SYNTHESIS OF NOVEL LINEAR MULTIVALENT PEPTIDE LIGANDS BASED ON THE TETRAPEPTIDE MSH(4)

by
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STATEMENT BY AUTHOR

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ABSTRACT

This thesis describes the synthesis of a novel multimeric peptide ligand targeted to the human melanocortin 4 receptor. The synthesis of the peptide was attempted both by solid phase peptide synthesis and by solution phase peptide synthesis, leading to the conclusion that the necessary C- and N- terminal substituents were much easier to install via the solution phase route. The bifunctional peptide was purified and then multimerized in both protected and active amino acid forms using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. The multimers were characterized using MS and UV-Vis spectroscopy. It was found that a large portion of the monomer cyclized under CuAAC conditions, though sufficient multimerization took place to form up to nonamers, as determined by mass spectrometry.

INTRODUCTION

The detection of cancerous cells and delivery of therapeutic agents are serious challenges in combating cancer. One strategy that can be pursued for some cancers is to target an overexpressed receptor on the cell surface. If a patient is presented with a therapeutic agent targeting such receptors, much of the agent will end up at the desired location; however, the agent may also bind elsewhere, potentially harming the patient. One approach to solving this problem is to use a weakly binding ligand and compensate for the low binding strength by attaching many of these ligands to a single molecule to form a multivalent ligand. Such ligands improve targeting by preferentially binding to cells overexpressing the intended receptor. There are two major modes by which multivalent ligands may improve binding: first, they can provide a second ligand moiety in close proximity to the first bound ligand moiety, improving chances of the multivalent construct as a whole remaining bound to the cell; second, they can bridge multiple receptors, keeping the multivalent construct tightly bound to the cell.[1] In both cases, these modes of binding are most effective for weak ligand moieties, as too strong a ligand may never dissociate from a receptor after binding.

Many different types of biological ligands have been made into multivalent ligands through attachment to some scaffold. These have included steroids[2] and peptide hormones.[3] In the cited cases, ligands are functionalized to hang from the scaffold, like charms on a bracelet. However, another proposed method is to string ligands together like beads on a necklace (Figure 1). To be effective, a peptide ligand in such a construct will need to be active even when functionalized at both the C- and N- termini.

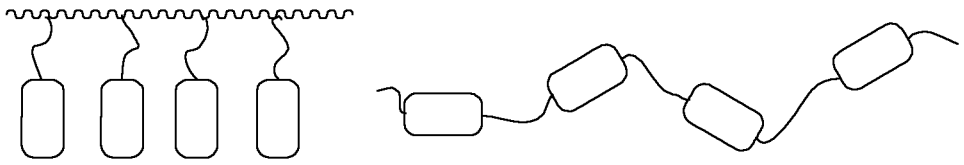


Figure 1. Approaches to forming a multivalent ligand. On the left, ligands are suspended from a scaffold like charms on a bracelet, on the right, ligands are incorporated into the main chain, like beads on a necklace.

In this thesis, a model multivalent ligand composed of weak agonists to the hMC4R receptor is synthesized and analyzed. This receptor is well studied, and is known to have a weakly binding tetrapeptide agonist, providing a simple synthetic target. While this receptor may not necessarily uniquely identify cancerous cells, it is useful as a model system for a proof-of-principle study.

The melanocortin family of receptors has five members, hMC1-5. These receptors play a number of roles in the body, including control of pigmentation, energy regulation, and sexual function.[4] The hMC4R receptor is expressed in brain tissue, and recent research has shown that it has a function in the regulation of energy homeostasis. Mutations in this receptor are also a possible cause of severe obesity.[4]

Although the receptor is a fairly recent discovery, the melanocortin peptides, agonists of the melanocortin receptors, have been known since 1916[5] and have been shown to directly affect pigmentation in the skin of frogs and lizards.[6] α-Melanocyte stimulating hormone (α-MSH), a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), is a strong melanocortin agonist[6]. More potent synthetic analogues have also been created. One of the best of these is [Nle^4, D-Phe^7]-α-MSH (NDP-α-MSH), which exhibits a potency 26 times greater than α-MSH in an adenylate cyclase assay, indicating stronger binding, better transduction, or both. This synthetic

peptide is also more resistant to cleavage by proteolytic enzymes[6], making it a good candidate for drug development. However, as previously stated, a strongly binding ligand is not suitable for use in testing a multivalent construct. Instead, the minimal active sequence of NDP-α-MSH would be a better ligand for this application.

The minimal active sequence of α-MSH was confirmed to be the tetrapeptide His-Phe-Arg-Trp (α-MSH_{6-9}) in 1987 by Hruby, et al.[7] They demonstrated that this tetrapeptide cannot be truncated and retain measurable activity in frog or lizard skin, nor can the amino acid sequence be shifted to α-MSH_{5-8} or to α-MSH_{7-10} and retain activity.[7,8] Upon the discovery and cloning of human melanocortin receptors, it was found that α-MSH and NDP-α-MSH are also strong agonists of the entire receptor family. However, NDP-α-MSH_{6-9} (MSH(4)) only showed binding and activity at two receptors, hMC1R and hMC4R.[9] Thus, MSH(4) and the hMC4R receptor are a good pairing for testing multivalent constructs.

MSH(4) has been used previously to study the effect of multivalency using a squalene derived scaffold.[10] This scaffold was functionalized with one or two pendant azide ethers, then MSH(4) functionalized on the N-terminus with an alkyne was coupled to the scaffold by a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (Figure 2). These constructs were tested using a europium-based competitive binding assay on cells overexpressing hMC4R. Compared to unmodified MSH(4), the monovalent MSH(4)-squalenol construct bound somewhat more weakly, but the construct with two MSH(4) ligands bound about three times more strongly, demonstrating a binding enhancement from multivalent effects.[10]

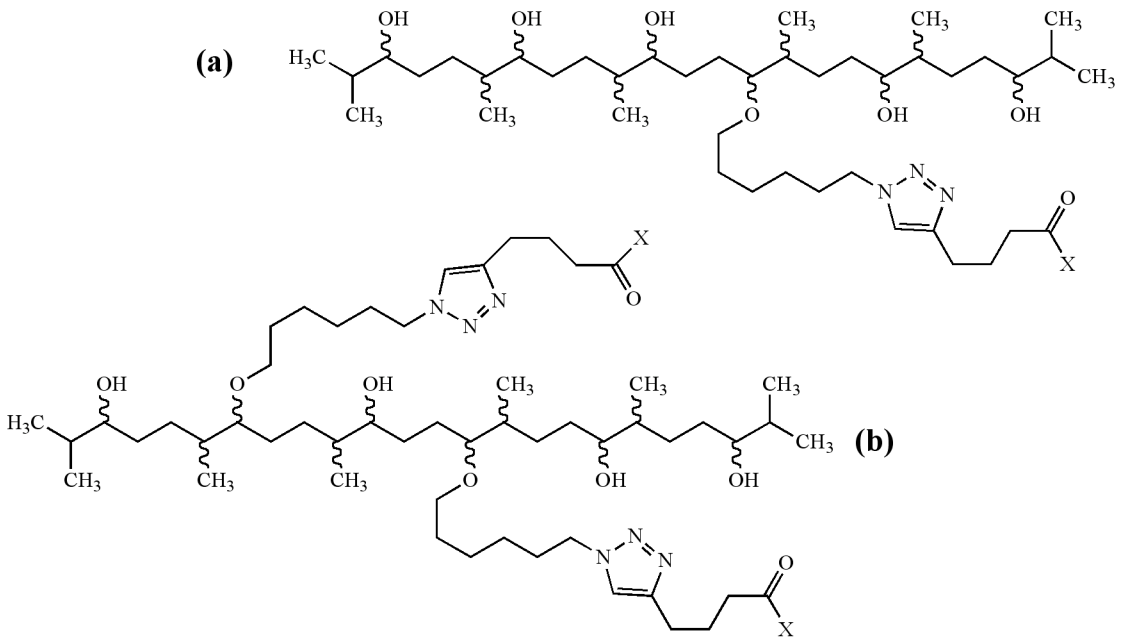


Figure 2. Squalene derived monovalent (a) and divalent (b) ligands. Compounds in which X was either MSH(4) or NDP-α-MSH were synthesized. The hydroxide sites to which ligands attached were arbitrary.

As in the case above, CuAAC is used to link the MSH(4) monomers produced herein. Azide and alkyne substituents are attached to the N- and C- termini, respectively, of MSH(4) so that [3+2] cycloaddition may be used to link the peptide ligand moieties. While thermal alkyne-azide cycloadditions have been known for some time,[11] a regiocontrolled copper-catalyzed methodology was developed by Sharpless.[12] This copper-catalyzed system limits triazole formation to the 1, 4 regioisomer, improves yields, and is usable in both polar and non-polar solvents; these qualities characterize it as the quintessential "Click reaction". The wide range of conditions under which this reaction can occur, and the fact that the triazole moiety is biologically inert, have led to an increasing use of CuAAC reactions to link structural elements in bioconjugates.[13]

The initial plan to synthesize the necessary C- and N- substituted MSH(4) monomer was based on creating an alkylated linker by solution phase chemistry, then attaching the new linker to a solid support. This approach was abandoned when it was found in literature that alkylation of the Rink linker had previously been accomplished on resin.[14] This solid phase method of synthesizing the target peptide was used; however, this method was found to produce extremely low yields, so alternative solution phase peptide synthesis methods for building the peptide from an alkynyl amine were also utilized. Once the peptide monomer was made, CuAAC multimerization reactions were carried out under a variety of conditions.

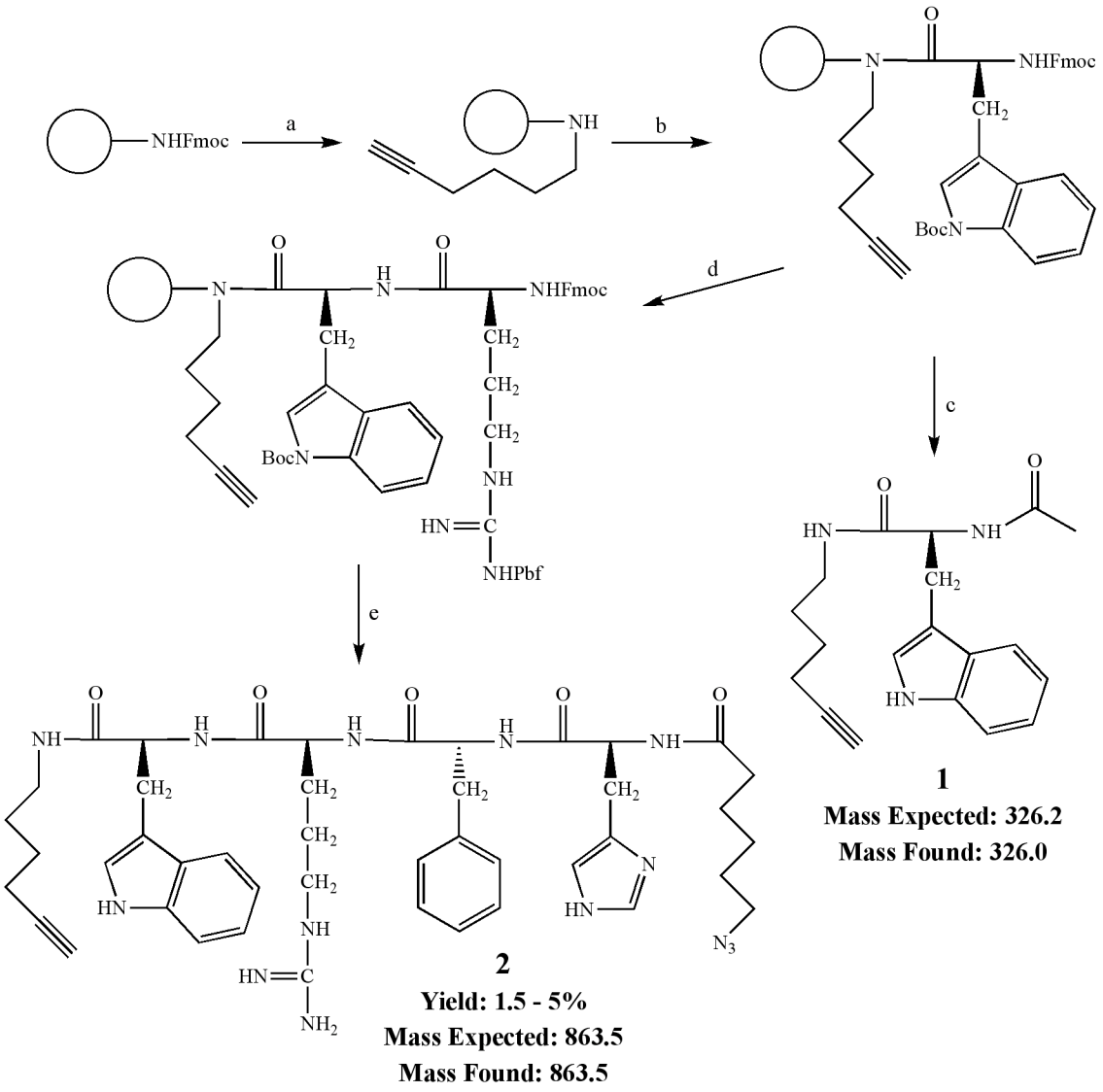
RESULTS AND DISCUSSION

Synthesis of N-(6-Azidohexoyl)-C-(5-hexynyl) MSH(4) by SPPS

The first step in making MSH(4) multimers was to make the desired monomer. To make it entirely by solid phase peptide synthesis (SPPS) required that the azide survive the decoupling/deprotection conditions, and that a method be found to alkylate the C terminus reliably.

To ensure the first condition, a batch of N-(6-azidohexoyl) MSH(4) amide was made by SPPS. This was a success, producing the desired compound in 44% yield after High Performance Liquid Chromatography (HPLC) purification.

To install the C- terminal hexynyl group, the initial plan was to modify the Rink Linker in solution, then attach it to a solid support and proceed with typical SPPS to make the desired compound. However, while searching for procedures on reductive alkylation, a method to modify the linker in situ on a polymer support was found.[14] The method was tested by starting a batch of beads, then removing a sample for analysis after coupling the first amino acid to the resin. This sample was deprotected, the N terminus acetylated, and the amino acid decoupled from the resin using 91:3:3:3 TFA/TIPS/H_2O/thioanisole cocktail and diethyl ether washes. The mass spectrum of this sample clearly showed that the compound N-(5-hexynyl) N^2-acetyltryptophan amide **(1)** (Scheme 1) was present as desired. Having evidence that the reductive alkylation was successful, the remaining three amino acids were coupled to the remaining resin, and then 6-azidohexanoic acid was used to cap the N terminus. After decoupling from the resin and HPLC purification, this synthesis yielded 4.5 mg of the desired MSH(4) derivative



Scheme 1. Reagents: (a) 1.piperidine, DMF 2. 5-hexynal, THF, AcOH, H_2O, $NaCNBH_3$. (b) Fmoc-Trp(Boc)-OH, DIC, HOBt, DMF. (c) 1. piperidine, DMF 2. Ac_2O, Pyridine. (d) 1. piperidine, DMF 2. Fmoc-Arg(Pbf)-OH, DIC, HOBt, DMF. (e) sequential deprotection and coupling using reagents in step d with the amino acids Fmoc-ᴅPhe-OH and Fmoc-His(Trt)-OH and 6-azidohexanoic acid.

(**2**), a 1.5% yield from the original mass of resin. Though the SPPS of the desired N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) was carried out in this manner multiple times, the yield was never greater than 5%.

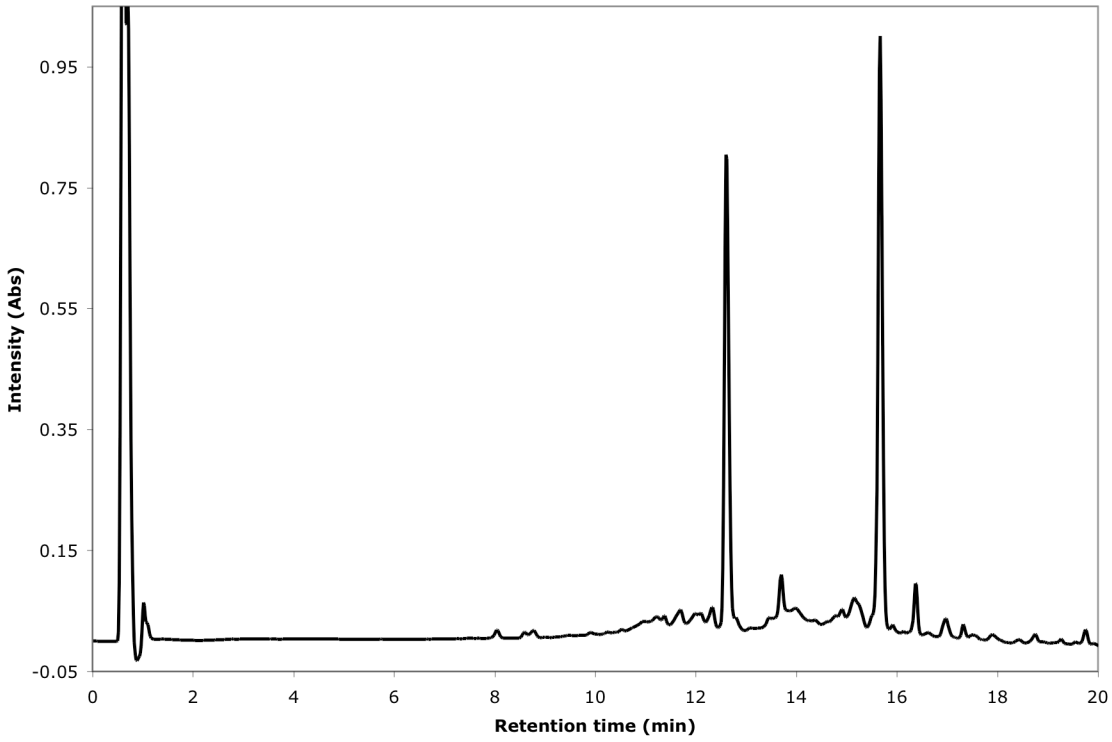


Figure 3. HPLC of crude SPPS product. Peak at approx. 15.75 min is the desired product with a C-terminal alkyne while the peak at approx 12.75 min is the undesired compound without C- terminal functionalization.

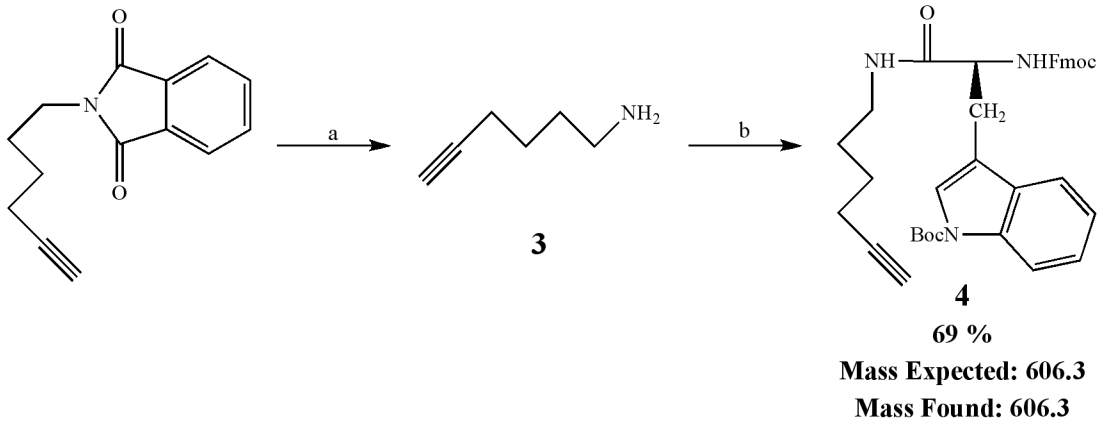
A significant problem with this approach was incomplete reaction of the resin with the aldehyde. This was most clearly indicated by HPLC, in which peptides with and without C- terminal alkylation had very different retention times (Figure 3). It is possible that the structure of the aldehyde plays a role in the effectiveness of the reaction; the majority of the aldehydes used by Brown and Nuss were aromatic compounds,[12] and the rest were relatively bulky, unlike the 5-hexynal used here. This may have contributed to the difference in yields between their results and those reported here. This problem was also exacerbated by the slow decomposition of the aldehyde, resulting in an increase in 6-azidohexoyl MSH(4) amide with every reaction done.

In the end, this method was found to work, but would have been an inefficient method for making the relatively large amounts of MSH(4) monomer needed to run multiple multimerization trials.

Synthesis of N-(6-Azidohexoyl)-C-(5-hexynyl) MSH(4) in Solution

Rather than pursuing further the above inefficient method to produce the desired monomer, solution phase peptide synthesis was used. This method had several advantages. First, gram-scale batches of monomer could be prepared, providing sufficient material for multimerization trials. Second, the compound could be handled as a normal organic compound, allowing use of chromatography on normal phase silica, and the purity and identity at every step could be confirmed by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Finally, the options of either deprotecting the peptide prior to multimerization, or using the peptide in a protected form afforded a larger range of CuAAC conditions under which multimers might form.

Starting from *N*-(5-hexynyl)phthalimide, 6-amino-1-hexyne (**3**) was made by reaction with hydrazine in ethanol, then the crude amine was coupled to Fmoc-Trp(Boc)-OH with diisopropylcarbodiimide (DIC) in DCM (Scheme 2), establishing the desired C-terminal alkyne group of the MSH(4) monomer. This compound was somewhat difficult to purify, as many impurities from the amine had carried over to the Fmoc amide, as evidenced by the multiple spots on TLC (not shown). Once the pure compound was isolated, MS and NMR were obtained, showing that it was the correct compound. Three characteristic features of this compound in the NMR are the 9H singlet of the Boc

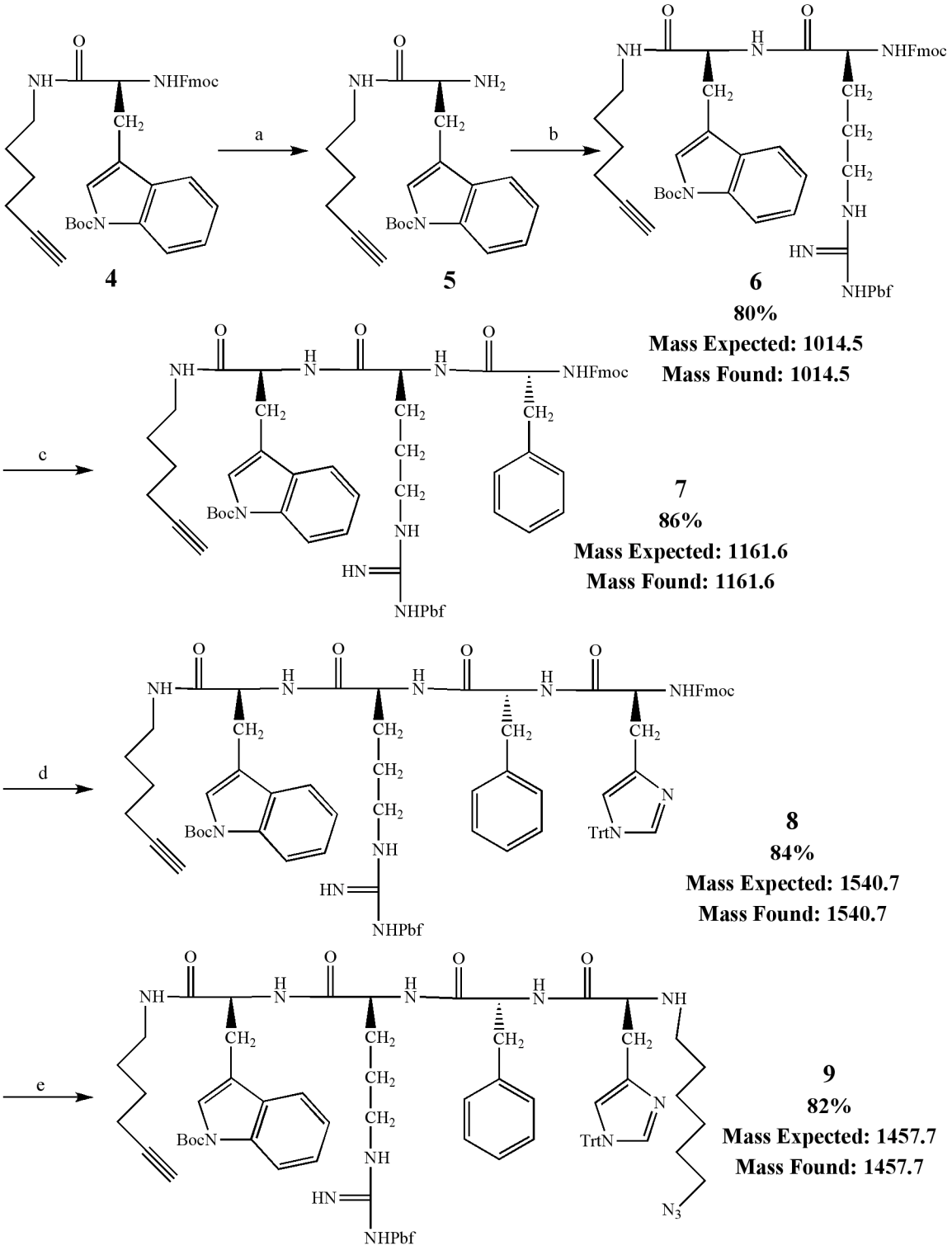


Scheme 2. Reagents: (a) N_2H_4, EtOH. (b) Fmoc-Trp(Boc)-OH, DIC, DCM.

protecting group at δ 1.64, the 1H singlet (triplet in spectra of some larger peptides) of the terminal alkyne at δ 1.87, and the characteristic doublet, triplet, triplet, doublet of the Fmoc protecting group in the aromatic region. Taken together, these prove that the desired compound was made, as these features do not all appear in either of the starting materials alone. MS also confirmed that the desired product was formed (Scheme 2).

The first attempt to couple the other 3 amino acids and the N-terminal azide followed a rapid solution phase synthesis scheme created by Carpino et al.[15] Using tris(2-aminoethyl) amine (TAEA), the Fmoc group was removed from the peptide in DCM, the solution was diluted with ethyl acetate, and then washed with a pH 5.5 phosphate buffer to remove the Fmoc-TAEA conjugate. The solution containing the deprotected peptide was evaporated, and the residue was redissolved in DCM or THF, then peptide couplings were carried out with DIC or HBTU/DIPEA. Although a product was isolated at the end, this method turned out to be a failure, yielding none of the desired product according to MS. This may have been caused by impurities introduced with each peptide coupling, as none of the materials were repurified, but rather used as purchased. It may have also been

Scheme 3. Reagents:(a) TAEA, DCM. (b) Fmoc-Arg(Pbf)-OH, DIPEA, HBTU, THF. (c) 1. TAEA, DCM. 2. Fmoc-DPhe-OH, DIPEA, HBTU, THF. (d) 1. TAEA, DCM. 2. Fmoc-His(Trt)-OH, DIPEA, HBTU, THF. (e) 1. TAEA, DCM. 2. 6-azidohexanoic acid, DIPEA, HBTU, THF.


a
b
c
d
e
4
5
6
7
8
9
80%
Mass Expected: 1014.5
Mass Found: 1014.5
86%
Mass Expected: 1161.6
Mass Found: 1161.6
84%
Mass Expected: 1540.7
Mass Found: 1540.7
82%
Mass Expected: 1457.7
Mass Found: 1457.7

the case that the coupling reagents used did not couple as cleanly as those used by Carpino, et al.[15]

As the rapid solution phase scheme was unsuccessful, a more traditional approach was adopted, in which the peptide was isolated and purified after each coupling (Scheme 3). Similar conditions were used as above, but after each coupling step the peptide was precipitated from EtOAc with hexanes, then chromatographed on silica, consistently giving yields over 80%, resulting in an overall yield of 47% from the alkynyl tryptophan amide **4**. NMR and MS of each synthetic intermediate demonstrated that the peptide was growing as desired.

Each step added several key peaks to the NMR. After coupling arginine, three broad singlets (δ 2.52, 2.60, 2.89), each of 3H, were added, coming from the phenyl methyl groups on the Pbf protection. Next, a large multiplet was added in the aromatic region after coupling D-phenylalanine, and then the coupling of histidine produced another set of aromatic peaks due to the trityl protection. Lastly, the Fmoc protecting group peaks disappeared with the coupling of 6-azidohexanoic acid. The identity of each synthetic intermediate was also determined by MS, as shown in Table 1. The mass of each compound increased by the mass of the new amino acid, until the Fmoc protecting group was removed and the N terminal azidohexoyl group was added. Taken together, these data provide a demonstration of the increasing size and identity of the protected peptide.

Table 1. MS and NMR characteristics of N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) and synthetic intermediates.

Compound	Mass Expected $(M+H)^+$	Mass found $(M+H)^+$	Major NMR peaks
Fmoc-Trp(Boc)-$NH(CH_2)_4CCH$ (**4**)	606.2962	606.2960	1.64 (s, 9H) - Boc CH_3 groups 1.87 (s, 1H) - terminal CCH 7.38 (t, 2H), 7.53 (t, 2H), 7.75 (d, 2H) - Fmoc aromatic hydrogens
Fmoc-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$ (**6**)	1014.4794	1014.4794	Above peaks plus 2.52 (s, 3H), 2.60 (s, 3H), 2.89 (s, 3H) - Pbf phenyl methyl groups
Fmoc-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$ (**7**)	1161.5478	1161.5488	Above peaks plus 7.05-7.2 (m, 9H) - Phe phenyl hydrogens mixed with previously present signals
Fmoc-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$ (**8**)	1540.7163	1540.7219	Above peaks plus 6.93-7.10 (m, 13H), 7.23-7.36 (m, 14H) - Trt phenyl hydrogens mixed with previously present signals
$N_3(CH_2)_5CO$-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$ (**9**)	1457.7227	1457.7232	Absence of Fmoc aromatic hydrogens, 2.14 (t, 2H) - $C\underline{H_2}CO$-His and other alkane signals from 6-azidohexoyl tail

Multimerization of Protected Monomer

The CuAAC multimerization reaction was first tried in MeOH using the catalyst combination of tetrakis(acetonitrile)copper(I) hexafluorophosphate and tris-(benzotriazolylmethyl) amine (TBTA) at room temperature. This combination of solvent and catalyst system gave extremely poor multimerization yields. Over multiple trials, the best yield according to MS was a tiny amount of trimer after a 7 day reaction period.

As the methanol-based system was not workable for the protected monomer, a system of CuBr and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DCM was tried, as DCM was known to be a good solvent for the protected peptide. In the first trial of this catalyst system, the very concentrated solution gelled in less than one hour, indicating

some multimerization had taken place. The mixture could be redissolved in chloroform, and it was determined that it contained monomer, dimer, and a small amount of trimer. Although trials with this catalyst system were performed several more times, no reaction formed multimers larger than tetramer, and all created turbid solutions. This suggested that multimers trimer and larger were not soluble in DCM at room temperature. To test this theory, another trial was carried out in a sealed tube at 40 °C. The product precipitated from the solution in this reaction and was again shown to be nothing larger than tetramer, although multimer yields were greater than in the room temperature reactions.

After solubility trials in various solvents, it was found that polar N,N'-dimethylformamide (DMF) was a good solvent for both monomer and multimers. A catalyst system of CuBr and DIPEA was used in DMF at room temperature to form multimers. This turned out to keep the multimers in solution, and formed product up to pentamer. A new method of separating multimers was also used with this system; any remaining monomer was soluble in EtOAc, but all multimers precipitated from the solution. Using this difference in solubility, a sample was obtained which contained no monomer, but a mixture of multimers from dimer to pentamer (Sample ID RS-1-100).

Multimerization of Free Monomer

After the success of creating multimers from protected starting material, the next logical step was to find a method for multimerizing active monomer. While the protected system worked well, there seemed to be a large amount of loss in deprotecting the

multimers, likely due to the various scavengers necessitated by the Pbf and tert-butyl protecting groups. It was expected that using active monomer would remove that issue, but instead leave residual monomer in the CuAAC reaction mixture.

The initial attempt to multimerize active monomer was done in a Biotage Initiator microwave using a similar concentration of monomer as the protected trials, but using methanol as the solvent and $Cu(ACN)_4PF_6$/TBTA as the catalyst system (Sample ID RS-1-103). The vial was heated at 100 °C in two periods, one of 2 hr, one of 12 hr. After 2 hr, no precipitate had formed, and the solution was still green in color, indicating the presence of Cu(I). After a total of 14 hr of irradiation, the entire sample was still soluble in methanol and green in color; a sample of the solution was submitted for MS. This MS demonstrated the presence of multimers up to and including nonamer, but the greatest portion remained monomer. This sample was worked up to remove copper and TBTA, and then purified by HPLC. After HPLC this mixture was found to contain up to heptamer. The ratio of monomer to multimer had also greatly shifted in favor of multimers, thus some monomer was removed from the multimers in performing the workup and/or HPLC.

A second trial of multimerization in the microwave was conducted, this time using about twice the concentration of monomer (Sample ID RS-1-105). It was treated with the same percentage of catalyst and the same total irradiation time, and again showed no precipitation or change in color. The work up was done immediately, then MS was obtained, showing up to heptamer in this sample, but again having a majority of monomer.

While it appeared at first glance that these two methods gave very similar results, they were quite different in the amount of multimer produced, even post work up. For example, when comparing the monomer and dimer peak intensities in each case after work up, the first reaction produced 16% dimer compared to monomer, while the second produced about 37% dimer. This improvement continued at least through tetramer, in which the first reaction produced 1.5% compared to monomer, but the second produced 6.8%, demonstrating a definite concentration dependence for the reaction.

Determination of Average Chain Length

The average number of MSH(4) repeats per chain is an important parameter that is needed for subsequent studies, especially demonstrations of activity. Two different approaches were used to determine this number, first by determining UV-Visible spectra of the mixtures and comparing the concentrations of triazole to MSH(4), and second by taking a weighted average of each multimer from the MS. It was thought that the UV-Visible method would give a fast, accurate measurement of multimer sizes, however, it was determined that there was a great deal of error in this method which rendered it considerably less reliable than the MS approach.

To determine the sizes by UV-Visible spectroscopy, calibration plots of concentration vs. absorbance were needed for both MSH(4) and triazole in methanol. A calibration plot of MSH(4) in methanol was obtained from Ramesh Alletti, a Mash Group member. Error in this equation was estimated by determining the slopes of the lines from each point in the data set to (0,0), then finding a standard deviation of these slopes. This

standard deviation was carried forward as error in the molar absorbtivity, $\varepsilon_{MSH(4)}$. Spectra of N-(6-(4-butyl-1,2,3-triazolyl)hexanoyl) MSH(4) amide dissolved in methanol were then obtained, and the calibration line of pure MSH(4) was used to find the concentration in these new samples. As this compound contains one triazole for each MSH(4) unit, the MSH(4) absorbance could be used to determine the concentration of triazole for each solution. From there, a plot of concentration vs. triazole absorbance allowed determination of a calibration line for triazole. Error in the slope of the MSH(4) calibration line was carried forward into this calculation, establishing an estimated error in the triazole calibration line. For additional details, see the experimental section.

Once both calibration lines were determined, a solution of each sample being submitted for biological testing was made, and the absorbance readings were placed on the calibration lines to determine the concentrations of MSH(4) and triazole. A ratio of the two concentrations was determined, then used to determine chain length, *n*, in the equation $\frac{n-1}{n} = \frac{C_{triazole}}{C_{MSH(4)}}$. As seen in Table 2, the values determined by this method without taking into account the error bars appeared to indicate a larger multimer size than was observed in the MS data, which indicated that the majority of species in these mixtures consist of one to three MSH(4) units. For comparison, values of *n* were also determined from MS by determining the percent of monomer and each multimer in the samples, then multiplying the length of each species by the percent found and summing those values. These are compared with the UV-Vis values in Table 2.

Table 2. Calculated chain length of selected multimerizations.

Sample ID	Description	$\frac{C_{triazole}}{C_{MSH(4)}}$	$n_{\text{UV-Vis}}$	n_{MS}
RS-1-100	Mult. done in protected form, residual monomer removed	0.721	3.58	3.12
RS-1-103	Mult. done in active form in microwave reactor, residual monomer present	0.859	7.09	1.79*
RS-1-104	Duplicate of RS-1-103	0.928	13.81	1.39
RS-1-105	Mult. done in active form in microwave reactor. residual monomer present. Conc. about twice that of RS-1-103.	1.044**	-22.67	1.66

* An attempt was made to remove monomer from this sample, resulting in a difference between the crude results discussed in the multimerizations section of the text and the numbers given here.
** Even in a fully cyclized peptide, there should be no more than one triazole per MSH(4) unit. As such, this value does not convert to a sensible chain length.

The large difference in n given by the two independent approaches prompted a careful re-examination of the approach used to obtain the chain length from UV-Vis, indicating a fundamental limitation in the use of this method in this case, along with other potential sources of error relating to the compound itself and impurities in the solution. The fundamental limitation arises from the fact that the equation being used to determine n is numerically unstable. Solving for n gives $n = \frac{1}{1 - C_{triazole} / C_{MSH(4)}}$ which becomes infinite when the concentrations are equal, and becomes negative when the concentration of triazole is greater than the concentration of MSH(4). Factoring error from the calibration plots into the ratio of concentrations does not fully correct these values to correlate with MS data (Table 3). As this error is not symmetric for n, it is most worthwhile to look at the lower bound of the ratio, which will correct the value of n toward that of n_{MS}. In the case of sample RS-1-100, the MS value is comfortably

Table 3. Error factored into concentration ratios used to determine chain lengths by UV-Visible spectroscopy.

Sample ID	$\frac{C_{triazole}}{C_{MSH(4)}}$ (min)	$n_{\text{UV-Vis}}$ (min ratio)	$\frac{C_{triazole}}{C_{MSH(4)}}$ (max)	$n_{\text{UV-Vis}}$ (max ratio)
RS-1-100	0.520	2.08	0.999	763.68
RS-1-103	0.620	2.63	1.190	-5.26
RS-1-104	0.670	3.02	1.285	-3.51
RS-1-105	0.754	4.05	1.446	-2.24

contained in this error determination. However, the other three samples still exhibit values considerably larger than n_{MS}. As the values of n_{MS} lie outside the error calculated for the UV-Vis methodology, it is likely there are other sources of error than simply the numerical instability of the equation.

A second contribution to the error in the UV-Vis determination of *n* may come from cyclization of the MSH(4) derivative being measured. The equation used to determine the chain length assumes that each multimer will be a linear chain that always contains one more MSH(4) unit than triazole unit. However, it is possible that monomer or any multimer species could cyclize upon itself, creating a one-to-one ratio of triazole to MSH(4). As noted above, a sample in which the ratio approaches a value of one leads to a very large, undefined value for n, which may become negative with the inclusion of error bars from the calibration curves. A small amount of cyclization in sample RS-1-100 could account for the difference in $n_{\text{UV-Vis}}$ and n_{MS} determined for this sample; however it could not explain the concentration ratio calculated for RS-1-105, in which it appears that there are more triazoles than MSH(4) units, which should be physically impossible.

Finally, the samples for UV-Visible spectroscopy may have contained impurities which absorbed at a similar wavelength as triazole, inflating the absorbance value and

creating an incorrect value for triazole concentration. Sufficient extra absorption at the triazole wavelength can result in the equation for *n* giving negative values, as was observed for sample RS-1-105.

Although UV-Visible Spectroscopy is typically a very sensitive technique, in this case limitations in the necessary equation and the inability to differentiate cyclized from uncyclized samples made it an unusable technique for determining chain lengths. Determining counts by MS, while somewhat more laborious, appears to be the more effective method of finding the average size of chains in these samples.

Investigation of Cyclization

As noted in the previous section, one likely cause of the unusual numbers from UV-Vis spectroscopy is cyclization of monomer and/or multimers. If some species in a multimerization reaction cyclize, the ratio of triazole to MSH(4) increases and can approach one, meaning that the equation for determining the size of multimers approaches infinity.

To test this idea, a sample of multimers was compared to monomer using MS-MS. A fragment peak of monomer-HN_3 was identified at 820 m/z in the MS-MS of unreacted monomer. If the remnant monomer in the reacted sample were cyclized, this peak should be greatly reduced or eliminated. This is in fact observed in Figure 4. In comparison to the peak at 818 m/z, the unreacted monomer has a peak 80-90 % as intense, but the intensity of the peak at 820 m/z was reduced to about 10 % that of the peak at 818 m/z in a sample of RS-1-105. This demonstrates that a large portion of the

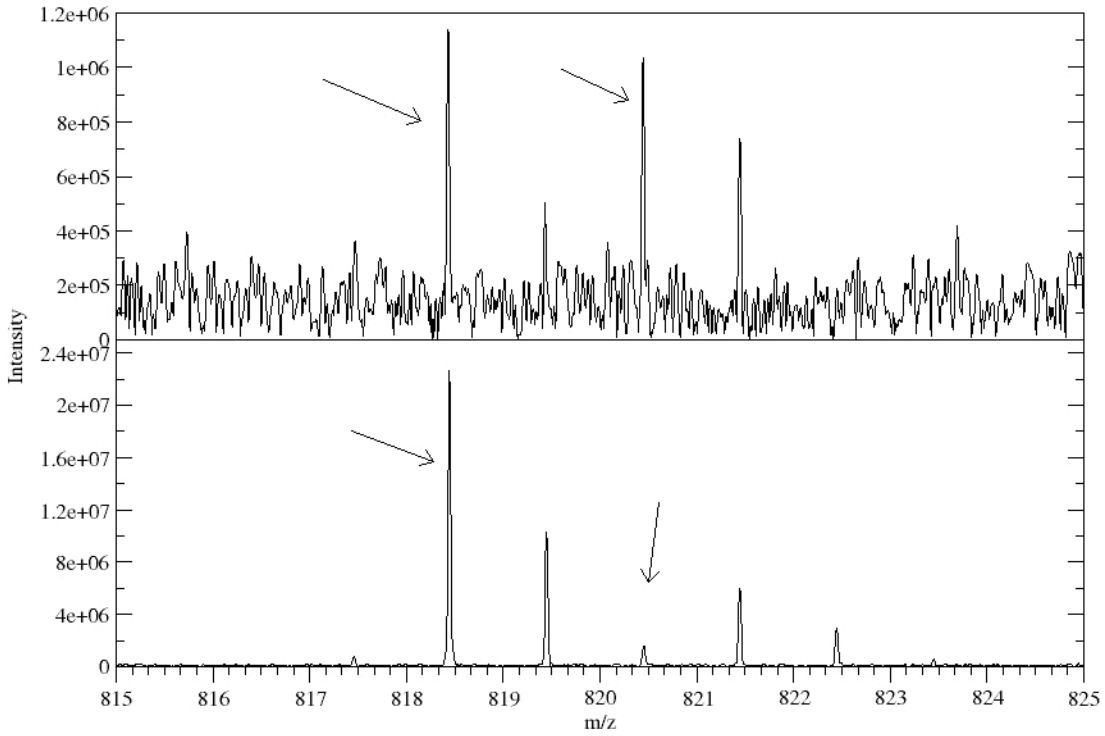


Figure 4. MS-MS of monomer before (bottom) and after (top) undergoing a multimerization reaction. As the peak at 820 m/z is a fragment missing HN_3, this strongly suggests that there is little free N_3 to fragment from monomer after multimerization. Arrows indicate the peaks of interest.

residual monomer is cyclized, and it may be fully cyclized if the triazole can ring open in the MS, creating a small portion of apparently unreacted monomer to fragment and create the peak at 820 m/z.

Additional evidence to support this theory came from the results of an analytical HPLC, in which it was observed that the retention time of the monomer peak had shifted to a slightly earlier time from the known uncyclized time (Figure 5). Other workers have demonstrated that peptides cyclized by CuAAC reactions will shift in HPLC[16], in the same way as observed in this work.

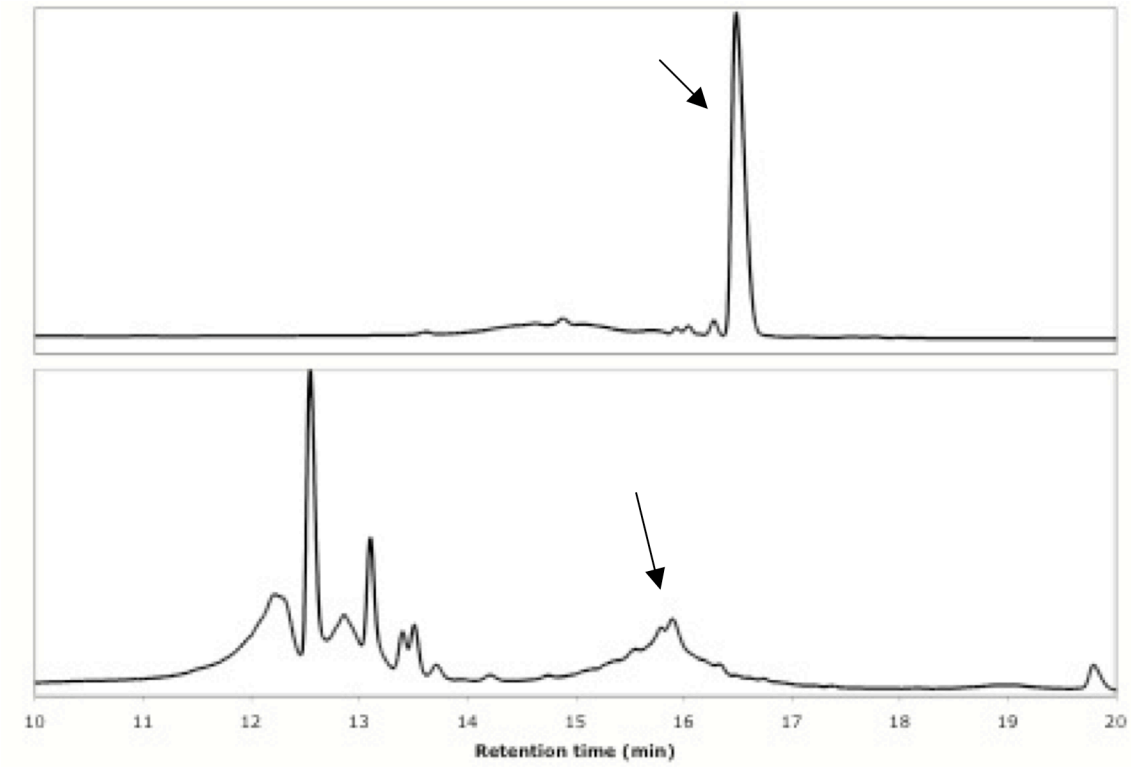


Figure 5. HPLC of active monomer (top) and a crude multimer sample (bottom). Arrows indicate peaks which were found to have the same mass by MS, but clearly different retention times.

CONCLUSION

This thesis demonstrates the synthesis of a novel C- and N- terminally functionalized MSH(4) derivative, and the CuAAC multimerization of that derivative. The functionalized MSH(4) derivative was most easily made by solution phase peptide synthesis, resulting in an overall yield of 47%. The compound was found to be reactive in several CuAAC conditions, in both protected and active forms. The best of these appear to be both the multimerization in DMF using DBU and CuBr as the catalyst system, and the multimerization in MeOH using TBTA and $Cu(ACN)_4(PF_6)$ in the microwave reactor, each for different reasons. The first reaction may be purified to remove all remaining, and likely cyclized, monomer, giving a mixture of multimers only. However, the microwave reaction produced larger multimers, which may show better behavior as multivalent ligands. Finally, the multimers were studied using UV-Visible spectroscopy in an effort to determine the average multimer length. This effort was only marginally successful, mainly due to a large degree of instability in the equation used, but also due to cyclized mono- and multimers, as demonstrated by MS-MS.

FUTURE DIRECTIONS

As shown above, cyclization is a major problem with this system. One way to reduce or eliminate cyclization effects could be by changing the lengths of the C- and N-terminal functional groups such that they could no longer reach across the peptide. These functional groups could also be changed, such that the multimerization is between two molecules: MSH(4) functionalized with either an alkyne at both ends or an azide at both ends. This would ensure that at least dimers form. The linear multivalent ligand could also be improved by co-polymerization of the MSH(4) moieties with some inactive spacer, such that the active peptides may form more bridges between receptors, rather than acting as spacers themselves. This may be especially important in aqueous media, as three residues of this peptide become protonated. Such a dense chain of positively charged species could be attracted to negatively charged cell surfaces, in turn counter-acting the binding and targeting enhancement desired from the ligands.

Although these are interesting synthetic challenges, the efficacy of the multimers created herein must also be tested. If they prove to be effective and selective in their binding, they could be a new way of finding certain cancers, and if cyclization problems are solved could also deliver imaging or therapeutic agents with high specificity.

EXPERIMENTAL

General Experimental: All Fmoc protected amino acids were obtained from Novabiochem and were used without further purification. Diisopropylcarbodiimide (DIC), 6-chloro-1-hydroxybenzotriazole (Cl-HOBt), and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Chem-Impex and were used without purification. All other reagents were purchased from Aldrich Chemical Company and were used as received. Silica gel for flash chromatography (230-400 mesh) was purchased from Silicycle. Preparative HPLC was performed on a Waters 600 HPLC equipped with a Waters 2487 UV-Vis Spectrophotometry detector and an XBridge 19 x 250 mm C18 column. Analytical HPLC was performed using a Symmetry 4.6 x 75 mm C18 column attached to the same instruments. ^{1}H NMR were acquired at 300 MHz on a Varian Unity spectrometer, at 500 MHz on a Bruker DRX-500 spectrometer, and at 600 MHz on a Bruker DRX-600 spectrometer. ^{13}C NMR were acquired at 125 MHz and at 150 MHz, respectively, on the DRX-500 and DRX-600 spectrometers. All mass spectrometry was performed by the Department of Chemistry Mass Spectrometry Facility at the University of Arizona. UV-Visible spectroscopy was performed on a Shimadzu UV-2401PC.

5-Hexynal[17]**:** 5-Hexyn-1-ol (5.0 g, 50.9 mmol) was dissolved in DCM (150 mL), and pyridinium chlorochromate (27.2 g, 126 mmol, 2.5 eq) was added to the solution at room temperature. After 2 hr TLC showed the reaction was complete, and the solution was diluted with ~150 mL ether and filtered through Florisil. The eluent was evaporated in

vacuo at ~750 mbar until ~125 mL remained, then distilled at ambient pressure, with product appearing at 130-135 °C. Product was a very pale yellow liquid (1.65 g, 17.1 mmol, 34 %). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.82 (quint., 2H, J = 7.0 Hz), 1.95 (t, 1H, J = 2.6 Hz), 2.24 (td, 2H, J_1 = 2.6, J_2 = 6.9 Hz), 2.58 (t, 2H, J = 7.2 Hz), 9.77 (s, 1H). These data are consistent with data in the literature.[18]

6-Azidohexanoic Acid[19]**:** 6-Bromohexanoic acid (5.09 g, 26.1 mmol) was dissolved in DMSO (150 mL) and sodium azide (16.4 g, 253 mmol, ~10 eq) was added. The mixture was stirred overnight (~12 h), then poured into water (~300 mL). The product was extracted with diethyl ether, then washed three times each with water and brine. The washed solution was dried over $MgSO_4$, the mixture was filtered, and the ether was removed in vacuo, leaving a pale yellow oil (2.19 g, 13.9 mmol, 53 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.41 (p, 2H, J = 6.6 Hz), 1.58 (sept, 4H, J = 7.3 Hz), 2.35 (t, 2H, J = 7.3 Hz), 3.25 (t, 2H, J = 6.7 Hz). These data are consistent with data in the literature.[19]

Solid phase Synthesis of N-(6-azidohexoyl) MSH(4) amide: Fmoc protected Rink amide resin (0.3 g, 0.2 mmol reactive sites) was placed in a fritted syringe reaction vessel, then swelled in THF for 1hr. The resin was deprotected by successive treatments (2 min, then 18 min) with 20 % piperidine in DMF (~5 mL) then washed with DMF (5 mL x 3), DCM (5 mL x 3), DMF (5 mL x 3), 0.5 M HOBt in DMF (5 mL), 0.5 M HOBt in DMF with a drop of bromophenol blue (4 mL) to provide an in situ reaction indicator, DMF (5 mL x 2) and finally DCM (5 mL) to swell the resin slightly. A coupling solution

of Cl-HOBt (210 mg, 1.2 mmol), DIC (190 μL, 1.2 mmol), and Fmoc-Trp(Boc)-OH (322 mg, 0.612 mmol) was added and the mixture was shaken for 1 hr. The resin was rinsed with DMF (4 mL multiple times) and THF (4 mL), and the subsequent three amino acids (Fmoc-Arg(Pbf)-OH (397 mg, 0.612 mmol), Fmoc-DPhe-OH (237 mg, 0.612 mmol), Fmoc-His(Trt)-OH (379 mg, 0.612 mmol) were coupled using the same procedure. The azide tail was added by following the deprotection and washing steps above, then adding a coupling solution of 6-azidohexanoic acid (96 mg, 0.61 mmol), Cl-HOBt (210 mg, 1.2 mmol), and DIC (190 μL, 1.2 mmol) in DMF (5 mL) and shaking for 1 hr. The peptide was decoupled from the resin and deprotected using 91:3:3:3 TFA/H_2O/triisopropylsilane/thioanisole (6 mL) over 4 hr. The TFA solution was then evaporated to ~1 mL, the crude peptide was precipitated with cold Et_2O, centrifuged, and the supernatant was removed. The precipitate was then washed 2 times with more cold Et_2O to remove all traces of the decoupling cocktail, and purified by prep HPLC using a gradient program going from 10 % ACN/0.1 % aq. TFA to 90 % ACN/0.1 % aq. TFA over 40 min at a 15 mL/min flow rate, yielding a fluffy white solid (70.9 mg, 0.0906 mmol, 44.4 %). MS calcd for $C_{38}H_{51}N_{14}O_5^+$: 783.41. Found 783.3.

Solid Phase Synthesis of N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4): Fmoc protected Rink amide resin (1.00 g, 0.68 mmol reactive sites) was placed in a fritted syringe reaction vessel, then swelled in THF for 1 hr. The resin was then deprotected by successive treatments (2 min, then 18 min) with 20 % piperidine in DMF (~10 mL), and then washed multiple times with DMF, DCM, and THF to remove all traces of piperidine

prior to reductive alkylation. A solution of 5-hexynal (80 mg, 0.832 mmol, 1.2 eq), THF (10 mL), AcOH (0.5 mL), and H_2O (0.5 mL) was added to the syringe, which was then shaken for 10 min. $NaCNBH_3$ (1 mL, 1.0 M in THF) was then added to the syringe solution, and the mixture was shaken for a further 3 hr. The solution was removed, and the resin was washed sequentially with THF (~10 mL, 3 times), H_2O (~10 mL, 3 times), MeOH (~10 mL, 3 times), THF (~10 mL, 3 times), and DCM (~10 mL, 3 times). A coupling solution of Cl-HOBt (0.350 g, 2.06 mmol), DIC (0.630 mL, 4.06 mmol), and Fmoc-Trp(Boc)-OH (1.08 g, 2.05 mmol) in 10 mL DMF was then added and allowed to react with the alkylated beads for 1 hr. The coupling solution was removed, and a solution of HBTU (0.775 g, 2.04 mmol), DIPEA (0.710 mL, 4.08 mmol), and Fmoc-Trp(Boc)-OH (1.08 g, 2.05 mmol) was added and allowed to react overnight (~14 hr) to ensure full coverage. The coupling solution was removed, and 50:50 Ac_2O/pyridine (4 mL) was added and the syringe was shaken for 5 min to cap all unreacted amines. The resin was then washed with DMF (~10 mL, 3 times) and DCM (~10 mL, 3 times) to remove residual acetic anhydride. The remaining amino acids (Fmoc-Arg(Pbf)-OH, 1.325 g; Fmoc-DPhe-OH, 0.790 g; Fmoc-His(Trt)-OH, 1.270 g) were added using the following cycle. Deprotection was effected by 20 min (2 min, 18 min) treatment with 20 % piperidine in DMF (~10 mL), then the resin was washed with DMF (10 mL, 3 times), DCM (10 mL, 3 times), DMF (10 mL, 3 times), and THF (10 mL, once). The amino acid was then coupled to the resin in repeated couplings to ensure full reaction of all amine sites. The first coupling solution was Cl-HOBt (0.350 g, 2.06 mmol), DIC (0.630 mL, 4.06 mmol) and the amino acid (Fmoc-Arg(Pbf)-OH: 1.33 g; Fmoc-DPhe-OH: 0.790 g;

Fmoc-His(Trt)-OH: 1.27 g; 2.04 mmol) in DMF (10 mL). This was allowed to preactivate for 5 min, then added to the resin and shaken for 1 hr. The second coupling solution was HBTU (0.775 g, 2.04 mmol), DIPEA (0.710 mL, 4.08 mmol) and the amino acid (2.04 mmol), again in DMF (10 mL), and again allowed to react with the resin for 1 hr. Once the couplings were complete, a Kaiser test was performed to determine if an appreciable amount of free amine was still present. This test was performed by taking a small portion of the resin (~5-10 beads) and placing them in a small test tube, then adding 2 drops each of (A) 5% ninhydrin in EtOH, (B) 80% phenol in ethanol, (C) 2% 1mM aq. KCN in pyridine, and heating at 100 °C for 5 min. If neither the beads nor the solution were blue, then there were no detectable free amines. If the Kaiser test was positive, another 1 hr HBTU based coupling was performed; if not, 50:50 Ac_2O/Pyridine (4 mL) was added to the resin and shaken for 5 min to ensure that any non-apparent free amines were capped. The resin was then washed with DMF, DCM, and THF, and the cycle was repeated for the next amino acid. The azide tail was added by following the deprotection and washing steps above, then adding a coupling solution of 6-azidohexanoic acid (0.320 g, 2.04 mmol), Cl-HOBt (0.350 g, 2.06 mmol), and DIC (0.630 mL, 4.06 mmol) in DMF (10 mL) and shaking for 1 hr. The peptide was decoupled from the resin using 91:3:3:3 TFA/H_2O/triisopropylsilane/thioanisole (~10 mL) over 4 hr. The TFA solution was then evaporated to ~4 mL, the crude peptide was precipitated with cold Et_2O, centrifuged, and the supernatant was removed. The precipitate was then washed 2 times with more cold Et_2O to remove all traces of the decoupling cocktail, and purified by semi-prep HPLC using a gradient program going from 10 % ACN/0.1 % aq. TFA to 90 % ACN/0.1 % aq.

TFA over 40 min at a 15 mL/min flow rate, yielding a fluffy white solid (19.6 mg, 3.3 %). MS calcd for $C_{44}H_{59}N_{14}O_5^+$: 863.47. Found 863.5.

6-Amino-1-hexyne[20]: *N*-(5-Hexynyl)phthalimide (5.00 g, 22.0 mmol) was dissolved in ethanol (250 mL) under argon. Hydrazine monohydrate (6.05 mL, ~121 mmol, 5.5 eq) was added and the solution was heated at reflux for 2.5 h, at which time TLC indicated the reaction was complete. The reaction was allowed to cool for 2 h, during which time a solid byproduct precipitated. The solvent was removed in vacuo and the product was redissolved in DCM (100 mL), then filtered to remove the byproduct. The DCM solution was washed with sat. $NaHCO_3$ (3x10 mL), brine (3x10 mL) then dried over $MgSO_4$. The solvent was removed to leave a pale yellow oil (1.00 g, 10.3 mmol, 46 %). No data was collected and the crude compound was used in the next experiment.

Fmoc-Trp(Boc)-NH(CH_2)$_4$C≡CH: 6-Amino-1-hexyne (1.00 g, 10.3 mmol) was dissolved in DCM (100 mL) and Fmoc-Trp(Boc)-OH (5.97 g, 11.4 mmol, 1.1 eq) and DIC (1.76 mL, 11.4 mmol, 1.1 eq) were added. The solution was stirred overnight, and the precipitate (presumably N, N'-diisopropyl urea) was removed by filtration. The solvent was removed in vacuo giving a crude cream colored solid in >100% yield (6.58 g). The crude product was purified by flash chromatography using 10-25 % EtOAc/hexanes as eluent, giving a white solid (4.33 g, 7.15 mmol, 69 %). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.31 (broad m, 2H), 1.39 (broad m, 2H), 1.64 (s, 9H), 1.87 (s, 1H), 2.07 (s, 2H), 3.12 (broad m, 3H), 3.22 (broad m, 1H), 4.19 (t, 1H, J = 6.9 Hz), 4.40 (m,

2H), 5.60 (broad s, 1H), 5.71 (broad s, 1H), 7.23-7.33 (m, 4H), 7.38 (t, 2H, J = 7.4 Hz), 7.45 (s, 1H), 7.53 (t, 2H, J = 7.1 Hz), 7.62 (broad s, 1H), 7.75 (d, 2H, J = 7.5 Hz), 8.12 (broad s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.2, 17.9, 21.1, 25.4, 28.19, 28.23, 28.6, 39.0, 47.1, 53.4, 55.3, 60.4, 67.2, 68.8, 83.8, 115.3, 119.1, 120.0, 122.8, 124.3, 124.7, 125.0, 127.1, 127.7, 130.1, 141.3, 143.7, 149.4, 170.6. HRMS(ESI$^+$) calcd for $C_{37}H_{40}N_3O_5^+$: 606.2962. Found: 606.2960.

Fmoc-Arg(Pbf)-Trp(Boc)-NH(CH$_2$)$_4$C≡CH: Fmoc-Trp(Boc)-NH(CH$_2$)$_4$C≡CH (1.00 g, 1.65 mmol) was dissolved in DCM (10 mL) and tris(2-aminoethyl) amine (TAEA, 2 mL) was added. The solution was stirred for 20 min, then EtOAc (20 mL) was added and the solution was washed with pH 5.5 phosphate buffer (2x10 mL) and with brine (2x10 mL). Volatiles were removed in vacuo and the residue dissolved in THF (10 mL). Fmoc-Arg(Pbf)-OH (1.18 g, 1.82 mmol, 1.1 eq), DIPEA (320 μL, 1.1 eq), and HBTU (0.691 g, 1.82 mmol, 1.1 eq) were added, then the solution was stirred for 3 h, at which point TLC with ninhydrin staining indicated the reaction was complete. The solution was diluted with EtOAc (~20 mL) then washed with aq. citric acid (2x10 mL), and aq. Na_2CO_3 (2x10 mL). Volatiles were removed and the residue was dissolved in a minimal amount of EtOAc. Hexanes was then used to precipitate the product, giving a crude compound in >100 % yield (1.99 g). This product was purified by column chromatography on silica gel 60 using 90% EtOAc/hexanes as eluent to yield a white solid (1.35 g, 1.33 mmol, 80 %). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.17 (m, 2H), 1.24 (m, 2H), 1.40 (s, 6H), 1.58 (s, 9H), 1.81 (t, 1H, J = 2.5 Hz), 1.83 (s, 3H), 1.96 (m, 2H), 2.05 (s, 3H), 2.52 (s, 3H), 2.60 (s,

3H), 2.89 (s, 3H), 3.11 (m, 4H), 4.09 (t, 1H, J = 7.0 Hz), 4.23 (t, 1H, J = 9 Hz), 4.29 (t, 1H, J = 9 Hz), 4.48 (broad s, 1H), 4.59 (q, 1H, J = 7.4 Hz), 5.98 (d, 1H, J = 5.3 Hz), 6.11 (broad s, 1H), 6.33 (broad s, 1H), 6.45 (broad s, 2H), 7.13 (t, 1H, J = 7.5 Hz), 7.22 (m, 4H), 7.32 (m, 2H), 7.42 (s, 1H), 7.51 (m, 3H), 7.69 (d, 2H, J = 7.4 Hz), 7.91 (broad s, 1H), 8.02 (broad s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.5, 17.9. 18.0, 19.4, 25.3, 27.7, 28.0, 28.2, 28.6, 29.9, 38.6, 39.0, 43.2, 47.1, 53.9, 54.6, 67.1, 68.8, 83.8, 86.3, 115.2, 115.7, 117.5, 119.0, 119.9, 122.7, 124.3, 124.5, 124.6, 125.1, 127.0, 127.6, 130.1, 132.3, 132.7, 135.3, 138.4, 141.2, 143.6, 143.8, 149.5, 156.4, 156.6, 158.7, 171.4, 172.5. HRMS(ESI$^+$) calcd for $C_{56}H_{68}N_7O_9S^+$: 1014.4794. Found: 1014.4794.

Fmoc-DPhe-Arg(Pbf)-Trp(Boc)-NH(CH$_2$)$_4$C≡CH: Fmoc-Arg(Pbf)-Trp(Boc)-NH(CH$_2$)$_4$C≡CH (1.02 g, 1.00 mmol) was dissolved in DCM (10 mL) and TAEA (2 mL) was added. The solution was stirred for 20 min, then EtOAc (20 mL) was added and the solution was washed with pH 5.5 phosphate buffer (2x10 mL) and with brine (2x10 mL). Volatiles were removed in vacuo and the residue was dissolved in THF (10 mL). Fmoc-DPhe-OH (430 mg, 1.11 mmol, 1.1 eq), DIPEA (195 µL, 1.1 eq), and HBTU (420 mg, 1.11 mmol, 1.1 eq) were added, then the solution was stirred for 3 hrs, at which time TLC with ninhydrin staining indicated the reaction was complete. The solution was diluted with EtOAc (~20 mL) then washed with aq. citric acid (2x10 mL), and aq. Na_2CO_3 (2x10 mL). Volatiles were removed and the residue was dissolved in a minimal amount of EtOAc. Hexanes was then used to precipitate the product, giving a crude compound in >100 % yield (1.26 g). The crude product was purified by column

chromatography on silica gel 60 using 90 % EtOAc/hexanes as eluent to give a white solid (1.01 g, 0.866 mmol, 86 %). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.25 (m, 4H), 1.36 (m, 2H), 1.40 (s, 6H), 1.55 (s, 9H), 1.80 (s, 1H, C≡CH), 1.87 (s, 3H), 1.99 (m, 2H) 2.02 (s, 3H), 2.04 (s, 3H), 2.46 (s, 3H), 2.55 (s, 3H), 2.86 (s, 3H), 2.93 (m, 2H), 3.1 (m, 4H), 3.3 (m, 1H), 4.03 (t, 1H, J = 6.7 Hz), 4.13 (broad s, 1H), 4.22 (m, 1H), 4.37 (broad s, 2H), 4.63 (d, 1H, 6.3 Hz), 6.23 (broad s, 1H), 6.71 (broad s, 1H), 7.05-7.2 (m, 9H), 7.32 (t, 2H, J = 7.3 Hz), 7.42 (m, 3H), 7.51 (d, 1H, J = 7.8 Hz), 7.68 (d, 2H, J = 7.5 Hz), 7.80 (broad s, 1H), 7.96 (broad s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.5, 18.0, 19.3, 24.9, 25.5, 27.4, 28.1, 28.2, 28.6, 37.9, 39.1, 43.3, 47.0, 53.4, 54.3, 67.4, 68.7, 83.9, 84.1, 86.4, 115.2, 117.6, 119.3, 119.9, 122.7, 124.0, 124.5, 124.7, 125.0, 127.0, 127.1, 127.7, 128.7, 129.2, 130.4, 132.3, 135.2, 136.2, 138.3, 141.20, 141.22, 143.5, 143.7, 149.8, 156.2, 158.8, 171.1, 171.7. HRMS(ESI^+) calcd for $C_{65}H_{77}N_8O_{10}S^+$: 1161.5478. Found: 1161.5488.

Fmoc-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-NH(CH$_2$)$_4$C≡CH: Fmoc-DPhe-Arg(Pbf)-Trp(Boc)-NH(CH_2)$_4$C≡CH (1.00 g, 0.866 mmol) was dissolved in DCM (10 mL) and TAEA (2 mL) was added. The solution was stirred for 20 min, then EtOAc (20 mL) was added and the solution was washed with pH 5.5 phosphate buffer (2x10 mL) and with brine (2x10 mL). Volatiles were removed in vacuo and the residue was dissolved in THF (10 mL). Fmoc-His(Trt)-OH (591 mg, 0.954 mmol), DIPEA (170 μL), and HBTU (361 mg, 0.952 mmol) were added, then the solution was stirred for 3 h, when TLC with ninhydrin staining indicated the reaction was complete. The solution was diluted with

EtOAc (~20 mL) then washed with aq. citric acid (2x10 mL), and aq. Na_2CO_3 (2x10 mL). Volatiles were removed and the residue was dissolved in minimal EtOAc. Hexanes was then used to precipitate the product, giving a crude compound in >100 % yield (1.35 g). This product was purified by column chromatography on silica gel 60 using 95:5 EtOAc:hexanes as eluent to yield a white solid (1.12 g, 0.728 mmol, 84 %). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.25 (m, 7H), 1.39 (m, 10H), 1.51 (s, 12H), 1.78 (s, 1H), 2.01 (m, 10H), 2.29 (m, 3H), 2.43 (s, 3H), 2.50 (s, 3H), 2.66 (broad s, 1H), 2.77 (s, 1H), 2.85 (m, 5H), 3.05 (m, 4H), 3.18 (broad s, 2H), 4.25 (m, 4H), 4.60 (broad s, 1H), 6.13 (broad s, 1H), 6.40 (broad s, 1H), 6.93-7.10 (m, 13H), 7.15 (broad s, 4H), 7.23-7.36 (m, 14H), 7.47 (broad s, 4H), 7.65 (d, 2H, J = 7.3 Hz), 7.91 (broad s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.5, 13.4, 13.7, 13.9, 17.3, 17.9, 18.0, 19.1, 19.2, 19.3, 21.0, 22.0, 25.0, 25.5, 27.2, 27.7, 28.1, 28.2, 28.52, 28.54, 29.7, 30.6, 30.7, 36.0, 39.1, 40.4, 43.2, 46.9, 53.9, 54.2, 55.9, 56.0, 64.3, 67.3, 68.6, 75.5, 83.8, 84.1, 86.2, 115.0, 117.3, 119.2, 119.9, 122.6, 123.9, 124.4, 124.5, 125.0, 125.1, 126.7, 127.0, 127.6, 128.1, 128.4, 129..0, 129.6, 130.4, 132.1, 133.0, 135.0, 135.9, 137.1, 138.2, 138.7, 141.1, 142.0, 143.5, 143.6, 149.8, 156.1, 156.8, 158.5, 171.0, 171.9, 172.7. HRMS(ESI$^+$) calcd for $C_{90}H_{98}N_{11}O_{11}S^+$: 1540.7163. Found: 1540.7219.

$N_3(CH_2)_5CO$-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4C\equiv CH$ (Protected MSH(4)): Fmoc-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$ (1.02 g, 0.659 mmol) was dissolved in DCM (10 mL) and TAEA (2 mL) was added. The solution was stirred for 20 min, then EtOAc (20 mL) was added and the solution was washed with pH 5.5

phosphate buffer (2x10 mL) and with brine (2x10 mL). Volatiles were removed in vacuo and the residue was dissolved in THF (10 mL). 6-Azidohexanoic acid (114 mg, 0.725 mmol), DIPEA (145 μL), and HBTU (315 mg, 0.830 mmol) were added, then the solution was stirred for 3 h, when TLC with ninhydrin staining indicated the reaction was complete. The solution was diluted with EtOAc (~20 mL) then washed with aq. citric acid (2x10 mL), and aq. Na_2CO_3 (2x10 mL). Volatiles were removed in vacuo and the crude residue was purified by flash chromatography in 5 % MeOH/EtOAc to yield a white solid (0.792 g, 0.543 mmol, 82 %). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.25 (m, 4H), 1.36 (m, 2H), 1.42 (s, 6H), 1.46 (m, 6H), 1.58 (s, 10H), 1.82 (s, 1H), 2.02 (s, 2H), 2.05 (s, 3H), 2.07 (m, 1H), 2.14 (t, 2H, J = 7.4 Hz), 2.45 (s, 3H), 2.52 (s, 3H), 2.58 (m, 1H), 2.80 (m, 2H), 2.90 (s, 2H), 2.95-3.1 (m, 3H), 3.14 (m, 4H), 3.28 (m, 2H), 4.25 (broad s, 1H), 4.31 (broad s, 2H), 4.66 (d, 1H, J = 5.9 Hz), 6.19 (broad s, 1H), 6.30 (broad s, 1H), 6.56 (broad s, 1H), 6.95 (m, 1H), 7.01-7.07 (m, 9H), 7.15 (t, 1H, J = 7.4 Hz), 7.22 (t, 1H, J = 8 Hz), 7.31 (m, 9H), 7.48 Hz (m, 2H), 7.58 (d, 2H, J = 7.8 Hz), 7.70 (m, 1H), 7.97 (broad s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.5, 13.7, 17.9, 18.0, 19.1, 19.3, 21.0, 24.8, 25.2, 25.5, 26.1, 27.2, 28.1, 28.4, 28.6, 29.1, 30.6, 30.7, 35.9, 36.1, 38.6, 39.1, 43.2, 51.1, 53.9, 54.3, 54.5, 54.6, 55.8, 64.3, 68.7, 75.4, 83.9, 84.0, 86.3, 115.1, 117.4, 119.1, 119.9, 122.3, 124.1, 124.5, 124.6, 126.6, 128.0, 128.1, 128.3, 128.8, 129.0, 129.7, 130.3, 132.1, 132.7, 134.6, 135.1, 135.9, 138.2, 138.4, 142.0, 156.4, 158.7, 171.0, 171.1, 172.1, 172.2, 172.4, 174.3. HRMS(ESI$^+$) calcd for $C_{81}H_{97}N_{14}O_{10}S^+$: 1457.7227. Found: 1457.7232.

N-(6-Azidohexoyl)-C-(5-hexynyl) MSH(4) from Protected MSH(4): Protected MSH(4) (0.286 g, 0.196 mmol) was placed in a 50 mL round bottom flask, and 20 mL of 91:3:3:3 TFA/H_2O/thioanisole/triisopropylsilane was added. The solution was stirred for 20 hrs, then the majority of the TFA was evaporated, leaving ~ 5 mL of solution. The deprotected peptide was precipitated using cold Et_2O (~20 mL), then centrifuged, and the supernatant was removed. The crude peptide was washed twice more with aliquots of cold Et_2O, then dried. It was purified by HPLC using the same conditions as in solid phase synthesis, yielding a white solid (0.100 g, 0.116 mmol, 59 %). HRMS(ESI^+) calcd for $C_{44}H_{59}N_{14}O_5^+$: 863.4793. Found: 863.4782.

Room Temperature Multimerization of Protected MSH(4) in MeOH: Protected N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) (39.7 mg, 27.4 μmol), tetrakis(acetonitrile) copper(1) hexafluorophosphate (1.0 mg, 2.7 μmol), and tris(benzotriazolyl) amine (TBTA) (1.5 mg, 2.7 μmol) were placed in a vial, then sealed with a septum. The vial was purged with argon, and then 0.25 mL degassed MeOH was added and the solution was stirred for 24 hr. At that time, TLC showed no change from starting material, so both tetrakis(acetonitrile) copper(1) hexafluorophosphate and TBTA were increased to 1 eq., and the mixture was stirred for another 48 hr. The mixture was dissolved in DCM, then washed with water and aq. citric acid. The crude mixture was then submitted for MS analysis. MS ($MALDI^+$): 1457 (monomer), 2978 (dimer + Cu^+).

Room Temperature Multimerization of Protected MSH(4) in MeOH: Protected N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) (38.8 g, 26.6 μmol) tetrakis(acetonitrile) copper(1) hexafluorophosphate (10.0 mg, 26.8 μmol), and TBTA (14.5 mg, 27.3 μmol) were placed in a vial, then sealed with a septum. The vial was purged with argon, and then 0.25 mL degassed MeOH was added and the solution was stirred for 7 days. The mixture was dissolved in DCM, then washed with water and aq. citric acid. The crude mixture was then submitted for MS analysis. MS ($MALDI^+$): 1457 (monomer), 2916 (dimer), 4437 (trimer).

Room Temperature Multimerization of Protected MSH(4) in DCM: Protected N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) (52.8 mg, 36.2 μmol), CuBr (0.8 mg, 5.57 μmol), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (1.95 μL, 13.1 μmol) were placed in a sealed, septum covered vial, and the vial atmosphere was replaced with Ar. DCM (0.5 mL) was added and the solution was stirred for 48 hr. At that time the solution was poured into a bilayer of aqueous citric acid and $CHCl_3$, the citric acid neutralizing the DBU before it could react with the chloroform. The $CHCl_3$ layer was washed another 3 times with citric acid to remove copper, dried over $MgSO_4$, and volatiles were removed in vacuo, resulting in 100 % recovery of the initial mass of protected peptide, now multimerized as demonstrated by mass spectroscopy. MS ($MALDI^+$): 1477.8 (monomer + Na^+), 2914.4 (dimer), 4434 (trimer), 5828.1 (tetramer).

Multimerization of Protected MSH(4) in DCM at 40 °C: CuBr (1.1 mg, 7.67 μmol) and DBU (3.7 μL, 24.8 μmol) were placed in a tube, and the air was removed. Protected N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) (0.120 g, 82.3 μmol) was dissolved in DCM (1.5 mL), and added to the tube. The tube was then flame sealed, and placed in a 40 °C oil bath for 48 hr. After reacting, the tube was opened, and mixture was poured into a bilayer of aqueous citric acid and $CHCl_3$. The solid remaining in the tube was rinsed with citric acid, then dissolved in $CHCl_3$. The chloroform solution was submitted for MS without any further workup. MS ($MALDI^+$): 2916 (dimer), 4438 (trimer), 5891 (tetramer).

Multimerization of Protected MSH(4) in DMF at Room Temperature: Protected N-(6-azidohexoyl)-C-(5-hexynyl) MSH(4) (59.6 mg, 40.8 μmol) and CuBr (0.7 mg, 4.9 μmol) were placed in a vial and covered with a septum. The vial was purged with Ar, then a solution of DIPEA (2.6 μL) in degassed DMF (0.25 mL) was added. The solution was stirred for ~72 hr, then precipitated in EtOAc, separating residual monomer (soluble in EtOAc) from multimers. Both fractions were redissolved in $CHCl_3$ and washed with aq citric acid to remove copper, then submitted for MS. MS ($MALDI^+$): 4438 (precipitate from EtOAc, trimer).

Microwave Heated Multimerization of MSH(4) in MeOH: N-(6-Azidohexoyl)-C-(5-hexynyl) MSH(4) (29.2 mg, 33.8 μmol), TBTA (3.7 mg, 6.97 μmol), and $Cu(ACN)_4(PF_6)$ (2.6 mg, 6.98 μmol) were placed in a 0.25 – 0.5 mL microwave reaction vessel equipped

with a stir bar, and the vessel was sealed. The vessel was purged by evacuating the air, and replacing it with Ar 3 times, then 0.25 mL MeOH was added. The contents were fully dissolved, then the vessel was placed in the microwave reactor, and run at 100 °C for 2 hr, then checked for any precipitation. Upon seeing none, the solution was reacted at 100 °C for a further 12 hr. A solution of dithizone (4.1 mg, 16.0 μmol) in CCl_4 (20 mL) was prepared, and the reaction mixture was poured into a bilayer of water (5-10 mL) and dark green dithizone in CCl_4 (2-3 mL). This was agitated to allow the dithizone to complex with any remaining copper, changing color to a reddish brown, and remove it in the CCl_4 layer. The aqueous layer was washed with dithizone solution until it exhibited no color change, then it was washed numerous times with CCl_4 to remove any residual dithizone. Any remaining CCl_4 and MeOH were then removed in vacuo, and the remaining aqueous solution was lyophilized to isolate the multimers (22.9 mg, 78 % recovery). MS ($MALDI^+$): 863 (monomer), 1726 (dimer), 2591 (trimer), 3454 (tetramer), 4317 (pentamer), 5180 (hexamer), 6043 (heptamer, seen in crude MS), 6903 (octamer, seen in crude MS), 7782 (nonamer, seen in crude MS).

Microwave Heated Multimerization of MSH(4) in MeOH: N-(6-Azidohexoyl)-C-(5-hexynyl) MSH(4) (57.0 mg, 66.0 μmol), TBTA (7.4 mg, 13.94 μmol), and $Cu(ACN)_4(PF_6)$ (5.2 mg, 13.96 μmol) were placed in a 0.25 – 0.5 mL microwave reaction vessel equipped with a stir bar, and the vessel was sealed. The vessel was purged by evacuating the air, and replacing it with Ar 3 times, then 0.25 mL MeOH was added. The contents were fully dissolved, then the vessel was placed in the microwave reactor,

and run at 100 °C for 2 hr, then checked for any precipitation. Upon seeing none, the solution was reacted at 100 °C for a further 12 hr. A solution of dithizone (8 mg, 32 μmol) in CCl_4 (20 mL) was prepared, and the reaction mixture was poured into a bilayer of water (5-10 mL) and dark green dithizone in CCl_4 (2-3 mL). This was agitated to allow the dithizone to complex with any remaining copper, changing color to a reddish brown, and remove it in the CCl_4 layer. The aqueous layer was washed with dithizone solution until it exhibited no color change, then it was washed numerous times with CCl_4 to remove any residual dithizone. Any remaining CCl_4 and MeOH were then removed in vacuo, and the remaining aqueous solution was lyophilized to isolate the multimers (0.0443 g, 77.7 % recovery). MS ($MALDI^+$): 863 (monomer), 1726 (dimer), 2588 (trimer), 3450 (tetramer), 4330 (pentamer), 5211 (hexamer), 6095 (heptamer).

Concentration Calibration of MSH(4) in DMSO by UV-Visible Spectroscopy: C-(6-(4-Butyl-1,2,3-triazolyl)hexanoyl) MSH(4) amide (17.2 mg, 19.9 μmol) was dissolved in DMSO (10.0 mL), and a series of dilutions were made from this 1.99 mM solution. The absorbance spectra of these solutions from 190 to 400 nm were recorded, and the absorbance at 283 nm was used to create a linear concentration calibration for MSH(4) compounds in DMSO (Figure 6). The calibration equation found was

$$A = (4.682 \text{ L mol}^{-1} \text{ cm}^{-1})\, l\, c$$

where A is absorbance, l is path length, and c is concentration.

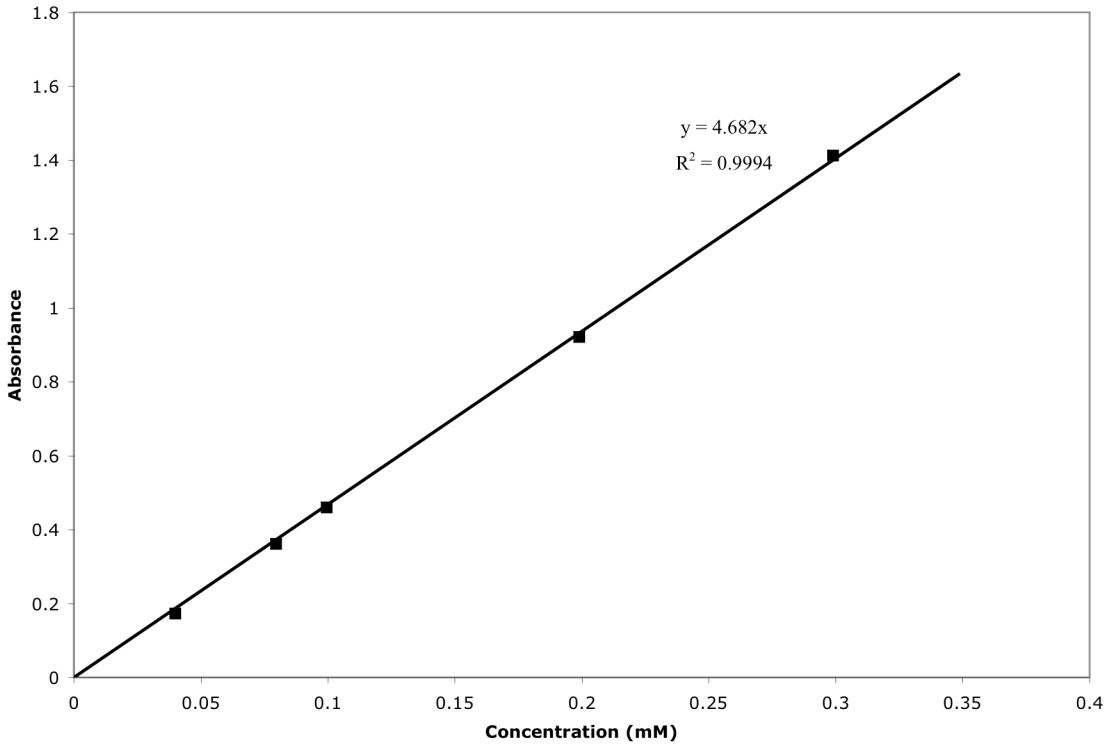


Figure 6. Calibration line for MSH(4) in DMSO.

Concentration Calibration of MSH(4) and Triazole in MeOH by UV-Visible Spectroscopy: A data set containing concentrations and corresponding absorbance values of C-acetyl MSH(4) amide was obtained from Ramesh Aletti, a Mash group member, and a calibration line of Concentration vs. Absorbance was created. Error in this line was estimated by determining the slopes of the lines from each point through (0, 0), then taking the standard deviation of these slopes (Figure 7). The equation found was

$$A = (3.9764 \pm 0.6424 \text{ L mol}^{-1}\text{cm}^{-1})\, l\, c$$

where A is absorbance, l is path length, and c is concentration, over the intended range of concentrations. C-(6-(4-Butyl-1,2,3-triazolyl)hexanoyl) MSH(4) amide (8.7 mg, 10.1 μ mol) was dissolved in MeOH (10.0 mL), and a series of dilutions were made from this

solution. The absorbance spectra of these solutions from 190 to 400 nm were recorded, and the absorbance at 283 nm was used determine the exact concentration of the MSH(4) species in solution, based on the above equation. This concentration and the absorbance at 219 nm was then used to create a linear concentration calibration for the triazole moiety in MeOH. Error was carried forward and applied to the calculated concentration values. The equation determined for triazole was

$$A = (28.404 \pm 4.589 \text{ L mol}^{-1}\text{cm}^{-1})\, l\, c$$

using the same variables as above.

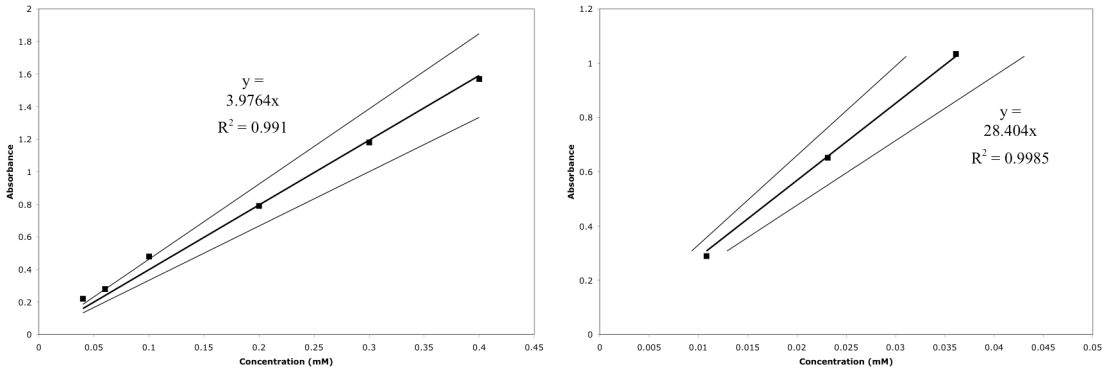


Figure 7. Calibration lines for MSH(4) (left) and triazole, both in methanol. Lighter lines denote error lines.

Determination of Multimer Length by UV-Visible Spectroscopy: 4 multimer samples (0.8-0.9 mg each) were each dissolved in MeOH (1 mL) to create ~1mM solutions. A 100 μL aliquot of each solution was diluted to 5.0 mL to create a set of solution of approx. 20 μM in MSH(4). The absorbance spectra of these solutions were recorded from 190 nm to 400 nm, and the absorbance at 283 nm and 219 nm were converted to concentrations using the linear calibrations determined above. As the ratio of concentrations (conc. triazole/conc. MSH(4)) should be proportional to the ratio of the number of MSH(4)s (*n*)

to the number of triazoles (n-1) in a linear chain, one can solve the equation $\frac{n-1}{n} = \frac{C_{triazole}}{C_{MSH(4)}}$ for n, the average number of MSH(4)s in a chain, $n = \frac{1}{1 - C_{triazole} / C_{MSH(4)}}$.

The data were plugged into this equation, and the values in Table 4 were found. The maximum and minimum ratios were also determined in two separate cases, first using the greatest value of $C_{triazole}$ and the smallest value of $C_{MSH(4)}$, then the smallest value of $C_{triazole}$ and the largest value of $C_{MSH(4)}$. These values, and the multimer size, n, calculated from them can be seen in Table 5.

Table 4. Calculated chain length of selected multimerizations.

Sample ID	Description	$\frac{C_{triazole}}{C_{MSH(4)}}$	$n_{UV\text{-}Vis}$
RS-1-100	Mult. done in protected form, residual monomer removed	0.721	3.58
RS-1-103	Mult. done in non-protected form in microwave reactor, residual monomer present	0.859	7.09
RS-1-104	Duplicate of RS-1-103	0.928	13.81
RS-1-105	Mult. done in non-protected form in microwave reactor. residual monomer present. Conc. about twice that of RS-1-103.	1.044**	-22.67

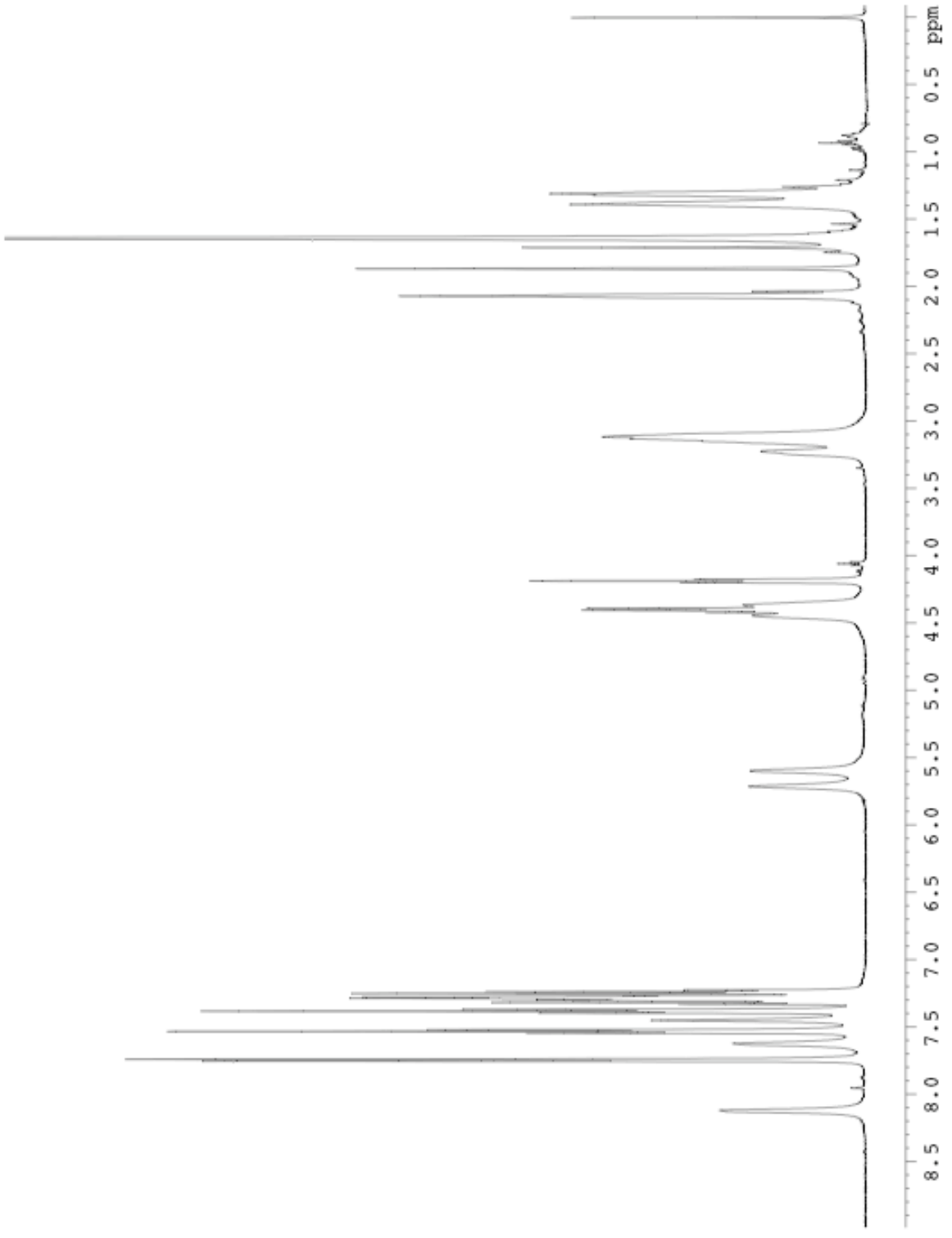
* An attempt was made to remove monomer from this sample, resulting in a difference between the crude results discussed in the multimerizations section of the text and the numbers given here.
** Even in a fully cyclized peptide, there should be no more than one triazole per MSH(4) unit. As such, this value does not convert to a sensible chain length.

Table 5. Error factored into concentration ratios used to determine chain lengths by UV-Visible spectroscopy.

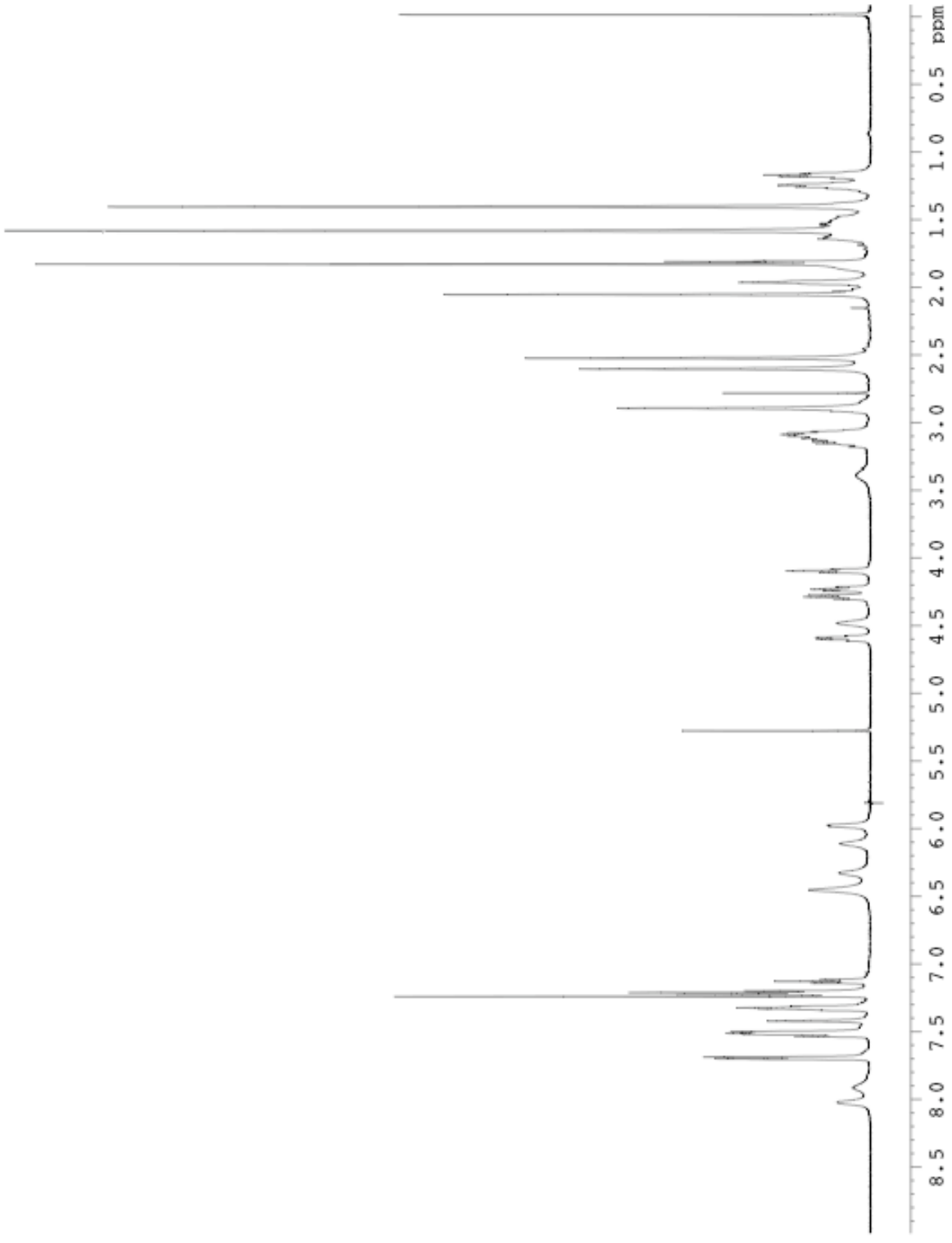
Sample ID	$\frac{C_{triazole}}{C_{MSH(4)}}$ (min)	$n_{UV\text{-}Vis}$ (min ratio)	$\frac{C_{triazole}}{C_{MSH(4)}}$ (max)	$n_{UV\text{-}Vis}$ (max ratio)
RS-1-100	0.520	2.08	0.999	763.68
RS-1-103	0.620	2.63	1.190	-5.26
RS-1-104	0.670	3.02	1.285	-3.51
RS-1-105	0.754	4.05	1.446	-2.24

APPENDIX A

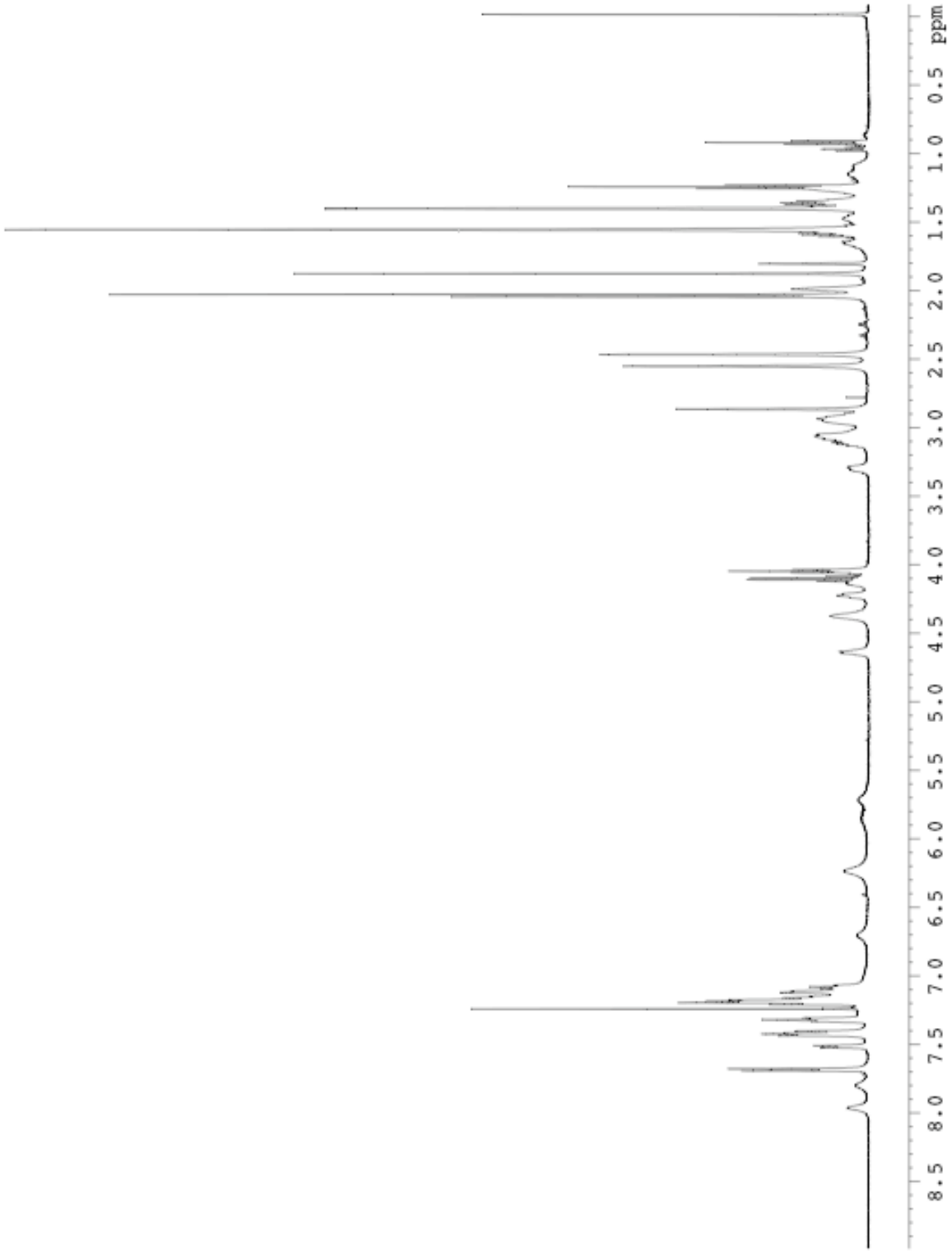
^{1}H NMR SPECTRA



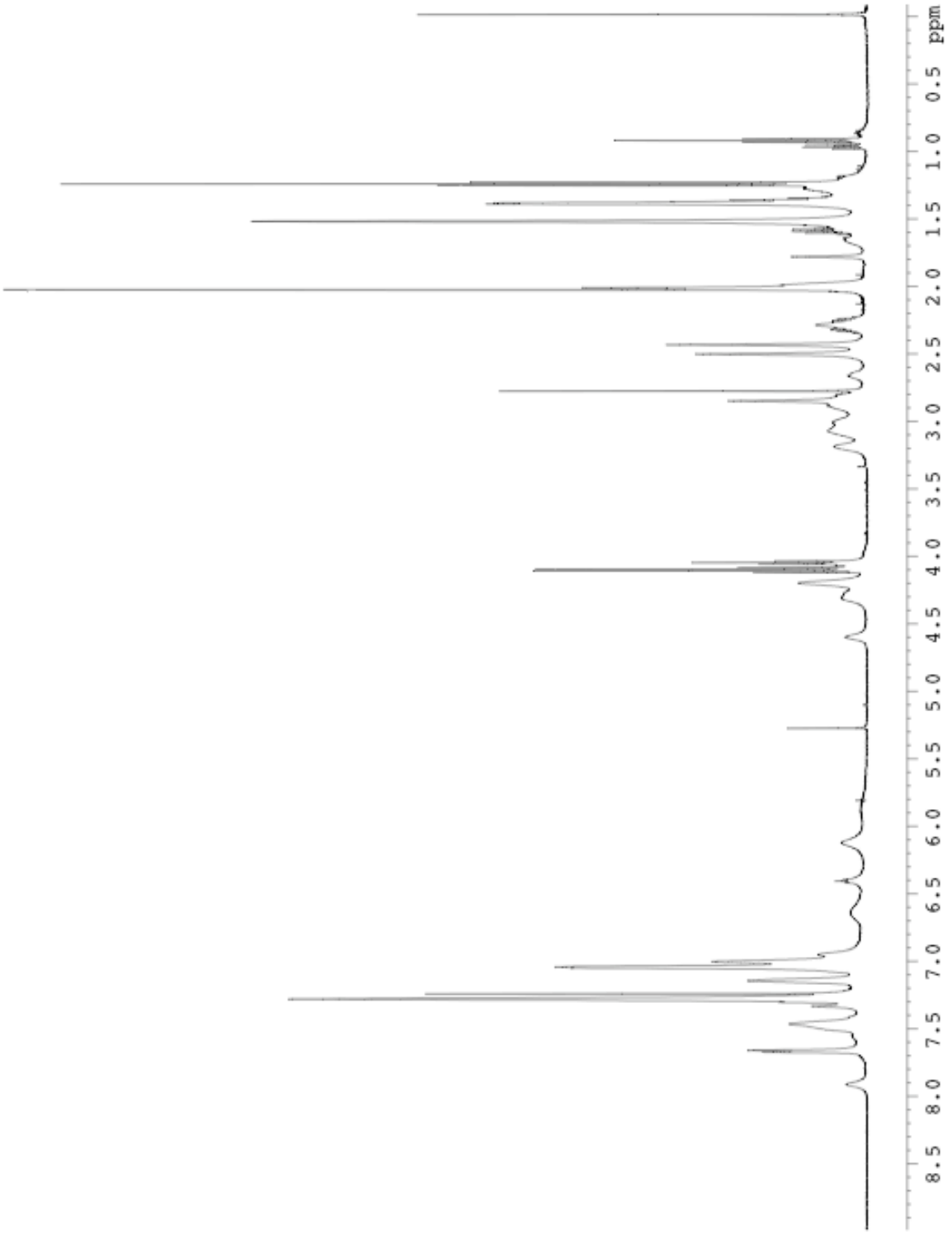
600 MHz ^{1}H NMR of intermediate **4**, Fmoc-Trp(Boc)-NH$(CH_2)_4$CCH



600 MHz ^{1}H NMR of intermediate **6**, Fmoc-Arg(Pbf)-Trp(Boc)-NH$(CH_2)_4$CCH

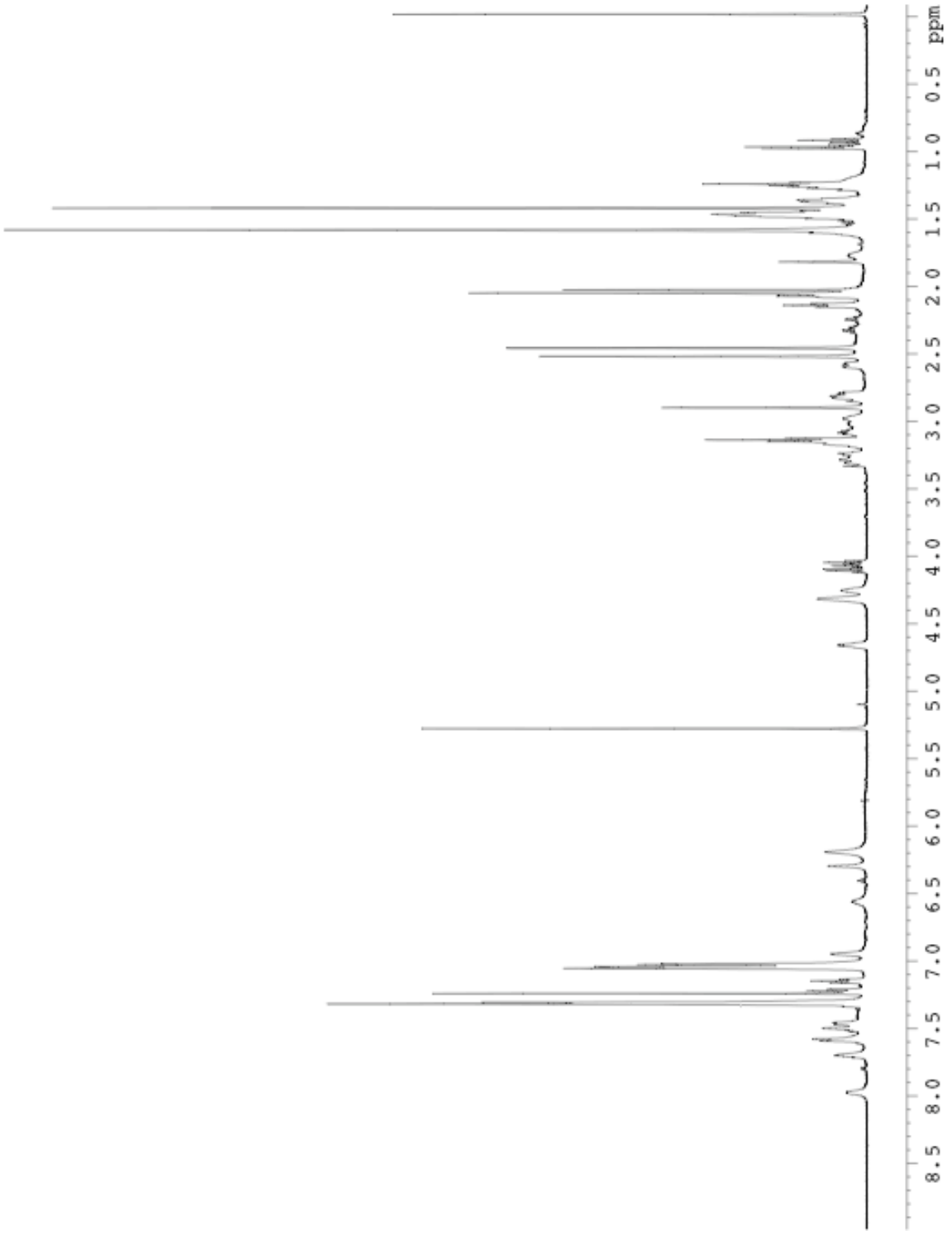


600 MHz ^{1}H NMR of intermediate **7**, Fmoc-DPhe-Arg(Pbf)-Trp(Boc)-NH$(CH_2)_4$CCH



600 MHz ^{1}H NMR of intermediate **8**,

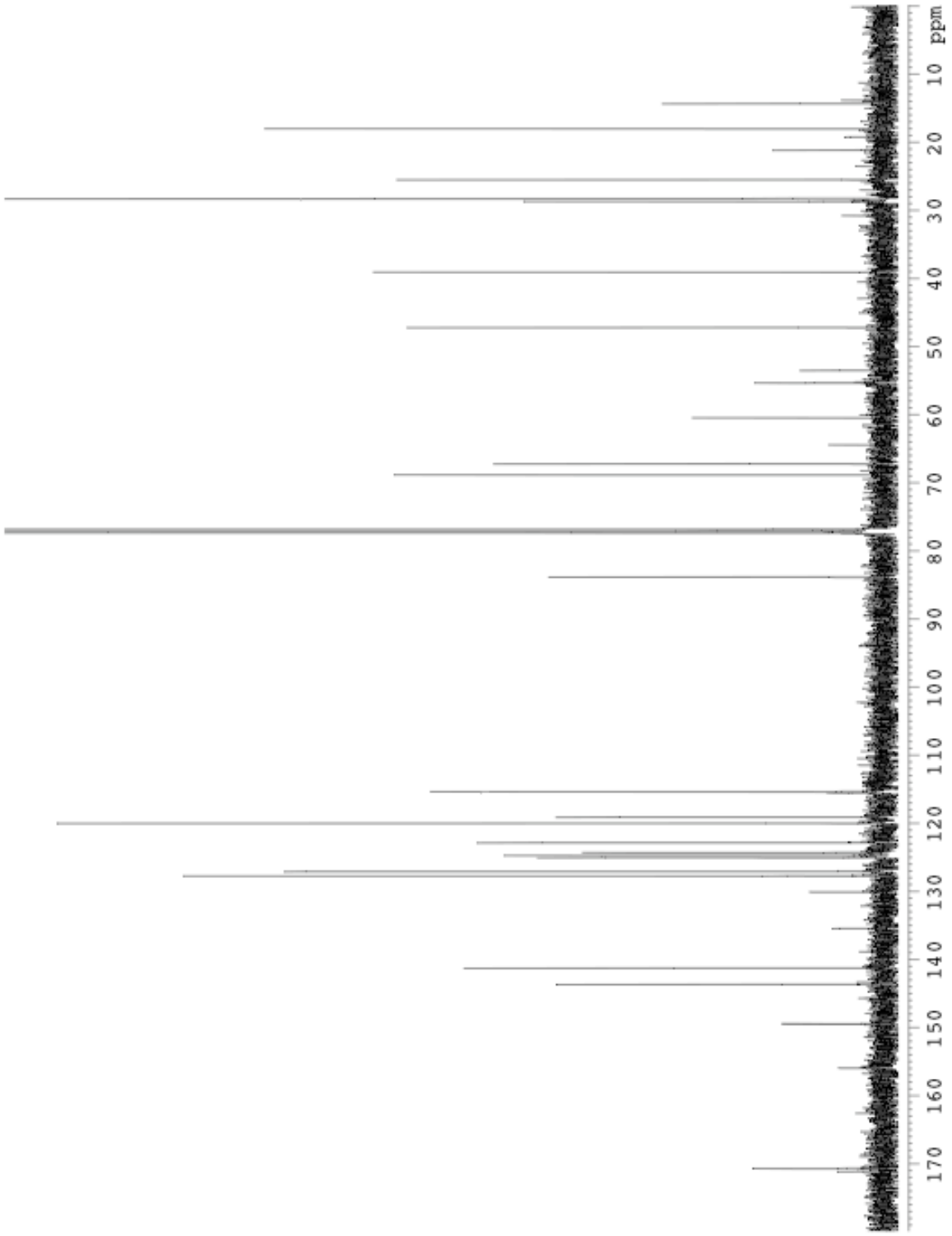
Fmoc-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-NH$(CH_2)_4$CCH



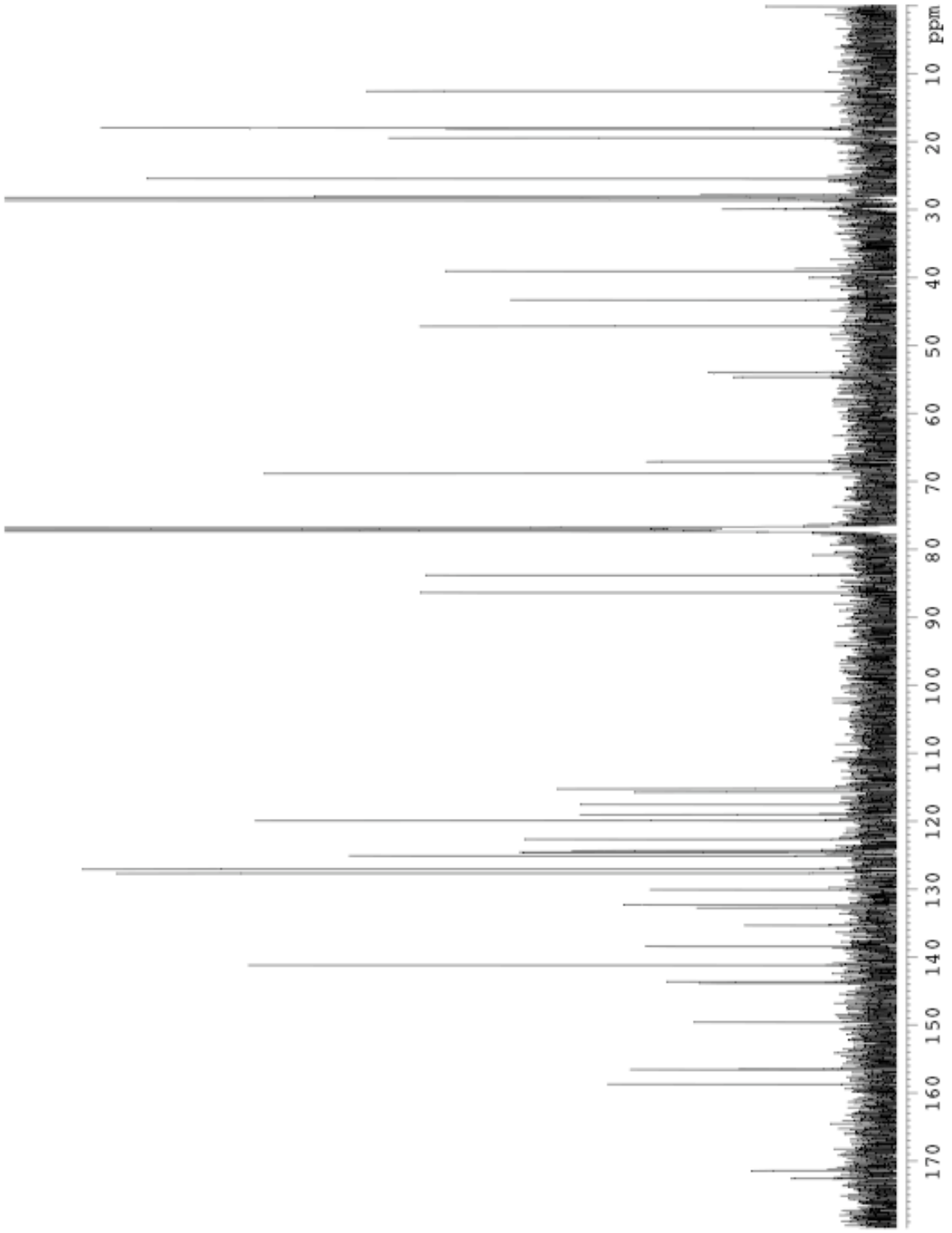
600 MHz ^{1}H NMR of compound **9**,
$N_3(CH_2)_5CO$-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$

APPENDIX B

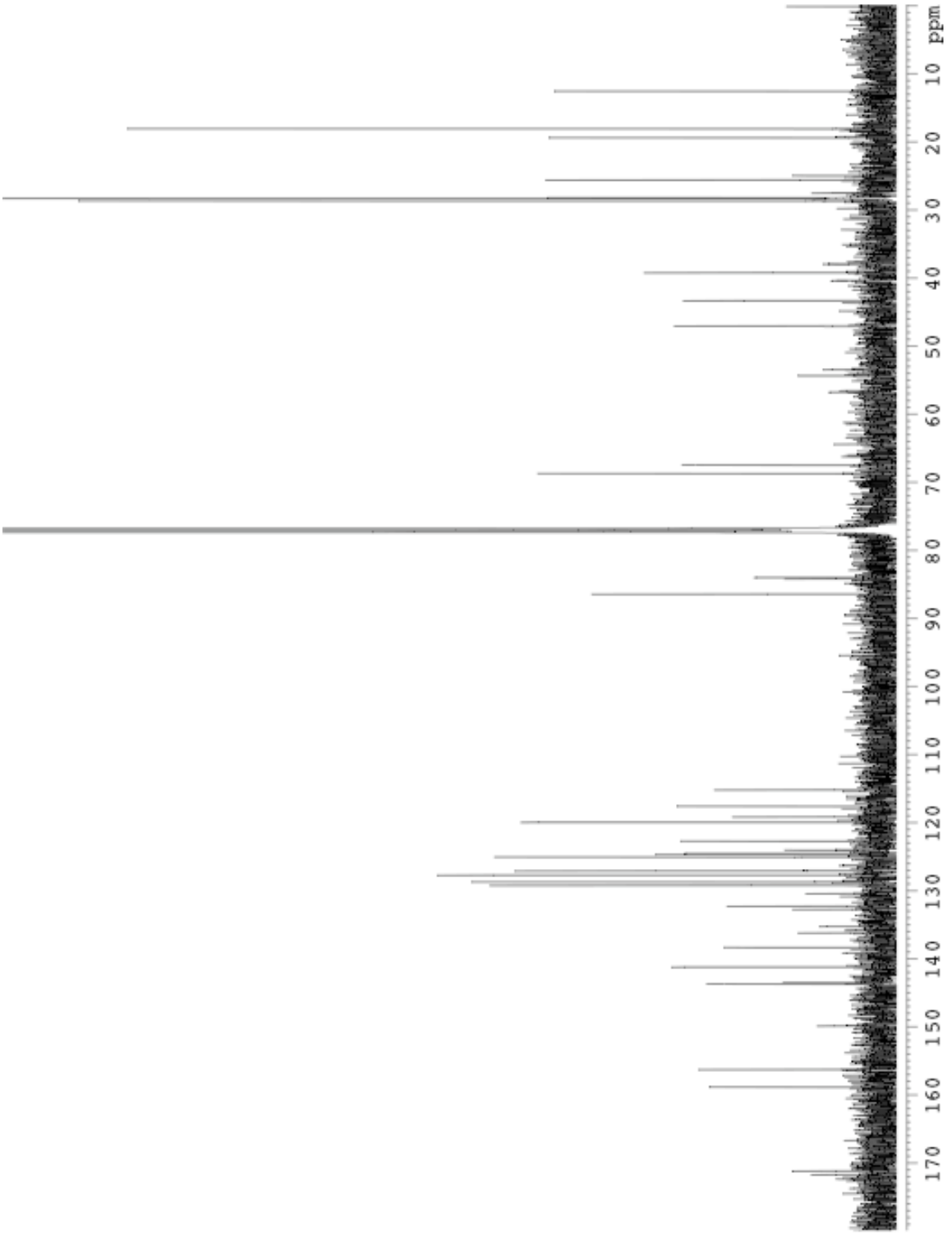
^{13}C NMR SPECTRA



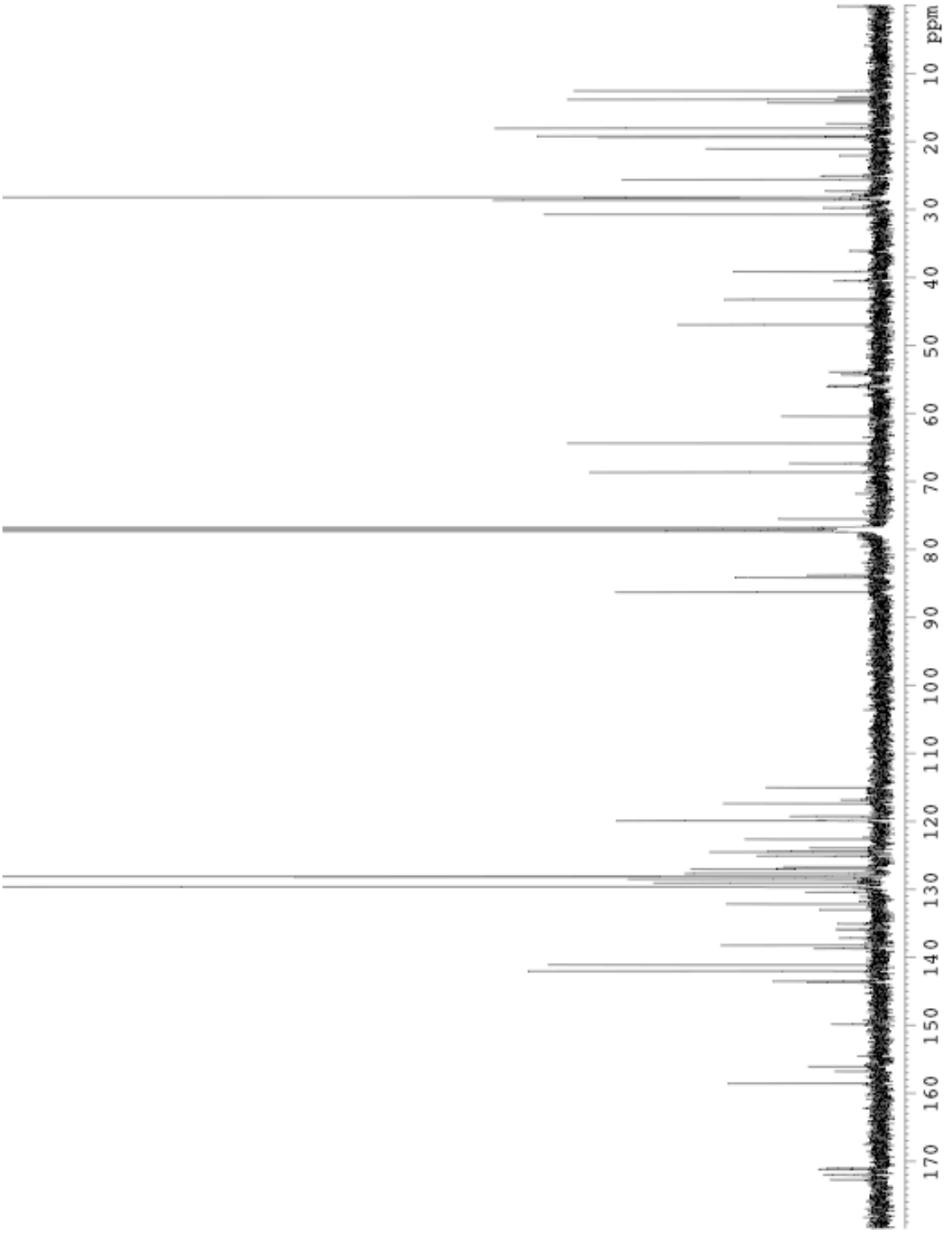
125 MHz ^{13}C NMR of intermediate **4**, Fmoc-Trp(Boc)-NH$(CH_2)_4$CCH



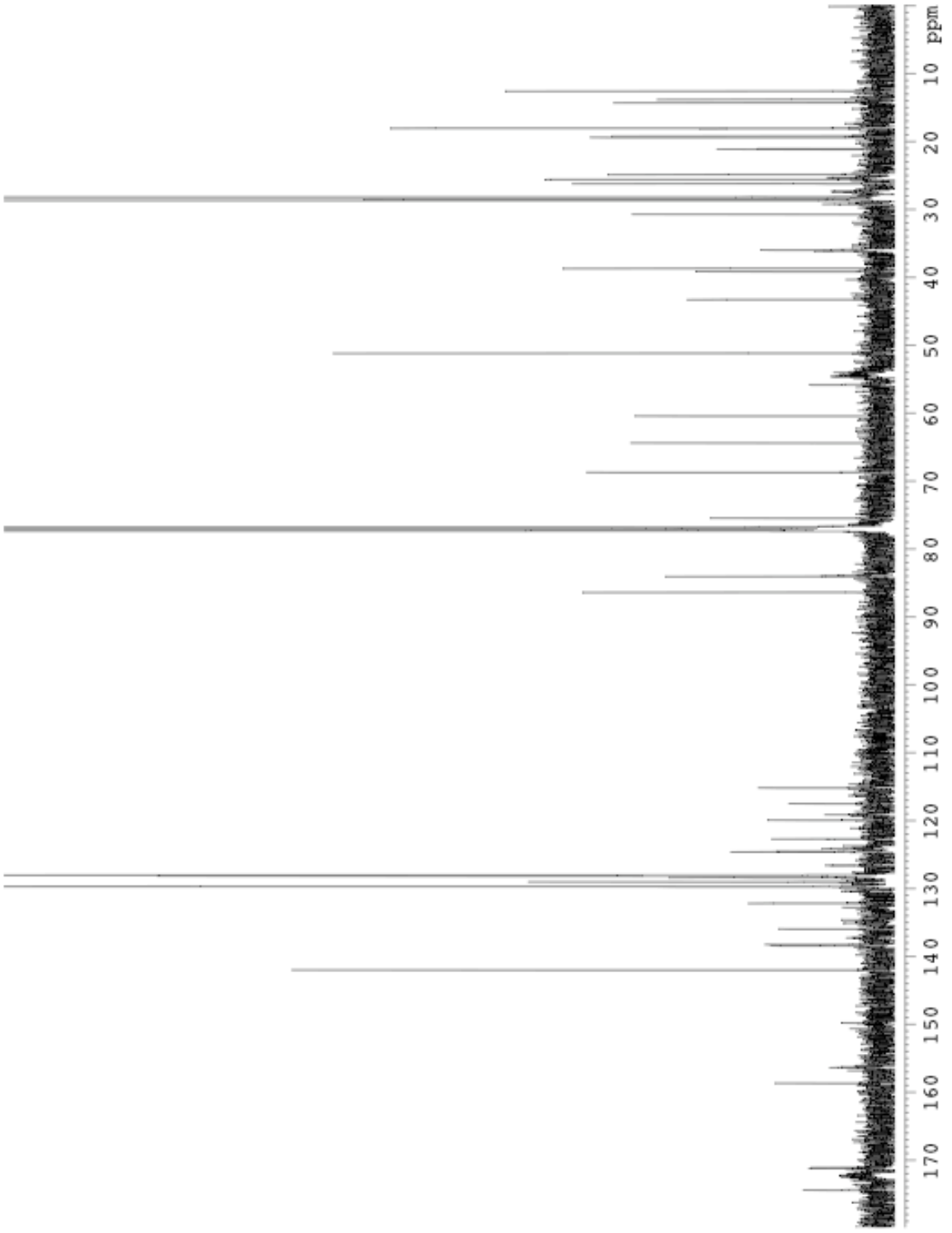
125 MHz ^{13}C NMR of intermediate **6**, Fmoc-Arg(Pbf)-Trp(Boc)-NH$(CH_2)_4$CCH



125 MHz ^{13}C NMR of intermediate **7**, Fmoc-DPhe-Arg(Pbf)-Trp(Boc)-NH$(CH_2)_4$CCH



125 MHz ^{13}C NMR of intermediate **8**,
Fmoc-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-NH$(CH_2)_4$CCH

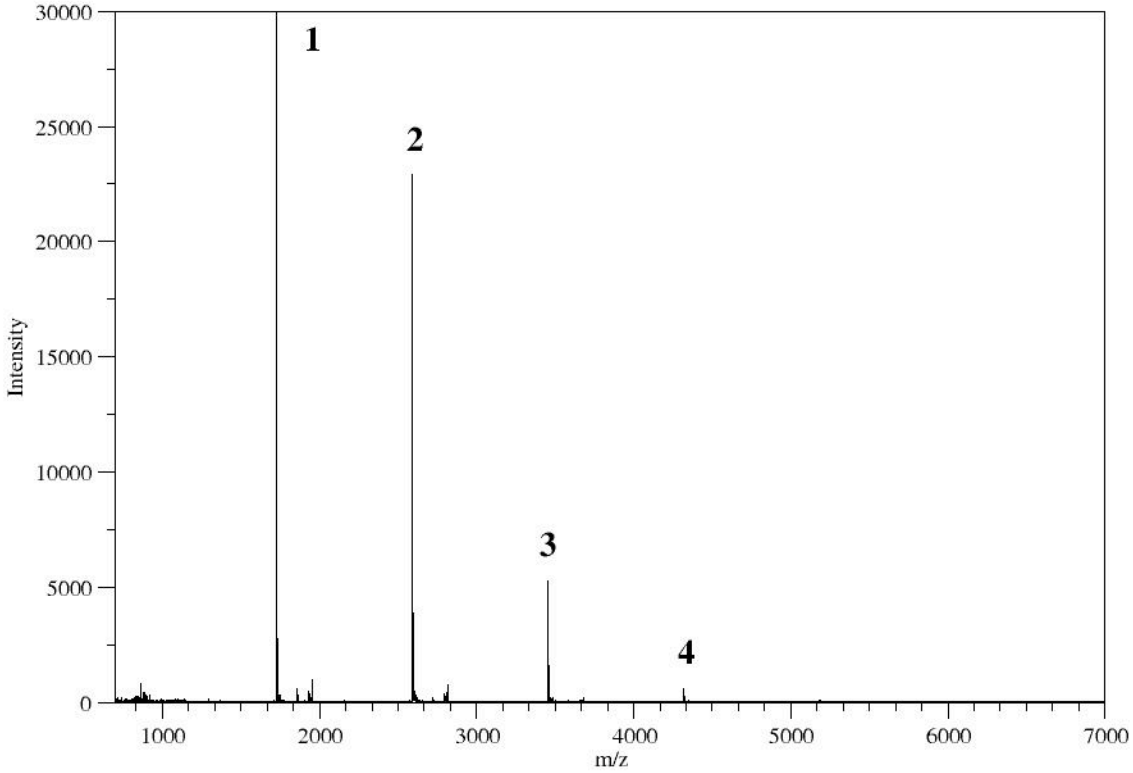


125 MHz ^{13}C NMR of compound **9**,
$N_3(CH_2)_5CO$-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-$NH(CH_2)_4CCH$

APPENDIX C

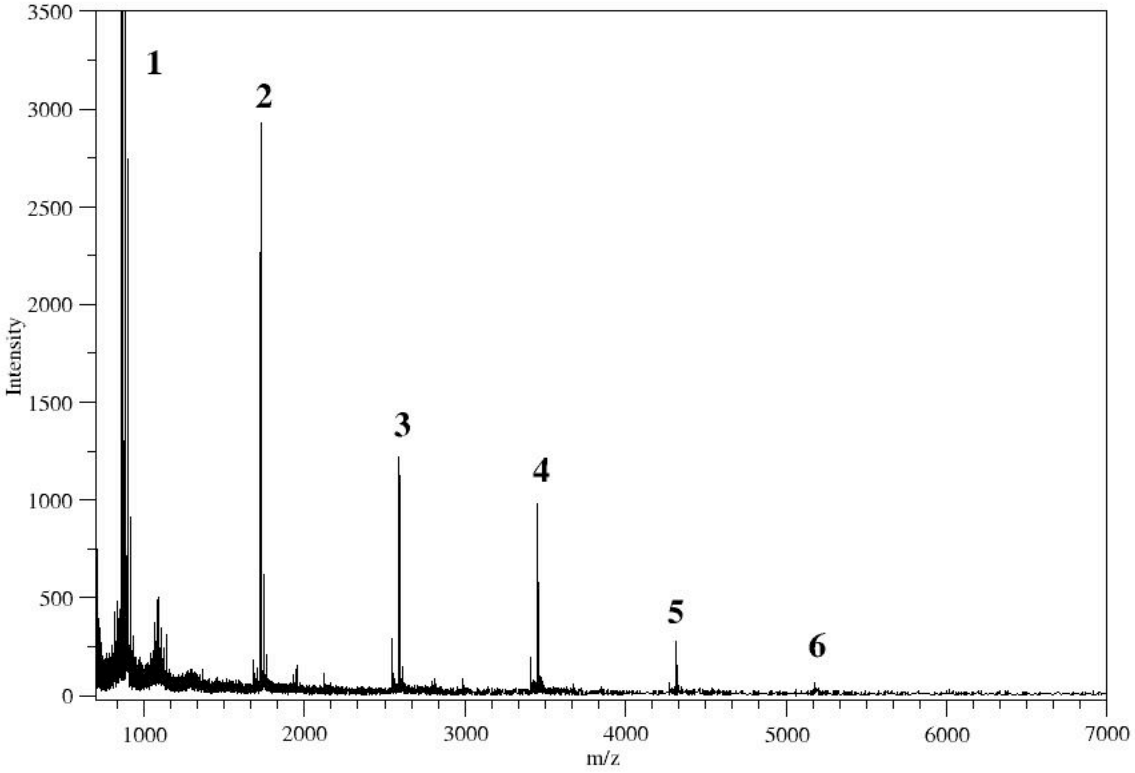
MS OF MULTIMERIZED SAMPLES

Multimerized Sample RS-1-100 post workup, ToF Mass Spectrum



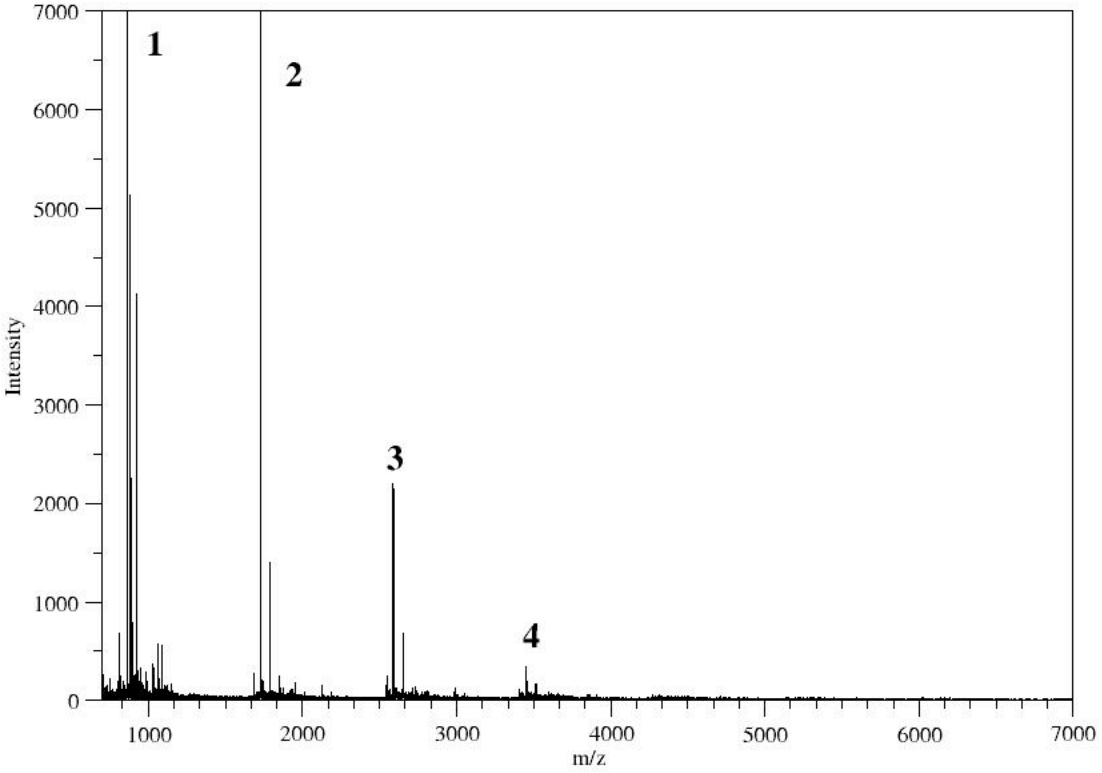
#	MSH(4) units	triazoles (uncyclized)	triazoles (cyclized)	$[M+H]^+$
1	2	1	2	1726
2	3	2	3	2589
3	4	3	4	3451
4	5	4	5	4314

Multimerized Sample RS-1-103 post workup, ToF Mass Spectrum



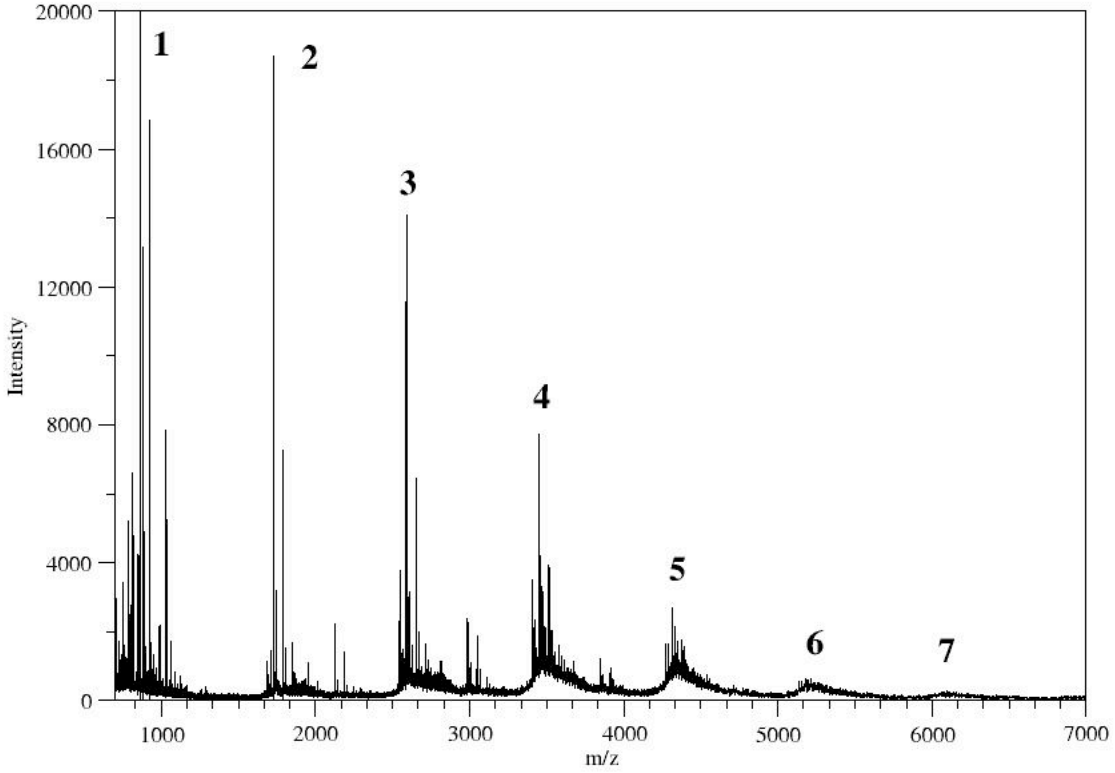
#	MSH(4) units	triazoles (uncyclized)	triazoles (cyclized)	$[M+H]^+$
1	1	0	1	863
2	2	1	2	1726
3	3	2	3	2589
4	4	3	4	3451
5	5	4	5	4314
6	6	5	6	5180

Multimerized Sample RS-1-104 post workup, ToF mass spectrum



#	MSH(4) units	triazoles (uncyclized)	triazoles (cyclized)	$[M+H]^+$
1	1	0	1	863
2	2	1	2	1726
3	3	2	3	2588
4	4	3	4	3451

Multimerized Sample RS-1-105 post workup, ToF Mass Spectrum



#	MSH(4) units	triazoles (uncyclized)	triazoles (cyclized)	$[M+H]^+$
1	1	0	1	863
2	2	1	2	1726
3	3	2	3	2588
4	4	3	4	3451
5	5	4	5	4313
6	6	5	6	5178
7	7	6	7	6061

REFERENCES

1. Kiessling, Laura L.; Gestwicki, Jason E.; Strong, Laura E. Synthetic Multivalent Ligands as Probes of Signal Transduction. *Angew. Chem. Int. Ed.* **2006**, *45*, 2348-2368.

2. Holub, Justin M.; Garabedian, Michael J.; Kirshenbaum, Kent. Peptoids on Steroids: Precise Multivalent Estradiol–Peptidomimetic Conjugates Generated via Azide–Alkyne [3+2] Cycloaddition Reactions. *QSAR Comb. Sci.* **2007**, *26*, 1175-1180.

3. Rosa Borges, A.; Schengrund, C.- L. Dendrimers and Antivirals: A Review. *Curr. Drug Tar: Infectious Disorders*. **2005**, *5*, 247-254.

4. MacNeil, Douglas J; Howard, Andrew D.; Guan, Xiaoming; Fong, Tung M.; Nargund, Ravi P.; Bednarek, Maria A.; Goulet, Mark T.; Weinberg, David H.; Strack, Alison M.; Marsh, Donald J.; Chen, Howard Y.; Shen, Chun-Pyn; Chen, Airu S.; Rosenblum, Charles I.; MacNeil, Tanya; Tota, Michael; MacIntyre, Euan D.; Van der Ploeg, Lex H. T. The Role of Melanocortins in Body Weight Regulation: Opportunities for the Treatment of Obesity. *Eur. J. Pharma.* **2002**, *450*, 93-109.

5. Smith, P. E. Experimental Ablation of the Hypophysis in the Frog Embryo. *Science*. **1916**, *44*, 280-282.

6. Sawyer, Tomi K.; Sanfilippo, Pauline J.; Hruby Victor J.; Engel, Michael H.; Heward, Christopher B.; Burnett, Jean B.; Hedley, Mac E. 4-Norleucine, 7-D-phenylalanine-α -melanocyte-stimulating Hormone: A Highly Potent α - melanotropin with Ultralong Biological Activity. *Proc. Natl. Acad. Sci. USA*. **1980**, *77*, 5754-5758.

7. Hruby, Victor J.; Wilkes, Brian C.; Hadley, Mac E.; Al-Obeidi, Fahad; Sawyer, Tomi K.; Staples, Douglas J.; DeVaux, Ann E.; Dym, Orin; de L. Castrucci, Ana M.; Hintz, Mary F.; Riehm John P.; Rao, K. Ranga. α-Melanotropin: The Minimal Active Sequence in the Frog Skin Bioassay. *J. Med. Chem.*, **1987**, *30*, 2126-2130.

8. Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; Al-Obeidi, F.; Staples, D. J.; de Vaux, A. E.; Dym, O.; Hintz, M. F.; Riehm, J. P.; Rao, K. R.; Hruby, V. J. α-Melanotropin: The Minimal Active Sequence in the Lizard Skin Bioassay. *Gen. and Comp. Endocrinology,* **1989**, 73, l57-163.

9. Haskell-Luevano, Carrie; Hendrata, Siska;North, Cheryl; Sawyer, Tomi K.; Hadley, Mac E.; Hruby, Victor J.; Dickinson, Chris; Gantzr, Ira. Discovery of Prototype Peptidomimetic Agonists at the Human Melanocortin Receptors MC1R and MC4R. *J. Med. Chem.* **1997**, *40,* 2133-2139.

10. Jagadish, Bhumasamudram; Sankaranarayanan, Rajesh; Xu, Liping; Richards, Reyniak; Vagner, Josef; Hruby, Victor J.; Gillies, Robert J.; Mash, Eugene A. Squalene-Derived Flexible Linkers for Bioactive Peptides. *Bioorg. & Med. Chem. Let.* **2007**, *17*, 3310-3313.

11. Huisgen, Rolf; Szeimies, Günter; Moebius, Leander. 1,3-Dipolare Cycloadditionen, XXXII. Kinetik der Additionen Organischer Azide an CC-Mehrfachbindungen. *Chem. Ber*. **1967**, *100*, 2494-2507.

12. Rostovtsev, Vsevolod V.; Green, Luke G.; Fokin, Valery V.; Sharpless, K. Barry. A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.

13. Lutz, Jean-François; Zarafshani, Zoya. Efficient Construction of Therapeutics, Bioconjugates, Biomaterials and Bioactive Surfaces Using Azide–Alkyne "Click" Chemistry. *Advanced Drug Delivery Reviews.* **2008**, *60*. 958–970.

14. Brown, Edward G.; Nuss, John M. Alkylation of Rink's Amide Linker on Polystyrene Resin: A Reductive Amination Approach to Modified Amine-Linkers for the Solid Phase Synthesis of N-Substituted Amide Derivatives. *Tet. Let.* **1997**, *38*, 8457-8460.

15. Carpino, Louis A.; Ghassemi, Shahnaz; Ionescu, Dumitru; Ismail, Mohamed; Sadat-Aalaee, Dean; Truran, George A.; Mansour, E. M. E.; Siwruk, Gary A.; Eynon, John S.; Morgan, Barry. Rapid, Continuous Solution-Phase Peptide Synthesis: Application to Peptides of Pharmaceutical Interest. *Org Proc. Res. & Dev.*, **2003**, *7*, 28-37.

16. Liu, Yaquin; Zhang, Lihui; Wan, Jieping; Li, Yesen; Xu, Yuhong; Pan, Yuanjiang. Design and Synthesis of cyclo[-Arg-Gly-Asp-ψ(triazole)-Gly-Xaa] Peptide Analogues by Click Chemistry. *Tetrahedron*., **2008**, *64*, 10728-10734.

17. Negishi, Ei-ichi; Boardman, Larry D.; Sawada, Hiroyuki; Bagheri, Vahid; Stoll, A. T.; Tour, James M.; Rand, Cynthia L. Novel Cycloalkylation Reactions of (ω-Halo-1-alkenyl)metal Derivatives. Synthetic Scope and Mechanism. *J. Am. Chem. Soc.*, **1988**, *110*, 5383-5396.

18. Böttcher, Thomas; Sieber, Stephan A. β-Lactones as Privileged Strutures for the Active-Site Labeling of Versatile Bacterial Enzyme Classes. *Angew. Chem. Int. Ed.* **2008**, *47*, 4600-4603.

19. Parrish, Bryan; Emrick, Todd. Soluble Camptothecin Derivatives Prepared by Click Cycloaddition Chemistry on Functional Aliphatic Polyesters. *Bioconjugate Chem.*, **2007**, *18*, 263-267.

20. Rozkiewicz, Dorota I.; Jan'czewski, Dominik; Verboom, Willem; Ravoo, Bart J.; Reinhoudt, David N. "Click" Chemistry by Microcontact Printing. *Angew. Chem. Int. Ed.,* **2006**, *45*, 5292 –5296.