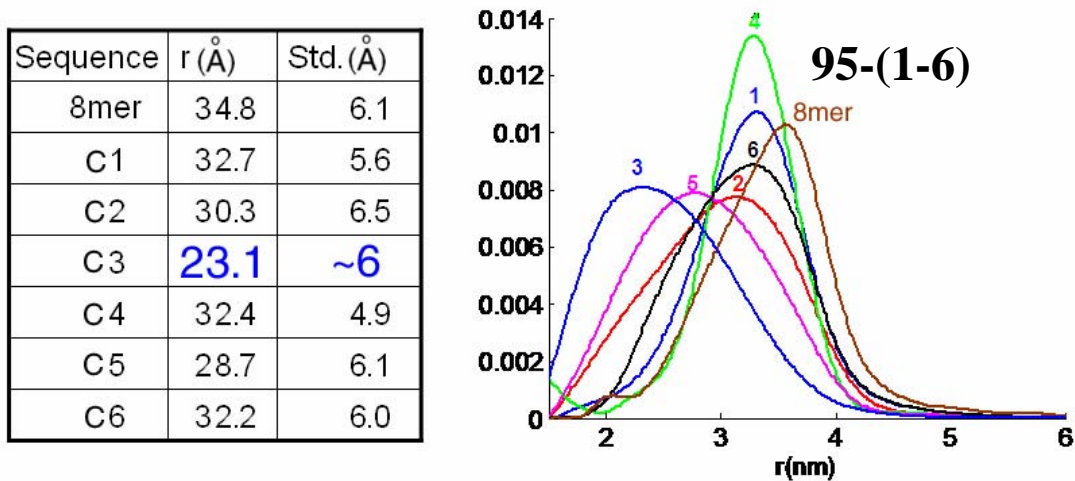


Figure 176 Distances measured for curved sequences (**95-1-6**) along with the all-SS 8mer for comparison

The conclusion of this experiment is that we *are* able to control shape. The most curved scaffold has a distance of 23.1Å while the totally straight scaffold has distance of 34.8Å. That is a difference of over 30%. This technique of measuring distance using ESR has really given us a great deal of insight into the behavior of our molecules. This should prove to be a very productive and fruitful collaboration long into the future.

6.3 CONCLUSIONS

In this chapter, it has been demonstrated that electron spin resonance (ESR) is effective at determining the long range distances of our water soluble *bis*-amino acid ladder oligomers. In order to develop these oligomers as rod-like structural elements for applications such as bivalent display of ligands and as elements of future nanoscale devices, quantitative information on the lengths and flexibility are required. We show that electron spin resonance provides a natural spectroscopic method to rapidly assay these structural parameters. The conclusion from these experiments is that we are able to control both shape and distance with our molecules, putting us in a category all to ourselves.

6.4 EXPERIMENTAL

General: Dichloromethane was distilled from CaH₂. All other reagents were used as received, unless stated otherwise. Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. N,N-diisopropylethylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and an

inert atmosphere over the reaction. HPLC-MS analysis was performed on a Hewlett-Packard Series 1050 instrument equipped with a Waters Xterra MS C₁₈ column (3.5 μm packing, 4.6 mm x 150 mm) and a diode-array detector, while the MSD-ESI was Series 1100. Preparative purification was done using a Varian Prostar 500 equipped with a Waters Xterra Prep MS-C₁₈ column (5 μm packing, 10 mm x 100 mm), equipped with a dual wavelength detector.

Synthesis of *bis*-spin labeled scaffolds of different lengths (93-(4-8)) or different shapes (94-(1-10)), (95-(1-6)).

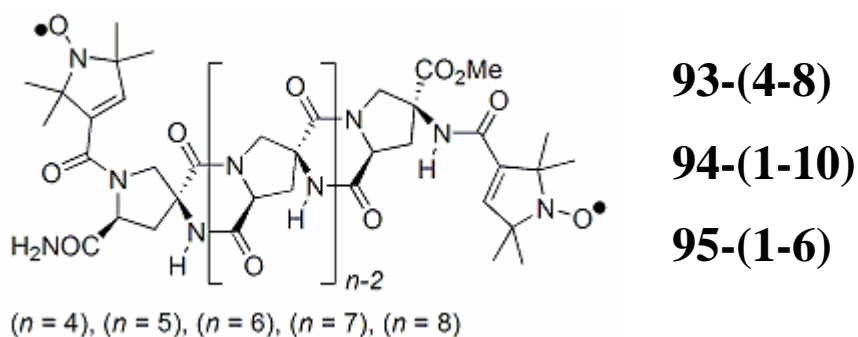


Figure 177 Five bivalent spin probes, lengths ranging from 4-8 building blocks, or 8 building blocks with different stereochemistries.

To five 1.5mL polypropylene solid phase peptide synthesis (SPPS) reaction vessels was added Rink amide AM Resin (Novabiochem) (5mg, 3.15μmol loading). The resin was swollen for 1hr in dimethylformamide (DMF). The terminal Fmoc-protected amine was deprotected in 0.5mL of 20% piperdine/DMF for 40min. By measuring the absorbance at 301nm of a 1/100 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, and DMF for 2min each.

In a 1.5mL microcentrifuge tube, the coupling solution was made by dissolving 17.1 mg (31.5 μmol) of Fmoc-(Boc) pro4(2X4X) building block[17] and 12.0 mg of HATU (31.5 μmol) in 160 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 11.0 μL of DIPEA (63.0 μmol) was added to make the active ester. After 10min activation time, an appropriate aliquot of the solution was added to each deprotected resin, and allowed to react for 30min. The resin was then washed 3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described

above. This process of coupling/deprotection was repeated from three to seven additional times as needed to make the desired number of monomer units.

After the final *nth* ($n=4-8$) building block was attached and Fmoc group removed, the first spin label was attached. A solution of 29.0 mg (158 μmol) of 2,2,5,5-Tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid and 60.0 mg of HATU (158 μmol) in 800 μL of 20% DCM/DMF was mixed using a micropipettor, after which 55.0 μL of DIPEA (315 μmol) was added to make the active ester. After 10min activation time, an appropriate aliquot of the solution was added to each resin, and allowed to react for 30min. After the second coupling, the resin was prepared for cleavage by washing with DMF, isopropanol, DMF, isopropanol, DCM, methanol, DCM, methanol, and DCM for 2 min each. The reactors were then put in vacuum tube, and dried, *in vacuo*, overnight.

The resins were cleaved in 1mL of 2.5% water, 2.5% triisopropylsilane, in trifluoroacetic acid (TFA), with stirring for 2 hours. The solution was filtered away from the resin beads, and 2 x 1 mL was used to wash the beads. The solutions were pooled and the solvent was removed under a stream of dry nitrogen. Residual solvent was removed, *in vacuo*, for one hour, yielding a colorless residue.

The cleaved products were dissolved in 125 μL of 20% piperidine/ N-methylpyrrolidinone (NMP). After 48hrs at room temperature, the products were precipitated by dripping into 2 mL of ether stirring in a 2.2mL polypropylene microcentrifuge tube. The precipitates were collected by centrifugation at 10000x g, 4°C, for 5min. The pellets were washed with 2mL of fresh ether, sonicated, and the centrifugation was repeated. The ether was removed and the pellets were allowed to dry. The pellets were dissolved in a solution of the active-ester of the spin probe, using identical amounts and volumes as above. This reaction was allowed to proceed for 30 min. The final, crude, *bis*-spin labeled oligomers were precipitated by dripping into 2 mL of ether stirring in a 2.2mL polypropylene microcentrifuge tube, washed and collected as above, with most of the excess spin-label conveniently being soluble in the ether. The precipitates were dissolved in 30uL of 30% acetonitrile in water with 0.1% trifluoroacetic acid. This crude material was purified on the analytical HPLC identified in the General Methods section. A small aliquot of each final product was re-injected on the LC-MS and confirmed by mass analysis. In all cases, the desired product was cleanly synthesized, with the only impurity being a small amount of HOAt.

Synthesis of *bis*-spin labeled 8-mer scaffolds with different shapes (96-(1-2)).

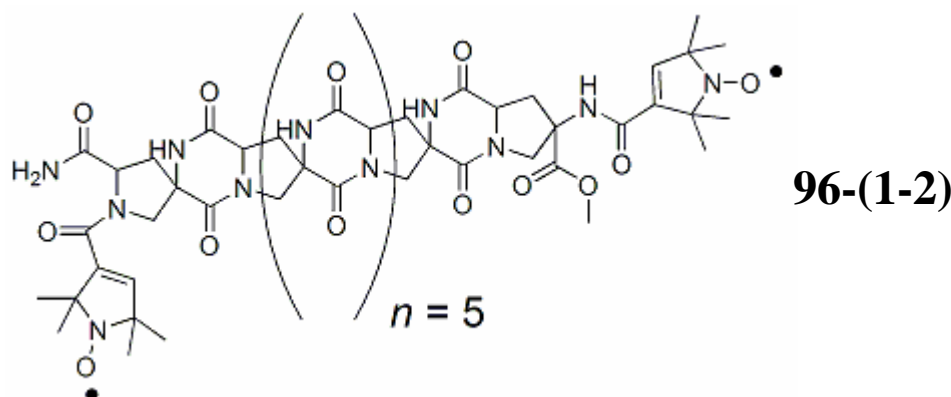


Figure 178 Bivalent spin probes, with different shapes having different stereochemistries.

Synthesis of this most curved motif containing (SS-RR)₃-SS-SS was identical to that described above except for the three following changes:

- (1). Employment of the regular Rink Amide resin (NOT AM).
- (2). After last building block is attached, the Boc groups were removed using TMSOTf-lutidine, as follows-- The Boc groups on the building blocks were removed using procedure from the Burgess lab.[42] The beads were washed with DCM for 5min. A solution of 5mL of 1M trimethylsilyl triflate (Aldrich-TMSOTf-from a Schlenk bottle) and 1.5M 2,6-lutidine in DCM was prepared. By adding 3 x 0.75mL x 5min, with slight bubbling, the Boc groups were removed while still attached to the resin, with DCM washes in between. After deprotection, the resin was washed vigorously with DCM, followed by 5 cycles of MeOH / DCM washes, ending up with swelling in DMF for 5min. The DKPs were then closed on the resin using a stirred solution of 0.5mL of 20% piperidine in NMP for 20hrs at 35°C-40°C. Two other choices were employed for closing the DKPs (a) 20% piperidine/NMP with 0.5M *ortho*-hydroxypyridine. (b) 0.5M piperidine, 0.5M liquefied phenol, in NMP. After 20hrs, the resin was washed extensively with DMF and IPA.
- (3). Spin labels were attached to both amines, while still attached to the resin, 2 x 20eq of both HATU and spin label-CO₂H, 40eq DIPEA, 0.2M in DMF, for 2 x 30min. Cleavage with 95% TFA and HPLC purification are as described above.

Electron Spin Resonance Spectroscopy

Sample preparation

For ESR experiments, 0.2 mM of the double labeled molecules were dissolved in 70% buffer (50 mM phosphate buffer, pH 7.4, 200 mM NaCl, 3 mM NaN₃, 1 mM EDTA) and 30% glycerol. Each sample (~10 μ l) was placed in a ~1.5 OD mm. pyrex capillary tube and flash frozen in liquid nitrogen immediately before insertion into the cavity.

FT-ESR Spectroscopy

The ESR experiments were performed using Bruker EleXsys E580 CW/FT X-band ESR spectrometer equipped with a Bruker X-band ER 4118X-MS2 split ring resonator for compounds n=4,6 and 7, and 4118X-MS3 for compound n=5 and 8. Both resonators provided identical results for the same measurement. The temperature was controlled by an Oxford ITC605 temperature controller and an ER 4118CF gas flow cryostat. All experiments were performed at the temperature of 80 K.

Four-pulse DEER experiments were obtained with a resonator $Q \leq 100$ and an ASE TWTA with an output power of 1 KW. The pulse sequence for generating the dipolar time evolution data is shown in the figure below. Two-step phase cycle was used for baseline correction. The observer frequency, ν_A , was set at the central field of the spin label peak which is around 9.5-9.6 GHz, and the pump frequency, ν_B , was set at 70 MHz higher. The length of the $\pi/2$ and π pulses was 24 and 48, respectively. The interpulse delays were 200 ns for τ_1 , 2200 ns for τ_2 . The increment of time T after the second pulse was 16 ns for 128 points. For each step of the phase cycle 500 averages were collected at a repetition rate of 1 KHz. The acquisition time used for each sample was roughly 24 hours.

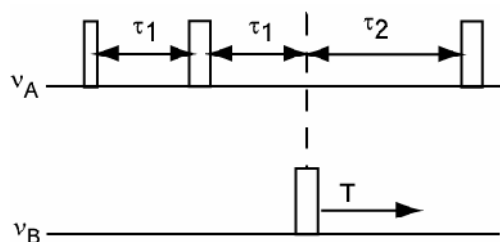


Figure 179 Four-pulse DEER sequence.

Analysis of ESR Data

The 4-pulse DEER time domain data were processed before acquiring the distance distribution functions. The background decay due to intermolecular interaction was subtracted by fitting the last 75% of the data with a first order polynomial function. For compounds 3-5 (n=6-8) the time domain data was smoothed by hamming function and zero-filled to 512 points before analyzing the data. Fourier Transform of the processed data provided the frequency spectra.

The time domain data was analyzed using DEERAnalysis 2004 program which is freely available on the web [www.mpip-mainz.mpg.de/~jeschke/distance.html]. The distance distribution functions were obtained using the Tikhonov regularization method with the regularization parameter of 4.0 for compounds 1-2 (n = 4-5), and 50.0 for compounds 3-5 (n = 6-8). In this analysis no constraints on the maximum possible distance was imposed. The experimental and simulated time domain signal and spectra are shown in the figure below.

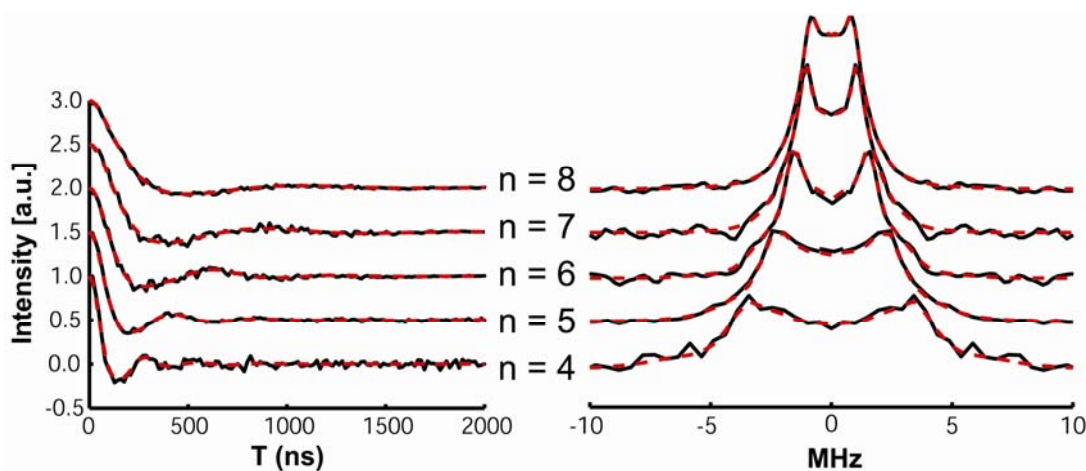


Figure 180 a) 4-Pulse DEER time domain. b) Fourier Transform of the DEER distribution functions.

The distribution functions for compounds 1-2 (cf. Figures S3-S4) appear to have physically unreasonable lobes on the upper end (large r-values). These are possibly due to the fact that the Tikhonov procedure is mathematically ill-posed in the presence in the noise. The use of a better regularization parameter of 10, obtained by using an L-curve criterion, was successful in suppressing these lobes.

In an alternative approach the ESR data was also inverted using the Tikhonov regularization method with limits imposed on the maximum allowable distance. Both methods yielded virtually identical distance distribution.

Molecular Modeling

Molecular dynamics simulations were carried out using the Amber94 force field.[101] Five-nanosecond *in vacuo* molecular dynamics simulations at 300 Kelvin were carried out on each compound. Histograms of the distance between the two nitroxide nitrogens were calculated and overlaid on the population distributions determined by ESR.

7. SCAFFOLD DETERGENT FOR SOLUBLIZATION OF MEMBRANE PROTEINS

7.1 INTRODUCTION

The structure and function of membrane proteins are central to many areas of biomedical research. Integral membrane proteins act as channels for transport of ions and small molecules into and out of the cell and as receptors for communication of signals and stimuli from outside the cell. As the first potential messenger in complex signal transduction pathways, membrane proteins are a prime target for perturbation by pharmaceuticals. However, in spite of the need for membrane protein structural information, only about 100 unique membrane proteins have been structurally characterized. Contrast that with the thousands of soluble proteins whose structures have been solved. Estimates of 1 out every 3 genes coding for a membrane protein indicates that, potentially, there could be 10,000 membrane proteins,[102] necessitating the urgent need for exponentially improved methods and techniques

In order to maintain the folded structure of a membrane protein outside of its native lipid bilayer, it must be stabilized by detergents which form micelles that mimic the lipid bilayer. Ideally, the detergent-protein complex needs to form small micelles, although even in these cases the mass of the complex doubles. The idealized situation would entail 2-D and 3-D contacts between individual membrane proteins, while the detergent simply fills the interstitial space between the membrane proteins. An alternative over-simplification may envision a smooth compact micelle providing shelter for a small number of membrane proteins. Both situations are inaccurate. A more accurate depiction would be that the micelle-protein complex is not well ordered or efficiently packed. Packing defects are introduced when other detergents or solutes are incorporated into the pure detergent micelle. Additional heterogeneity is inherent in the fluidity of the micelle itself. Although the average radius or lipid composition may not change

much, there still exists a great deal of diversity in micellar structure.[103] Diversity and heterogeneity are two features antithetic to well-ordered crystals capable of high resolution characterization.

In the search for detergents capable of forming well-ordered complexes able to solubilize membranes, amphipathic peptides potentially fit the mold. An early example of this potential was the *de novo* design of an amphipathic peptide that was found to fold into a four-helix bundle.[104] Hydrophobic contacts between the leucines and alanines of adjacent peptides are the driving force behind the stable fold of this short peptide (Figure 180).

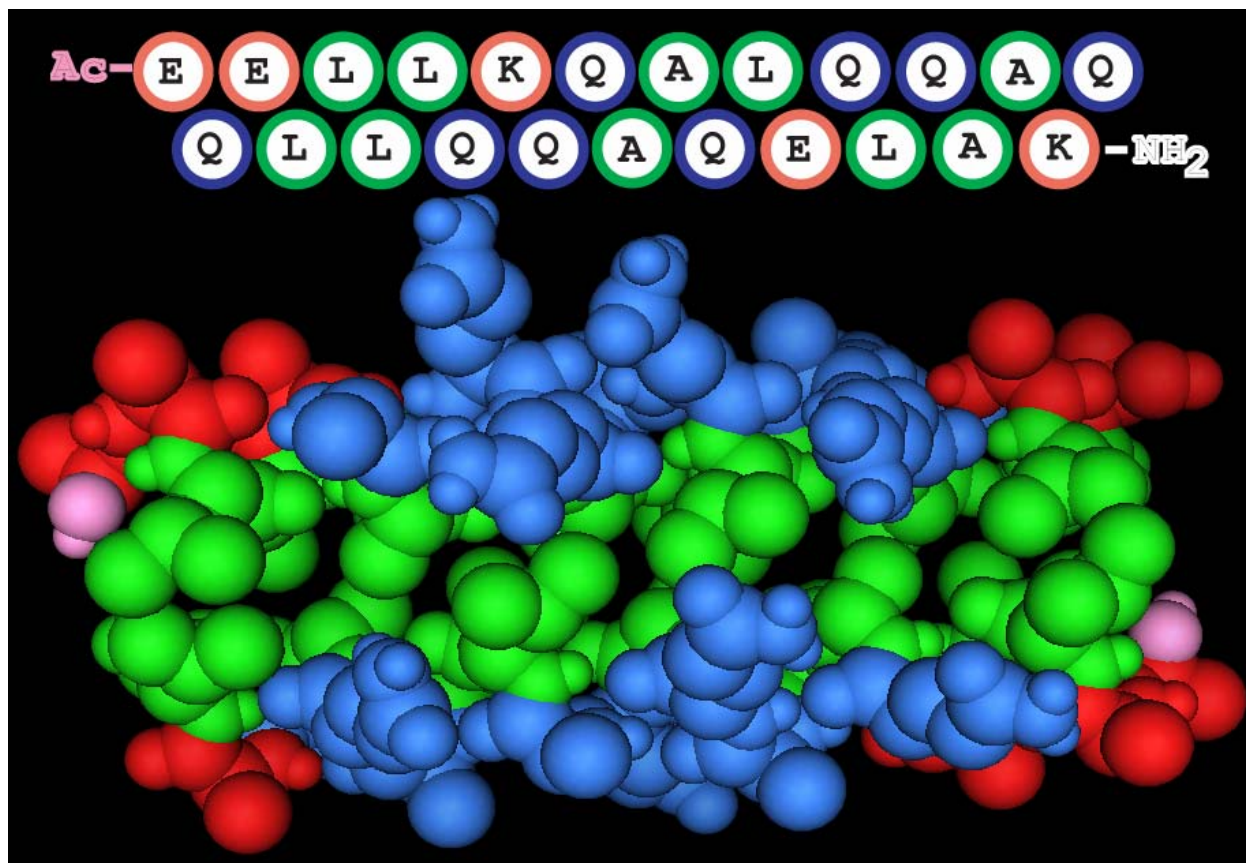


Figure 181 PDB structure of the 4HB1 as a dimer.

It was found that this peptide acted as a detergent, or a “peptitergent,” as judged by its ability to stabilize the folded structure of bacteriorhodopsin and rhodopsin, both α -helical even when the initial detergent concentration was diluted to 1/100th of the critical micelle concentration. However, over the period of days to weeks, the peptitergent-membrane protein

complex was unstable. Unfortunately, this is the same time frame needed for the growth of large, high-quality crystals.

The same concept of alternation of polar/charged and non-polar amino acids in order to sequester hydrophobicity on a single face of a peptide was again used to elaborate on the premise of peptitergents.[105] In this case, simply by incorporating two ornithines at either end of the peptide, fatty acid chains of lengths (C12, C14, C16, C18, and C20) were able to be attached. It was found that acyl chain length had an effect on the micelle size but not on the maintenance of the folded state of membrane proteins, both α -helical and β -barrel. It was found that these peptitergents were capable of stabilizing the folded state of bacteriorhodopsin for periods in excess of 1 month, while the aggregational propensity of the particularly sensitive membrane protein, *lac* permease, was found to be less in the presence of a peptitertgent than in any other detergent mixture known to date.

There is conceivably one area for improvement and that is in lowering the mass. The mass of the lipopeptitertgent is around 3000 Da.[105] This has consequences for protein structural characterization by NMR in two areas. First, the larger the mass of the detergent, the more unwanted peaks there are in the NMR spectra of the membrane protein. Second, detergents with larger masses lead to detergent-protein complexes that have a longer rotational correlation time, which can lead to peak broadening and the blurring of interactions used for NOESY and ROESY spectral characterization. A “scaffold” detergent would have about half of the mass of a peptitertgent, making clear the potential benefits that our techniques and methods could bring to the field of membrane protein structural characterization.

7.2 RESULTS AND DISCUSSION

It was proposed that a scaffold detergent should have similar properties to previous peptitertgents by exploiting the amphipathic nature of the polar scaffold backbone and non-polar long chain, fatty acids. Methods and procedures had just been developed for the synthesis of nanoscale molecules,[20] and the synthesis of the scaffold detergent would stand to validate the generality of these solid phase techniques, as well introduce some new approaches towards making functional, nanoscale molecules. The number of building blocks (seven) was chosen because a

scaffold of this length should have an end-to-end distance of 30Å, which is the distance of a typical membrane cross-section.

Using the Rink Amide resin, the first residue, orthogonally protected Fmoc-ornithine(methyltrityl)-OH, was attached using HBTU / HOBt. Double couplings of 5 equivalents, relative to initial resin loading, allowed for quantitative acylation in 2 x 30min reaction times, as judged by subsequent fluorenylmethyloxycarbonyl (Fmoc) release and small scale cleavages at each step. Due to the large scale of this synthesis, illustrated in Figure 181, and the corresponding amount of building block that was being consumed, or wasted if a coupling failed for some reason, it was thought wise to proceed slowly and very cleave small amounts of resin to ensure reaction completion. The Kaiser test for resin bound free amines is an excellent way to monitor reaction progress and is very sensitive for even small amount of free amines, producing a vivid blue-purple color in the presence of amines, however, due to the presence of an amine on a quaternary center, the Kaiser test is unreactive towards this type of amine. Cleavage and analysis by LC/MS before each Fmoc release, in all cases, confirmed that 2 x 2eq building block, along with the more reactive coupling reagent HATU were sufficient to quantitatively acylate the resin bound amine. After the seventh building block had been incorporated, the second ornithine was added to complete the linear sequence. Removal of this Fmoc was allowed to go for 2hr instead of the usual 30-40min. Reaction between the liberated amine and the methyl ester occurred at this time to form a diketopiperazine (DKP). Formation of this end-DKP spontaneously occurs any time an amino acid follows a building block. Allowing this reaction to go for any extra period of time ensure that no free amines remained, which had significance for the following steps.

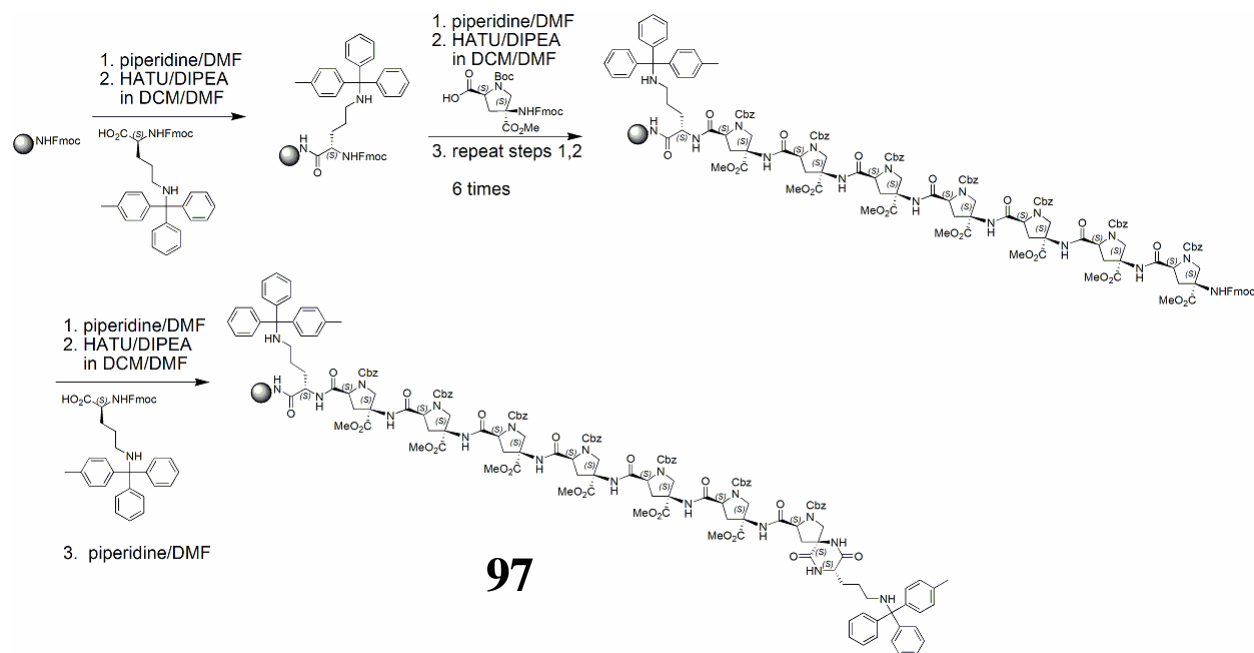


Figure 182 Synthesis of the fully protected scaffold prior to side chain derivatization.

At this time the resin, now weighing in excess of 450mg, was dried in vacuo and small portions (~5mg) were used to work through the remaining steps. Treatment of the resin with 1% trifluoroacetic acid (TFA), 5% triisopropylsilane, in dichloromethane DCM served to selectively remove the methyltrityl (Mtt) protecting group while preventing cleavage of the linker (Figure 182). As judged by LCMS, initial attempts at Mtt removal followed by acetylation with acetic anhydride resulted in the observance of a significant amount of product still containing a free amine. It was judged that it was likely that the removal of the Mtt group was incomplete, as acetylation was known to be fast and quantitative when used in such large (>100eq) excess. It was found that the vendor's recommended procedure of 3 x 1mL x 2min was insufficient, because when the reaction was extended to 10 x 1mL x 3min followed again by acetylation, complete conversion to the amide was achieved. Several lengths of fatty acid chains, first C8, C12, and C16 were chosen initially as these lengths would have been capable of spanning the lengths of the scaffold. Later, lengths of C4 and C6 were included for the purpose of exploring the effects on the solubility properties.

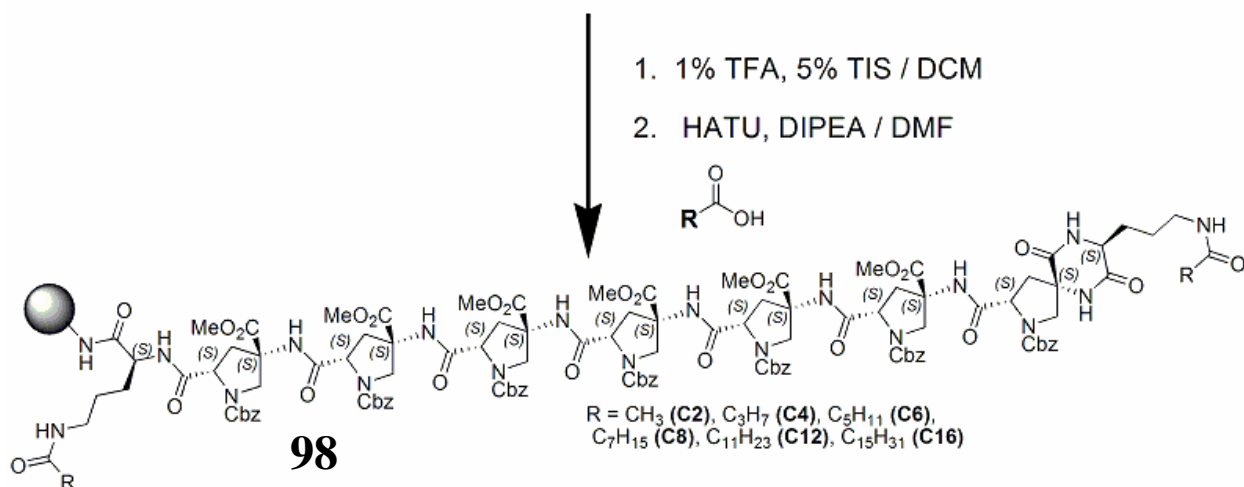


Figure 183 Synthesis of the fully protected scaffold with alkyl chains (R-groups).

Improved cleavage protocols were developed that allowed for cleavage and Cbz deprotection in a single step. Previously, cleavage was done using 95%TFA, followed by hydrogenation of the Cbz groups using H_2 , Pd/C in MeOH:H₂O:AcOH (7:3:1). Difficulties can ensue because of the drastic polarity change upon conversion of multiple benzyl groups into protonated amines. Especially when long fatty acid chains were attached to the scaffold, difficulties in recovery of product were observed, perhaps due to interactions between the alkyl chains and carbon. In light of these difficulties, alternative conditions were explored utilizing stronger acid cleavage cocktails capable of protonating off the Cbz groups and cleaving the linker as well. Several sets of conditions were tested, employing a variety of scavengers and trimethylsilyl trifluoromethanesulfonate (TMSOTf) or trifluoromethanesulfonic acid (TfOH). It was found that ideal conditions consisted of 4% TfOH, 4% ethanedithiol, and 8% thioanisole in TFA. These conditions reliably removed all Cbz groups, while keeping other spurious impurities or oxidation products to a minimum, as judged by LC/MS (Figure 183).

With these improved conditions in hand, initial attempts at synthesizing a scaffold detergent were made by acylating the scaffold with fatty acids of chain length C2, C8, C12, and C16. In spite of the lack of a good chromophore, the amide bonds provided sufficient absorbance at 220nm, to prove that the desired products were successfully synthesized as judged by LC/MS. On a 5% - 95% acetonitrile (0.05% TFA), water (0.1% TFA) gradient over 30min

(3% change per minute), it was clear that elution time was directly related to fatty acid chain length. C2 eluted around 5min, C8 around 10min, C12 around 15min and C16 around 20min, all containing 7 free secondary amines. Initial attempts at closing the DKP proved frustrating. At this time, the knowledge base for clean and efficient closure of multiple DKP rings in 20% piperidine in DMF, while keeping potential epimerization to a minimum, was not very developed. In most cases, complete closure of all 7 DKP took at least 48hrs, while the sight of a single LCMS peak containing the desired final product mass was a rarity. More often, several peaks of identical mass would be present, as well as multiple products clearly having one or more DKP still in the open form were also present. In the case of the C16 alkyl chain, hydrophobicity increased so much that it would not even elute during the normal 5-95% ACN gradient, as the only way to see it would be to pump 100% ACN for several minutes at the end of the gradient. Because of this, there was zero potential for precise characterization of the extent of complete closure as well as any possible epimerization, nor would preparative purification have been able to resolve any impurities. C12 had similar problems of late elution on a 5-95% ACN gradient. C8 provided a DKP closed product eluting at around 15min which was prepurified, albeit not to total homogeneity.

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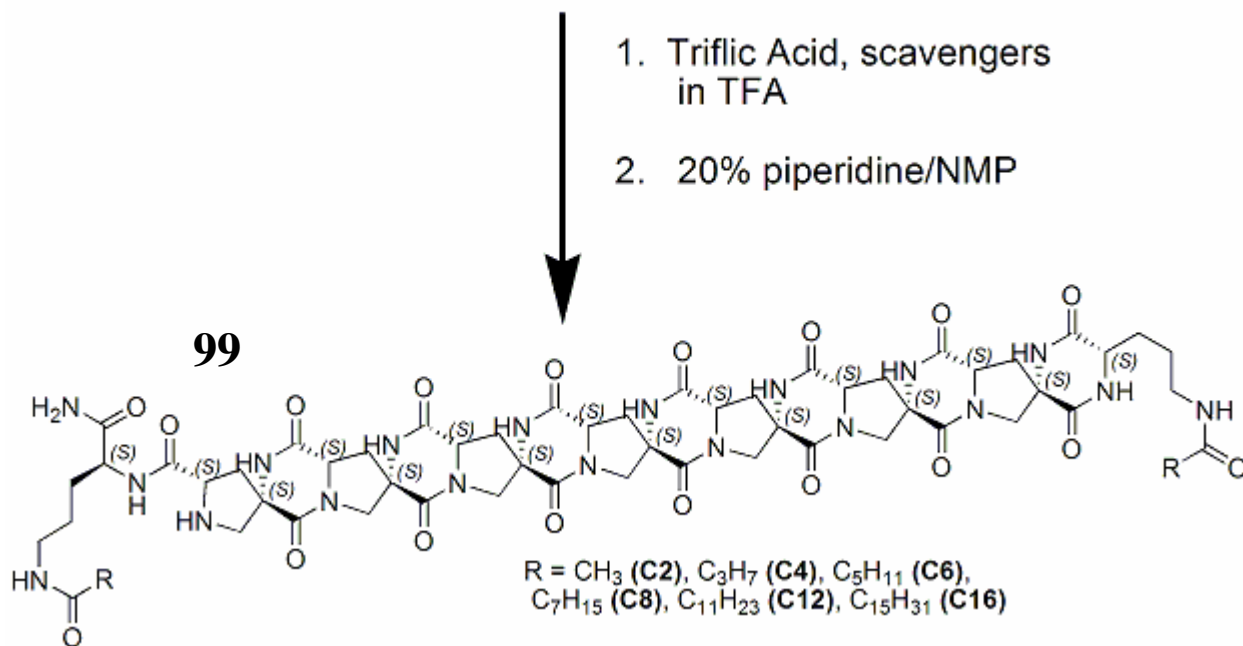


Figure 184 Synthesis of DKP closed scaffold with alkyl chains (R-groups).

Additional scaffold detergents, having C4 and C6 alkyl chains were synthesized. Likewise these lengths eluted between 5-10min on a 30min 5-95% ACN gradient, and could be prep purified. Again difficulties were encountered in getting total DKP closure and minimizing the appearance of epimerization.

Solubility tests ensued to evaluate the extent the length of the alkyl chain played on aqueous solubility. C2, C4, C6, and C8 all were dissolved in 25% ACN / water. All were soluble at about 10mg/mL as judged by LCMS, however, when the organic solvent was diluted to 2.5% - 5% by addition of water, only the C2 and C4 remained soluble at this organic solvent composition. Even at this relatively dilute concentration of ~1mg/mL, solubility was not possible for C6 and C8. Other organic solvents such as DMF or DMSO were evaluated, as well as different ways of enhancing solubility, such as sonication or addition of buffers or small amounts of urea or guanidinium, however, similar results were found. At this point there were three deficiencies in this attempt at making a scaffold detergent capable of self-associating into micelles. The first was the presumed insolubility of the longer chains such as C12 and C16. These lengths were needed, as they were modeled to be of the appropriate length to fully span the scaffold from end to end. Even shorter lengths that would be unable to span the scaffold, such as C6 and C8 were soluble in aqueous solutions only with large amounts of organic cosolvent. Secondly, the absolute solubility in water, with minimal amounts of organic solvent, was low to non-existent, for any fatty acid of potential utility. Solubility in excess of 5-10mg/mL was thought to be a requirement of any detergent molecule, as these concentrations are required for NMR structural analysis or crystal growth for X-ray structural determination. Thirdly, it can be inferred that the lack of high solubility in primarily aqueous solvent was a result of a failure to associate into micelles. This last point was the more glaring. Formation of micelles was an absolute requirement for any potential to solubilize a membrane protein. There obviously was not a driving force for the two fatty acid chains to associate on a single face of the scaffold, which could lead to self-association into cylindrical micelles. It turned out to be a quite an irony: an attempt was made to make a molecule that could maintain the water solubility of a membrane protein, but this molecule itself was not even water soluble.

Another attempt two years later was made on a second version of the scaffold detergent that was the result of the accumulation of knowledge gained in those two years. This version of

the scaffold detergent was designed to form the full 8 DKPs, instead of the previous 7 DKPs. This extra rigidity was hypothesized to aid in the pre-organization of the two fatty acid chains on the same face. In the analysis of the 3-D conformation of this molecule versus the previous attempt, it was obvious that the new attempt was going to be more rigid at both ends, while the previous version was highly flexible at one end. This decrease in conformational flexibility was thought to potentially lead to pre-organization of the alkyl chains on one face. By forcing the two side chains to point off of the same face, perhaps this could result in enhanced solubility in water.

This scaffold detergent with 8 DKPs required a different approach, because a methyl ester would need to present at the end of the scaffold that was attached to the resin, while in the previous version that end was a primary amide. Based on previous knowledge developed, this was accomplished by using a resin that produces a carboxylic acid upon cleavage. This was then converted to the methyl ester selectively. In order for this to be selective, all other protecting groups needed to still be intact. This required that the resin linker be particularly sensitive to cleavage, ideally a weak acid cleavable linker. The chlorotriptylchloride resin fits these requirements. Cleavage is accomplished by treatment with acetic acid, which would maintain the integrity of the Boc protected building blocks, now used exclusively for scaffold synthesis. However, this presented another issue to consider: the methyltrityl side chain protecting groups that were used in the previous version would be incompatible with these cleavage conditions. Orthogonality was found in the ivDde (isovaleryl-dimethyl-dioxocyclohexylidene) protecting group for amines. This protecting group forms a cyclic aromatic indazole upon treatment with hydrazine, thus by using diaminobutanoic acid (Dab) with an ivDde protecting, this scaffold detergent was assembled in a similar manner.

Attachment of the first residue to the trityl resin was accomplished by dissolving Fmoc-Dab(ivDde)-OH in dry dichloromethane, with 5eq of base (Figure 184). The first step resembles an S_N1 reaction, where the chloride leaves the triphenylmethyl position of the resin and the carboxylate anion acts as the nucleophile. Because of this, DMF could not be used, because it would tend to solvate the ions, thus making the reaction less favorable. Also, because of the mechanism, coupling reagents were not necessary. After 2hrs the reactor was washed and the Fmoc was removed in the normal fashion. Additionally, any electrophilic sites left unreacted on the resin would likely have been attacked by the piperidine, effectively capping the resin and

preventing the synthesis of truncated sequences in subsequent steps. Attachment of the subsequent building blocks was exactly as for the first version. Likewise when the last amino acid was attached, closure of the end DKP was facilitated by extending the Fmoc release for 2hrs. After this time, there were no free amines (**100**).

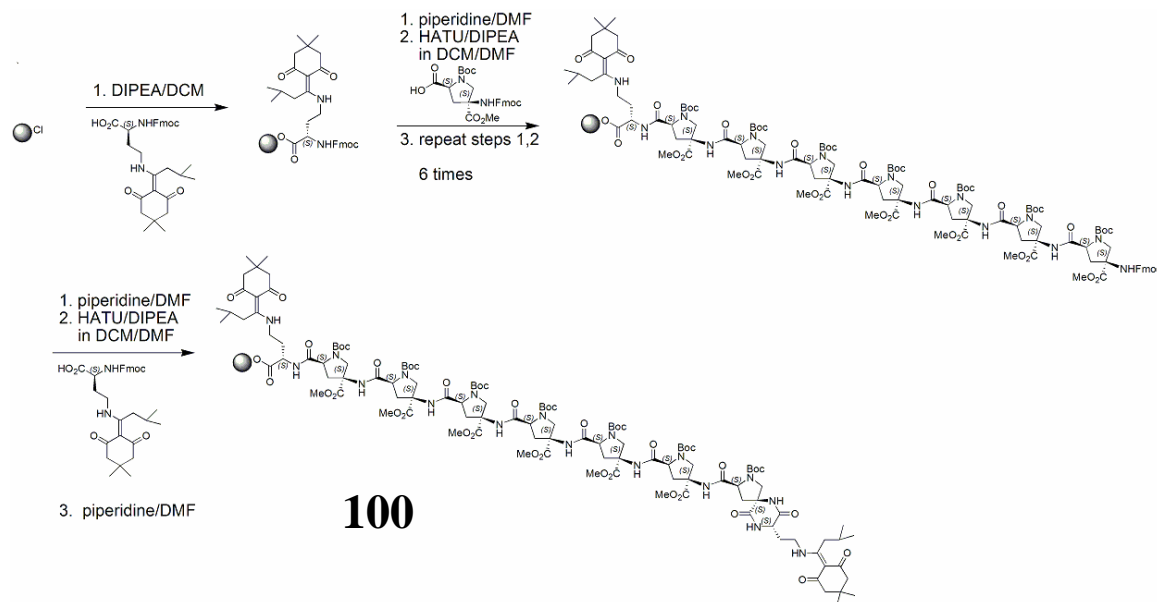


Figure 185 Synthesis of the fully protected scaffold prior to side chain derivatization.

Removal of the ivDde was done in 5 x 3min x 1mL of 3% hydrazine in DMF. At this point the resin was portioned into smaller amounts for the coupling of different lengths of fatty acid chains. Coupling of C2, C8, C12, and C16 was done the same way (Figure 185).

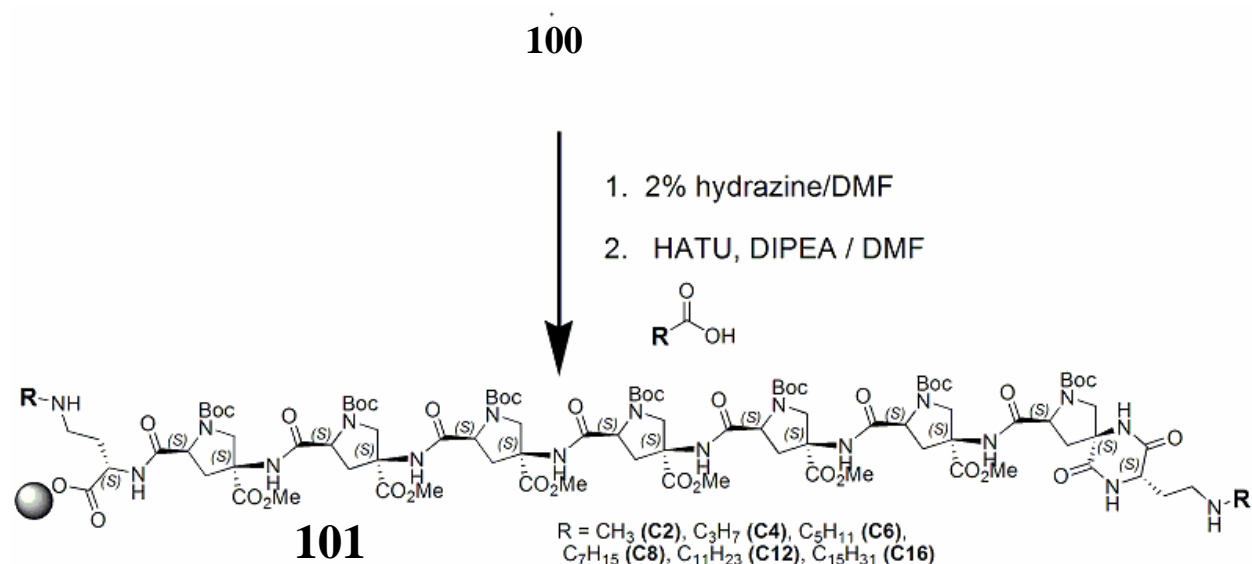


Figure 186 Synthesis of the fully protected scaffold with alkyl chains (R-groups).

Cleavage was carried out in 0.5mL of AcOH:trifluoroethanol:DCM (1:1:3) for 2hrs, and then the resin beads were again cleaved with 0.5mL of fresh cleavage cocktail, for 3 x 20min additional time (Figure 186). The cleavage solutions were pooled and the solvent was removed in a SpeedVac. By addition of DCM:hexanes (3:1) to the oily residue followed by removal of the solvent in the SpeedVac, and repeating this two more times, the oily residue turned into a white pellet. This indicated that all of the AcOH had been removed, which would prove to be important for the success of the next step. Dissolving the pellet in MeOH and treatment of the pellet with 25eq (based on initial resin loading and amounts) of trimethylsilyldiazomethane (TMSCHN₂) in ether, resulted in the conversion of the carboxylic acid to a methyl ester, while not reacting anywhere else in the molecule. Quenching of the excess TMSCHN₂ with TFA, followed by removal of the solvent in the SpeedVac resulted in an oil that was subsequently treated with TFA:DCM (1:1) to remove the Boc groups. Solvent was again removed in the SpeedVac. LCMS analysis of intermediates up to this point was troubled by two factors: lack of any free amine for MS and lack of good chromophore for LC analysis. However, quality MS chromatograms could be generated at this point to prove the success of reactions up to this point (Figure 187), **102-2**, **102-8**, **102-12**, **102-16**.

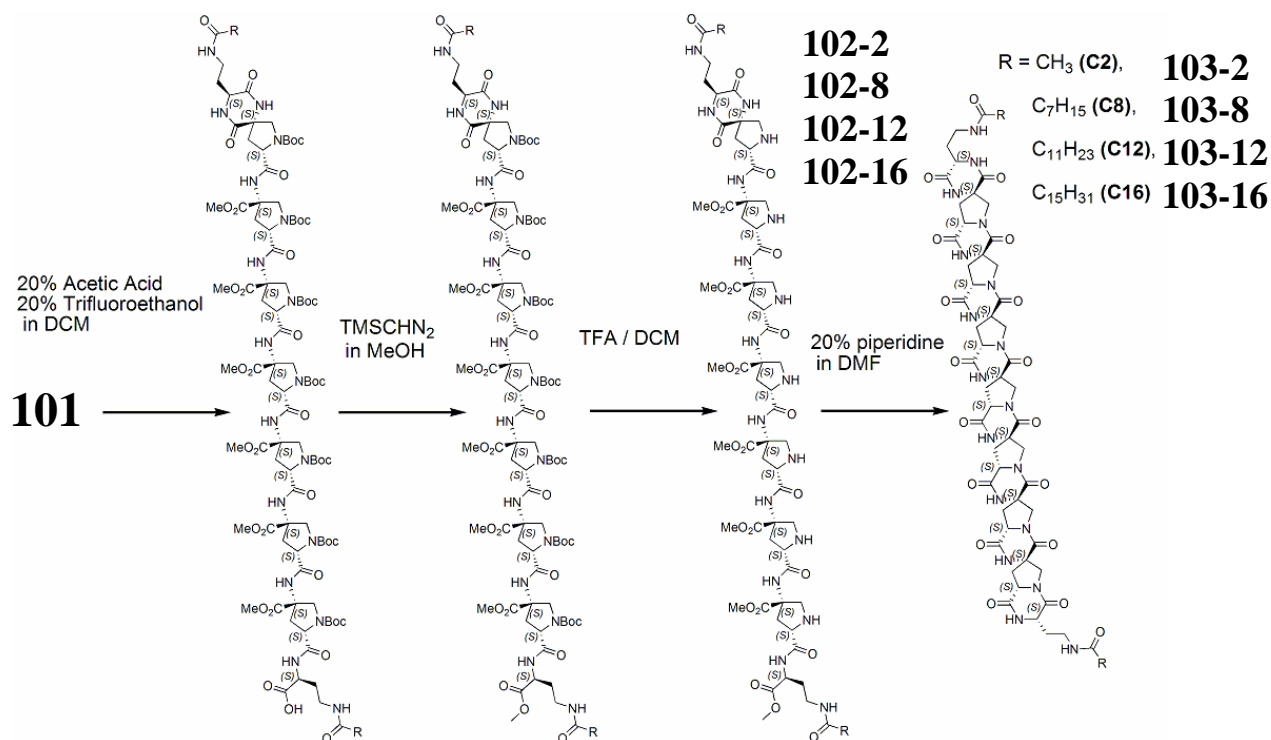


Figure 187 Cleavage, methyl ester formation, Boc deprotection, and DKP closure of this scaffold detergent.

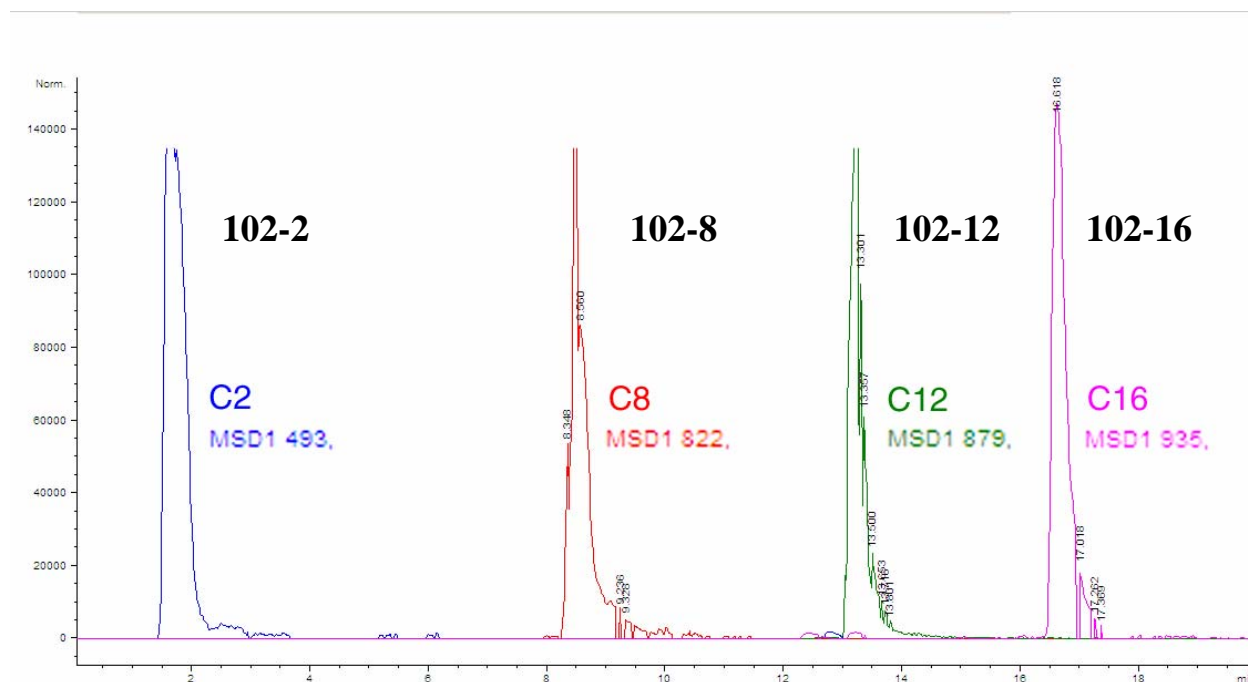


Figure 188 Extracted ion chromatograms of C2, 8, 12, and 16, after removal of Boc groups.

Treatment of this oil with a solution of the 20% piperidine in NMP, both from dry sure-seal bottles, resulted in the closure of the DKPs. Precipitation into ether yielded the final product. The solubility of the final product, C2, C8, C12, and C16 (**103-2**, **103-8**, **103-12**, **103-16**) in water with 10% or less of organic composition, again yielded the same results. The C2 was readily soluble, while the other sequences were totally insoluble, as judged by the absence of ions with expected masses. Again despite extensive sonication, nothing more lipophilic than an acetyl group was able to be dissolved in water with minimal organic cosolvent.

7.3 CONCLUSIONS

Long chain fatty acids are easily incorporated onto orthogonally protected di-amino acids, upon completion of the synthesis of the scaffold. This demonstrates the versatility and adaptability of the solid phase methods that have been developed. Two different approaches to the synthesis of a scaffold detergent have been developed. Both proved to have little solubility in water, with any chain longer than C=2. The key to initiating the formation of a cylindrical micelle may be pre-

organization of the alkyl chains on one face. This is one key difference between the approach taken by Prive and our approach. Both approaches utilize Ornithine side chains spaced a similar distance apart as the point of attachment for long chain fatty acids. Prive's peptitergent accomplishes pre-organization presumably by hydrophobic interaction between the non-polar amino acid side chains on one face of the alpha helix and the methylenes of the fatty acid chain. These interactions serve to hold the fatty acid in one place, facilitating formation of a stable cylindrical micelle. In both of our attempts, there is no potential for pre-organization, beyond the simple inherent rigidity of the scaffold. In fact there are actually interactions forcing the fatty acids away from the scaffold backbone. The polar amide bonds of the backbone actually would be a driving force against organization into micelles.

Solutions to this problem would fall into two categories. Firstly, incorporation of hydrophobic groups along one face of the scaffold backbone may interact with the alkyl chains to hold the chains in a position that could form a micelle. However, this would involve advances beyond what is presently available. Secondly, ligating or locking the fatty acid chains in place would allow for pre-organization to take place. This could be accomplished in two ways. The first would be to one end of the chain to a particular face of the scaffold backbone. The second would be to lock the two ends of the fatty chains together. This could potentially be possible by means of olefin metathesis as seen in Figure 188.[106]

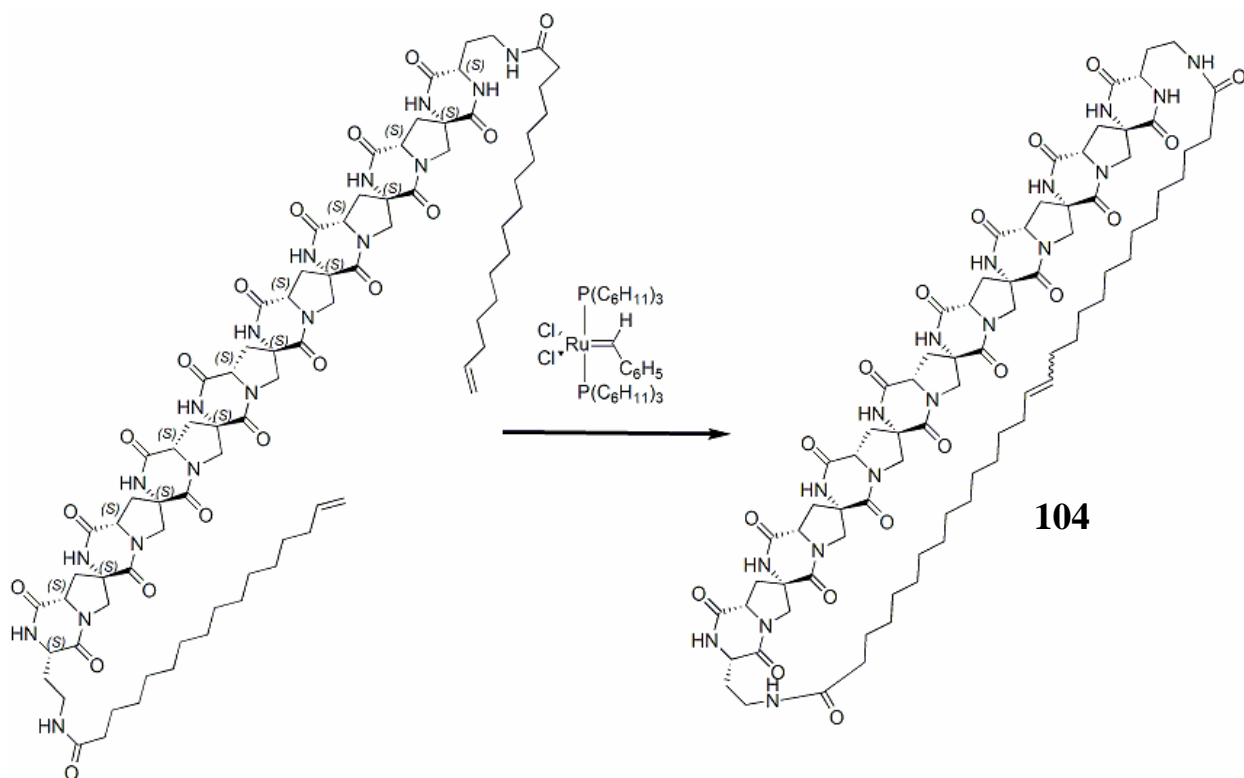


Figure 189 Proposed metathesis product.

Long chain, unsaturated, fatty acids are commercially available and could be converted to terminal olefins. These terminal olefin fatty acids could be incorporated in the same manner as the saturated fatty acid chains. Olefin metathesis would serve to “staple” the two chains together (**104**) in such a way that formation of cylindrical micelles may be formed.

7.4 EXPERIMENTAL

General: Solid phase synthesis was performed in a 1.5mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade of dimethylformamide (DMF) from Aldrich was used for coupling. Diisopropylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and

stored over molecular sieves. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and *N*-Hydroxybenzotriazole hydrate (HOBt) were obtained from Novabiochem. All solid phase reactions were mixed by bubbling argon up through reactor, allowing for mixing and an inert atmosphere over the reaction. HPLC analysis was performed using a Hewlett-Packard Series 1050 instrument equipped with a Varian Chrompack Microsorb 100 C₁₈ column (5μm packing, 4.6 mm x 250 mm) and a diode-array detector. HPLC-MS analysis was performed on a Hewlett-Packard Series 1100 instrument equipped with a Waters Xterra MS C₁₈ column (3.5μm packing, 4.6 mm x 100 mm) and a diode-array detector.

Synthesis of Scaffold Detergent, Open form version 1:

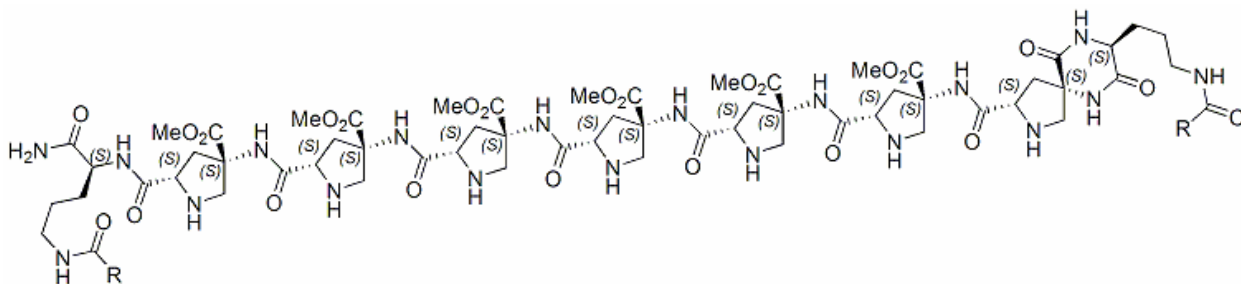


Figure 190 Scaffold detergent version 1, initial resin product.

To a 50mL polypropylene solid phase peptide synthesis (SPPS) reaction vessel was added Rink Amide AM Resin (Novabiochem) (165mg, 104μmol loading). The resin was swollen for 1hr in dimethylformamide (DMF). The terminal Fmoc-protected amine was deprotected in 5mL of 20% piperidine/DMF for 40min. By measuring the absorbance at 301nm of a 1/200 dilution, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$. The resin was washed with DMF, isopropanol, DMF, isopropanol, DMF, for 2min each.

In a 15mL polypropylene conical tube, the coupling solution was made by dissolving 317 mg (520 μmol) of Fmoc-L-Orn(Mtt)-OH (Novabiochem), 80mg of HOBt (520 μmol) and 198mg of HBTU (520 μmol) in 2600 μL of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 181 μL (1040 μmol) of *N,N*-diisopropylethylamine (DIPEA) was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react by argon mixing for 30min. The resin was then washed

3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release in the same manner described above.

Because this was such a large scale and this was the student's initial solid-phase experience, small very small portions of beads (~20beads, much less than 1 mg total) were sampled before each Fmoc-deprotection to confirm complete coupling. This was most necessary when probing the completion of double –coupling of build-block, because the presence of a primary amine on the quaternary center prevents utilization of the Kaiser/ninhydrin solid phase assay for the presence of free amines. Therefore beads were sampled in the following manner: Using a long bore, disposable glass pipette, the tip was gently touched to the pile of resin beads, until a visible amount of beads were adhered to the extreme tip. The pipette was moved to a small 1.5mL reactor column, and isopropanol was squirted down the inside and outside of the pipette, until all the beads were washed into the reactor. Preparation of the beads for cleavage was accomplished by sequential dichloromethane and methanol washes, for 1min each, repeating at least 5x. The reactor was then capped at the top, and placed in the under vacuum for at least 4hours. Then the beads were cleaved using 0.5mL of 2.5% triisopropylsilane, 2.5% water in trifluoroacetic acid for 2hours. LC/MS analysis was used to confirm complete coupling before subsequent Fmoc release and coupling of the next monomer.

In a 2mL microcentrifuge tube, the coupling solution was made by dissolving 113 mg (208 μ mol) of Cbz-protected pro4(2S4S) building block[20] and 79mg of HATU (208 μ mol) in 1040 μ L of 20% DCM/DMF. This solution was mixed using a micropipettor, after which 72.4 μ L (416 μ mol) of DIPEA was added to make the active ester. After 10min activation time, the solution was added to the deprotected resin, and allowed to react for 30min. The resin was then washed 3x 2min with DMF. Double couplings allowed for quantitative acylation, as judged by subsequent Fmoc release. This process of coupling/deprotection was repeated six additional times.

The final residue was attached in a manner identical to the first, except that the Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

It was at this point that the following steps proceeded slowly, first testing the conditions and behavior of subsequent steps on small scales.

The resin was swelled in DCM with slight bubbling for 30min. The Ornithine side chain protecting group, methytrityl, was removed using a 1% trifluoroacetic acid, 5% triisopropylsilane solution in DCM. 10 x 1mL, each reacting for about 2-3 minutes was sufficient to completely unmask the two side chain amines. Neutralization with 2 x 0.5mL of 5% DIPEA in DCM followed by swelling in DMF readied the resin for acylation. These amines were acylated with a variety of long-chain alkyl carboxylic acids. Approximately 10eq of fatty acid, 10eq HATU, 20eq DIPEA in a 0.2M 20% DMF/DCM, double coupling, was more than sufficient to ensure complete acylation. The resin wash extensively washed with DMF, IPA, DMF, IPA, DMF, MeOH, DCM, MeOH, DCM, MeOH, DCM, and then overnight drying, *in vacuo*.

The acylated scaffold was cleaved in 1mL of 4% trifluoromethanesulfonic acid, 4% ethanedithiol, 8% thioanisole in trifluoroacetic acid, with stirring for 2hours. This served to cleave the scaffold from the resin as well as remove the Cbz groups from the secondary amines. The cleavage cocktail along with 2 volumes of TFA for washing the beads, were dripped into 10 volumes ether, which was then spun at 3000rpm in a benchtop centrifuge. The pellet was washed with fresh ether and centrifuged again. The pellet was allowed to dry for an hour. The cleaved product was analyzed by LC/MS.

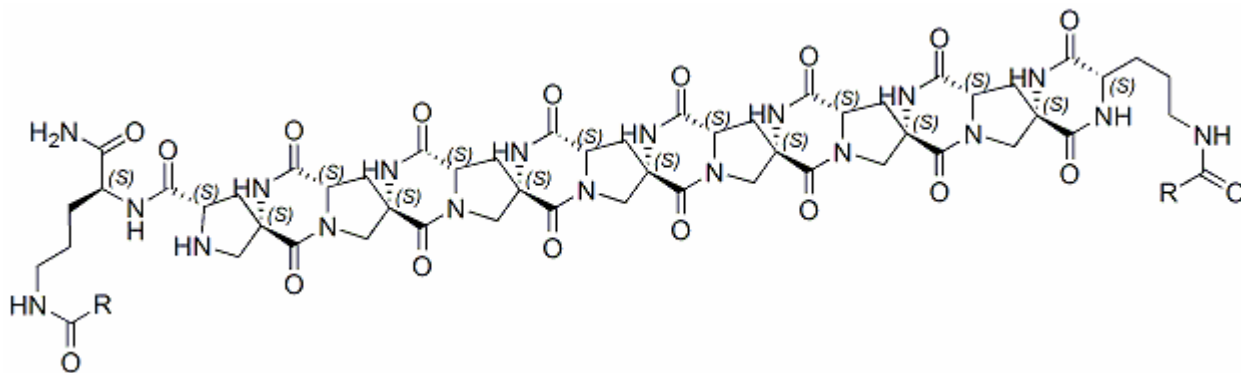


Figure 191 Scaffold detergent version 1, final closed product.

The diketopiperazines were closed by incubation for 48hr in 20% piperidine in DMF. After the reaction was complete as judged by LC/MS, the product was precipitated into ether, as described above. Solubility in a variety of solvents, such as water, methanol, and 25% ACN in water, was assessed by first sonicating the solution followed by centrifugation and LC/MS analysis to ascertain solubility.

by argon mixing for 30min. The resin was then washed 3 x 2min with DMF, and the coupling was repeated. The final Fmoc-deprotection was allowed to go for 2hr instead of 40min. This ensured that diketopiperazine formation was complete, thus all free amine was converted to an amide.

The resin was swelled in DMF for 30min. The Diaminobutanoic acid side chain protecting group, ivDde, was removed using a solution of 4% hydrazine hydrate in DMF. 4 x 1mL, each reacting for about 3-5 minutes was sufficient to completely unmask the two side chain amines. These amines were acylated with a variety of long-chain alkyl carboxylic acids. Approximately 10eq of fatty acid, 10eq HATU, 20eq DIPEA in a 0.2M 20% DMF/DCM, double coupling, was more than sufficient to ensure complete acylation.

The fully protected, acylated scaffold was cleaved in 1mL of 20% Acetic acid, 20% Trifluoroethanol in DCM, with stirring for 2hours. This was then drained and 3 x 1mL additional cleavage cocktail with stirring for 3 x 15min served to remove any residual product. The pooled cleavage solutions were transferred into a 13mm glass tube, and the solvent was removed in a SpeedVac. An additional ~4mL of DCM and ~2mL hexanes served to azeotropically distill off residual Acetic acid, and was repeated an additional three times, resulting in a dry white powder.

The white powder was dissolved in 1mL of dry MeOH, a stir bar was added and ~25eq trimethylsilyldiazomethane (TMSCHN₂), as a 2M solution in ether was added dropwise. Slight bubbling and yellow color resulted. The reaction was stopped after 10min by addition of an identical volume of trifluoroacetic acid, also dropwise. More bubbling was apparent and the color disappeared. The solvent was again removed in the SpeedVac. A stir bar was added and the residue was dissolved in 4mL TFA/DCM (1:1) and stirred for 2hrs. The solvent was removed in the SpeedVac, resulting in an oily residue.

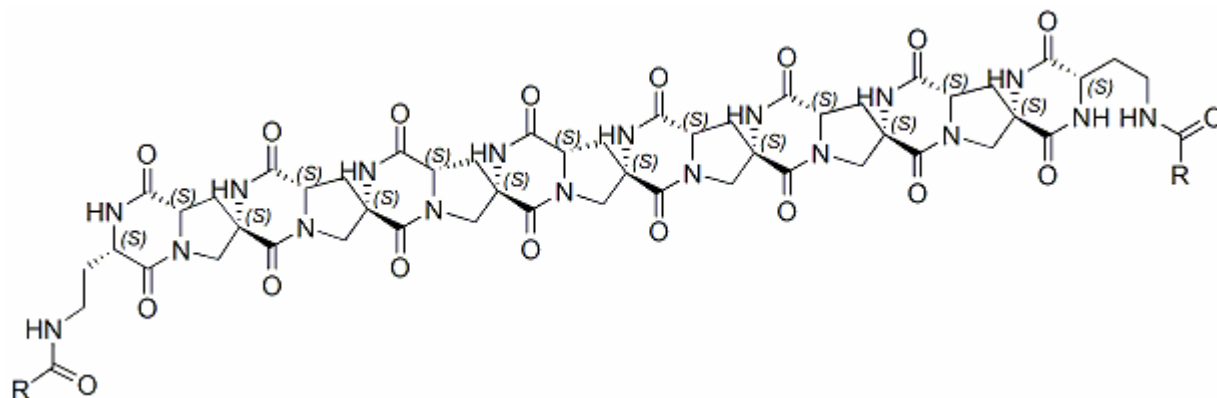


Figure 193 Scaffold detergent version 2, final closed product.

200 μ L of 20% piperidine in NMP was added to the residue. Incubation at room temperature for 36hrs served to close the diketopiperazines. After this time the tube was centrifuged for 20 min to remove any insolubles, the product was precipitated into 1.5 mL of ether, in a 2mL microcentrifuge tube. Solubility in a variety of solvents, such as water, methanol, and 25% ACN in water, was assessed by first sonicating the solution followed by centrifugation and LC/MS analysis to ascertain solubility.

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