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Progress Toward Controlled Disulfide Formation To Access Neuroactive Conotoxins

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PROGRESS TOWARD CONTROLLED DISULFIDE FORMATION TO ACCESS NEUROACTIVE *CONOTOXINS*

by

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DISSERTATION

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Advisor Date

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DEDICATION

I dedicate my dissertation work to my wonderful parents, Karunadasa and Anoma Kondasinghe who have made many sacrifices in their life to make me a better person. I could achieve this only because of your support, love and blessings. I also dedicate my work to my husband Manjula Jayawickrema for his endless love and continuous encouragement. Finally, I also dedicate this dissertation to Nuwan Kondasinghe, Ruchira Liyanage and Wathsala Bandara who have been my greatest strength and support.

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LIST OF ABBREVIATIONS

Acm	Acetamidomethyl
AcOH	acetic acid
ACN	acetonitrile
α	alpha
aq	aqueous
B.	Bacillus
BMC	*trans*-1,2-bis(2mercaptoacetamido) cyclohexane
Boc	*tert*-butoxycarbonyl
BPTI	bovine trypsin inhibitor
Bu_3SnH	tributyltin hydride
t-Bu	*tert*-butyl
Ca	calcium
°C	degrees Celsius
cat.	catalytic
calc'd	calculated
CCl_4	carbon tetrachloride
Cys	cysteine
DCM	dichloromethane or methylene chloride
DIPEA	iisopropylethylamine
DMF	dimethylformamide

DMSO	dimethylsulfoxide
dppf	1,1’-bis(diphenylphosphino)ferrocene
DTNB	5,5’-dithiobis(2-nitrobenzoic acid)
DTNP	2,2’-dithiobis(5-nitropyridine)
DTT	dithiothreitol
E.	Escherichia
equiv	equivalent(s)
ER	endoplasmic reticulum
ES-MS	electrospray ionization mass spectrometry
Et_3N	triethylamine
Et_3SiH	triethylsilane
Fmoc	fluorenymethyloxycarbonyl chioride
Fm	9-fluorenylmethyl
g	gram
GH	Glutathione
h	hour(s) or height
HCl	hydrochloric acid
HF	hydrogen fluoride
HPLC	high-performance liquid chromatography
I_2	iodine
K	potassium
$K_3Fe(CN)_6$	potassium ferricyanide

L	liter
LCMS	liquid chromatograph mass spectrometer
λ	wavelength
m	multiplet, meter, or milli
μ	micro or mu
*μ*W	microwave
M	mega, metal, or molar
m/z	mass to charge ratio
Me	methyl
Meb	p-methylbenzyl
Met	methionine
min	minute(s)
Mmt	4-methoxytrityl
Mob	4-methoxybenzyl
mol	mole(s)
mol%	percentage used based on moles
Na	sodium
nAChRs	nicotinic acetylcholine receptors
NCS	*N*-chlorosuccinimide
NET	norepinephrine transporter
NH_3	ammonia
NH_4OAc	ammonium acetate

NMM	N-methylmorpholine
NMP	N-methylpyrrolidinone
Npys	3-notro-2-pyridylsulfanyl
Pd	palladium
PDA	photodiode array detector
PDI	protein disulfide isomerase
PG	protecting group
pH	hydrogen ion concentration in aqueous solution
$Pd(OAc)_2$	palladium acetate
R	alkyl group
RNA	ribonucleic acid
RNase A	bovine pancreatic ribonuclease
sat.	saturated
sp.	Species
SPPS	solid phase peptide synthesis
S*t*Bu	*tert*-butylsulfanyl
$t_{1/2}$	half-life
temp	temperature
TEA	triethylamine
TFA	trifluoroacetic acid
TFMSA	trifluromethanesulfonic acid
THF	tetrahydrofuran

TIPS	triisopropylsilyl
Tmob	2,4,6-trimethoxybenzyl
Trp	tryptophan
Trt	triphenylmethyl
UV	ultraviolet
VGCCs	voltage-gated calcium channels
CGKCs	voltage-gated potassium channels
VGSCs	voltage-gated sodium channels
Vis	visual wavelength
Xan	9H-xanthen-9-yl

CHAPTER 1: INTRODUCTION TO PROTEIN FOLDING AND STRATEGIES TO DISULFIDE BOND FORMATION IN NEUROACTIVE PEPTIDES.

1.1 Disulfide Bonds in Proteins and peptides

In both proteins and peptides, disulfide bonds are formed as a reversible covalent bond between the side chain thiol groups of two cysteine residues. The cysteine amino acid residue is the only amino acid capable of forming disulfide bonds, and thus it become a unique residue among the proteinogenic amino acids.[1] Disulfide bonds enforce conformational constraints and stabilize the tertiary structure of peptides.[2] In addition, disulfide bonds prevent proteins from aggregation and subsequent degradation by cellular proteases, thus increasing their half-life.[3,4] The formation of disulfide bonds is a key post-translational modification in the folding and assembly of the extracellular domains of many membranes and secreted proteins.[5,6,7]

Most classes of extracellular proteins and peptides, including hormones, neurotransmitters, growth factors, toxins and enzyme inhibitors often have several disulfide bonds.[8] In nature, disulfide bonds are common in bacteria, microalgae, plant and animal peptide toxins.[9] Disulfide-rich mini-proteins derived from plants are known as cyclotides, which consist of 28-37 residues and display a cysteine knot motif composed of three disulfide bonds.[10,11] Cyclotides are exceptionally stable and serve a range of natural bioactivities such as antiviral and antimicrobial behaviors, which make them potential therapeutics.[12,13,14,15] The poisonous and venomous animals such as spiders, snakes, scorpions, shellfish, cone snails produce complex venom cocktails with thousands of unique bioactive compounds.[16,17] The disulfide-rich compact architectures

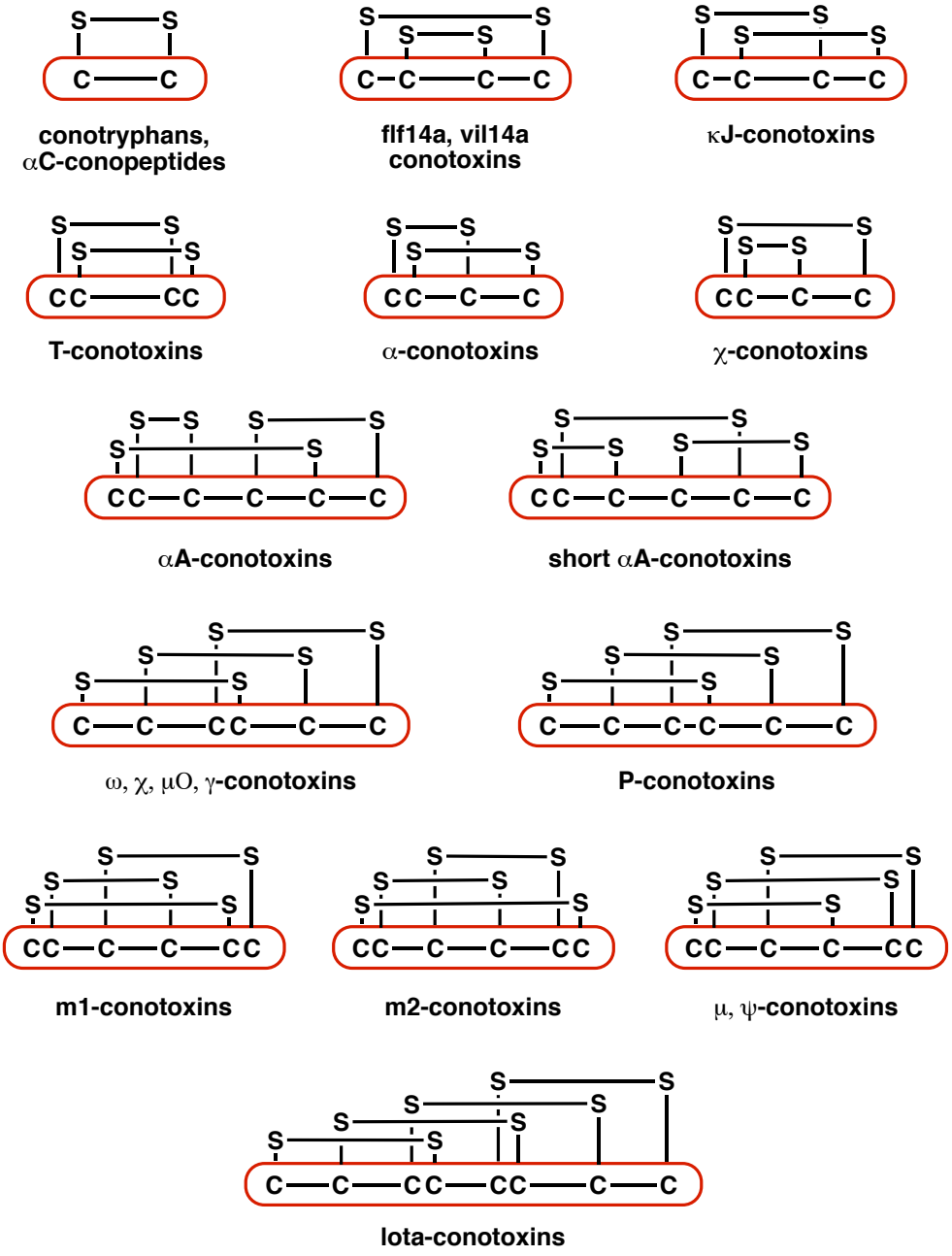
of these venoms make them resistant to extreme pH conditions and stable to high temperatures and organic solvents.[18,19] In addition, these disulfide-rich peptides have high binding affinity and specificity towards their cognate receptors, which make animal toxins extremely valuable pharmacological tools.[20,21,22]

Conus peptides are natural peptides with sufficiently complex and diverse disulfide bond patterns in their structure. Hence, in this study, among all toxins, conotoxins were chosen as excellent model system to study disulfide bond formation in peptides.

1.2 Conotoxins as Source of Disulfide-Rich Mini-peptides

Conotoxins are neurotoxic peptides isolated from venom of *Conus* marine snails.[23] They are mini-proteins consisting of 10 – 50 amino acid residues and typically have one or more disulfide linkages. Over 500 different species of cone snails have been identified and majority of them are carnivorous predatory sea snails and marine gastropod molluscs that use venom-based strategy to capture their prey.[24] All of the cone snails are venomous and produce conotoxins of 20 – 200 extremely complex components composed of modified peptides.[25] The exact composition of conotoxins varied widely from one species of cone snail to another; a rough estimate of the conotoxin diversity is that ~ 50,000 different peptides.[26] Generation of such a huge diversity of conotoxin is based on specific target macromolecules, mainly ion channels or receptors, and target animals, such as prey, predator or competitor. The huge diversity of conotoxins also evolved from various hyper mutations of the primary amino acid sequence, different cysteine patterns and disulfide scaffolds[27,28] (**Figure 1.1**).

Figure 1.1 Different cysteine patterns and disulfide scaffolds in conotoxins



Conotoxins have well-defined, compact structures, stabilized by different patterns of disulfide bonds. For two disulfide-bond conotoxins, either a ribbon or globular shape is more prominent.[29] For three disulfide-bond conotoxins, the structure varies from a globular shape to compact, cage-like structures.

Moreover, diversity of disulfide-bond mediated structural frameworks improve the stability, potency and selectivity of conotoxins.[30,31] Many of these conotoxins modulate the activity of ion channels. The action of conotoxins is fast, which can immobilize the injected prey by affecting its nervous system to cause massive membrane depolarization followed by an irreversible neuromuscular block.[32] This effect results from simultaneous inhibition of voltage-gated sodium and potassium channels.[33] This phenomena indicates the potential of conotoxins as inhibitors for specific ion channels (voltage-gated Na^+, K^+ and Ca^{2+} ion channels) and receptors (serotonin, acetylcholine) with high affinities and selectivities.

The conotoxins have been divided into a number of major classes based on their cysteine frameworks and physiologically relevant target (**Table 1.1**).[34] The high selectivity of conotoxins facilitates discrimination of closely related molecular isoforms of members of a particular ion channel family. For example, μ-conotoxins, which targets tissue specific sodium ion channel, inhibit voltage-sensitive sodium ion channels in muscles, with only minimum binding to neuronal sodium channel.[35] Some conotoxins show species-specific binding, where they have the ability to bind to same site of receptors, but produce different results. For example, δ-conotoxin such as conotoxin SVIA from *Conus striatus* bind with high affinity to voltage-independent sodium channels, and in contrast, *Conus purpurascens* which produce δ-conotoxin such as conotoxin PVIA have specific binding to voltage-sensitive channel.[36]

Table 1.1 Diversity conotoxins and their molecular targets

Conotoxins	Primary sequence	Molecular targets
A-superfamily		
α-GI	ECCNPACGRHYSC#	nAChRs
α-IMI	GCCSDPRCAWRC#	nAChRs
α-SI	ICCNPACGPKYSC#	nAChRs
T-superfamily		
χ−MrIA	NGVCCGYKLCHOC	NET
M-superfamily		
μ-GIIA	RDCCTOOKKCKDRQCKOQRCCA#	VGSCs
μ-PIIA	ZRLCCGFOKSCRSRQCKOHRCC#	VGSCs
μ-SmIIIA	ZRCCNGRRGCSSRWCRDHSRCC#	VGSCs
μ-SIIIA	ZNCCNGGCSSKWCRDHARCC#	VGSCs
μM-RIIIK	LOSCCSLNLRLCOVOACKRNOCCT	VGKCs
O-superfamily		
ω-MVIIA	CKGKCAKCSRLMYDCCTGSCRSGKC#	VGCCs
ω-MVIIC	CKGKGAPCRKTMYDCCSGSCGRRGKC#	VGCCs
ω-GVIA	CKSOGSSCSOTSYNCCRSCNOOYTKRCY#	VGCCs
μ-O-MrVIB	ACSKKWEYCIVPILGFVYCCPGLICGPFVCV	VGSCs
δ-SVIE	EASSSGGTFCGIHPGLCCSEFCFLWCITFID	VGSCs
δ-PVIA	EACYAPGTFCGIKPGGLCCSEFCLPGVCFG#	VGSCs
δ-TxVIA	CKQSGEMCNLLDQNCCDGYCIVLVCT	VGSCs

nAChRs - nicotinic acetylicholine receptors, VGSCs - voltage-gated sodium channels, VGCCs - voltage-gated calcium channels, VGKCS - voltage-gated potassium chennels, NET - norepinephrine transporter, O - hydroxyproline, Z - puroglutamatae, # - Amidated C-terminal,

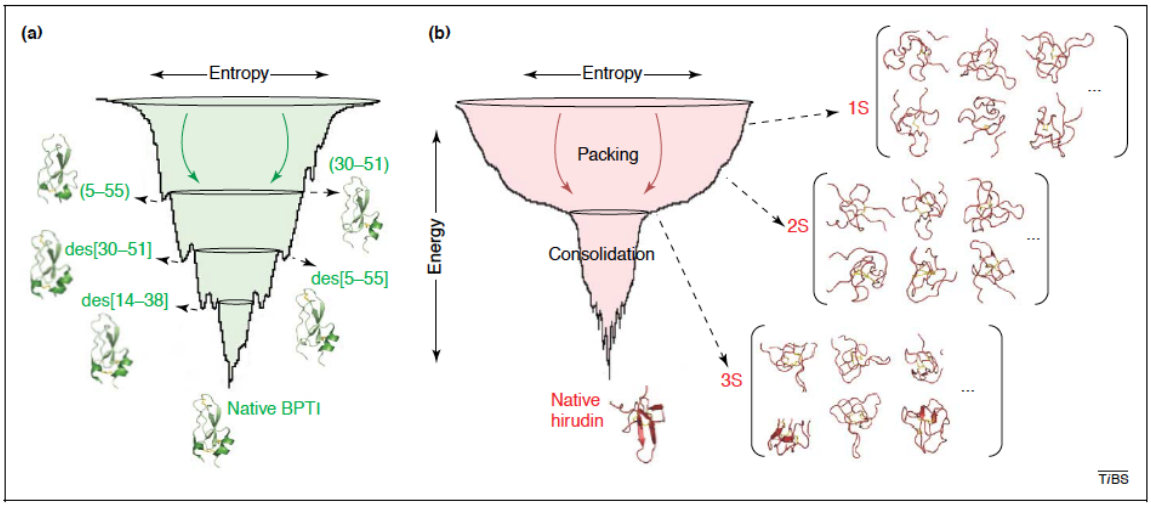
There are thousands of conotoxin-mediated selective bindings toward ion channels and receptors, which make conotoxins extremely valuable tools in biomedical research. The venom produced from *Conus magus* have shown promising activity as a non-addictive pain reliever 1000 times as powerful as morphine.[37] Furthermore, conotoxin AVCI from *Conus victoriae* has excellent potential for treating post-surgical and neuropathic pain, even accelerating recovery from nerve injury.[38] The first venom-derived painkiller, Ziconotide, was approved by the U.S. Food and Drug Administration in 2004 under the name "Prialt". It is an ω–conotoxin and acts by blocking calcium channels on the surface of the nerve cells that transmit the pain signal. Ziconotide can interfere with the transmission of pain signals within the spine, which aids to relieve pain.[29] There are many venom-based drugs in clinical and preclinical trials, which have potential in treatment of Alzheimer's disease, Parkinson's disease and epilepsy.[39]

1.3 Disulfide Bonds and Protein Folding

The mechanism by which the small, disulfide-rich peptides form their native disulfide bonds remain far from understood, and how these mini-proteins fold *in vivo* is one of the key unsolved problems in the field.[40] This is mainly due the challenges in identification and characterization of folding intermediates that are only very transiently present. However, majority of detailed in *vitro* folding studies have been done using model peptides such as BPTI,[41,42] Hirudin[43] and RNase A[44,45] These extensive studies have provided interesting insights for folding mechanism of disulfide-rich peptides. It was observed that most of the information needed to specify three-dimensional structure of a protein is encoded in its primary amino acid sequence and disulfide bonds greatly

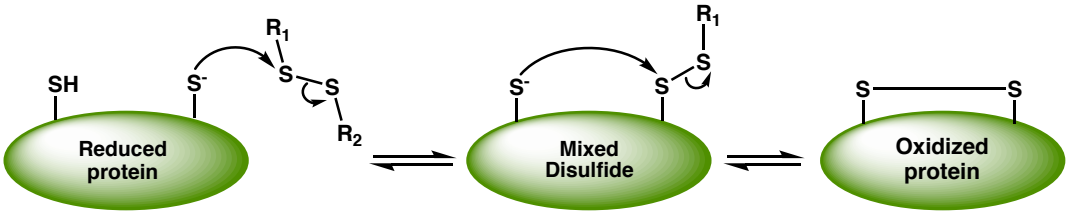
influence the thermodynamics of protein folding process.[46] Generally accepted method by which the disulfide bonds stabilize the native conformation of a protein is lowering the entropy of the unfolded form and making it less favorable compared to the folded form.[47] This process of protein folding can be seen as a folding funnel (**Figure 1.2**).[48]

Figure 1.2 Schematic overview of folding funnels for BPTI and Hirudin-like proteins



The folding process begins from a reduced, high energy, unfolded ensemble and the protein molecules work their way down a funnel of decreasing energy. For proteins such as BPTI, a limited number of native-like intermediates adopt protein conformation towards the native structure (**(a). Figure 1.2**). Hirudin-like proteins fold through an initial stage of disulfide bond formation, process known as packing, followed by the reshuffling of scrambled isomers to form the native protein (**(b). Figure 1.2**). Disulfide bonds in proteins are formed by two thiol exchange reactions (**Scheme 1.1**). First, the ionized thiolate form reacts with one of the sulfur atoms in the redox reagent to form a mixed disulfide bond between the protein and redox reagent. Then, the remaining thiol anion in the protein attacks the mixed disulfide to form the oxidized protein.[49]

Scheme 1.1 Thiol-disulfide exchange reaction between a protein and a redox reagent



The kinetics of protein folding are greatly affected by the location of disulfide bond relative to the folding nucleus. When disulfide bonds are in or near the folding nucleus, it can accelerate the folding process, whereas when disulfide bonds are far away from the folding nucleus, the folding process can decelerate up to three order of magnitude.[50] In addition, accessibility, proximity and reactivity of the thiol groups and disulfide bonds influence the formation of disulfide bonds.[51]

Disulfide bond formation is one of the major rate-limiting steps in protein folding in *vivo*. Therefore, protein folding catalysts called oxidoreductases are required to form disulfide bonds in *vivo*. Oxidoreductases are consist of catalytic disulfides at the active site and these disulfides are serve as the oxidant for disulfide formation. The reduced form facilitates the reduction or isomerization of disulfide bonds. In addition, oxidoreductases accelerate oxidative folding by eliminating misfolding in protein folding.[52] Formation of disulfide bonds in the bacterial periplasm has been extensively studied over past decades.[53] Those studies reveal that disulfide bonds in the periplasm of *Escherichia coli* are introduced by the Dsb family of proteins. The family includes DsbA and DsbB, which are involved in disulfide bond formation, and DsbC and DsbD, which are involved in disulfide bond isomerization.[54] In eukaryotic cells, disulfide bond formation occurs in the lumen of the endoplasmic reticulum (ER) and two proteins, protein disulfide isomerase (PDI) and Ero1p, are primarily responsible for controlling the process of oxidative folding.

These folding catalysts reduce and shuffle non-native disulfide bonds at early stages of folding to avoid misfolding.[55] As mentioned earlier, there is little known about the *in vivo* folding of small, disulfide-rich proteins, hence more future research should be focused on analyzing disulfide-mediated folding processes under physiological conditions.

With the attractive potential of conotoxin-like small, disulfide rich-proteins as valuable tools in pharmacological and biological studies, there is a huge need for isolation and/or synthesis of these bioactive molecules. As mentioned earlier in this chapter, the marine snails produce relativity small quantities of the toxins, and they are a complex mixture of thousands of bioactive compounds. Therefore, isolation of each interested bioactive compound is very difficult and the amount recovered after lengthy isolation protocol is not sufficient for those experimental studies. Moreover, production of these disulfide-rich bioactive peptides by recombinant methods using suitable host organisms is very challenging. The major obstacles are low expression rates, high susceptibility for degradation by the host cell proteases and a significant toxicity for the host organism.[56] In order to access these disulfide-rich mini-proteins in large quantities, many methods have been developed to perform chemical synthesis of these bioactive molecules.

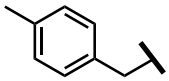
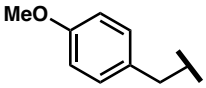
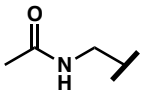
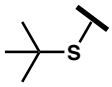
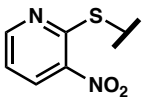
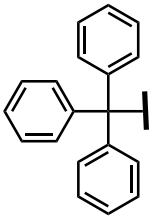
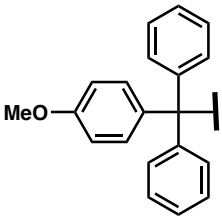
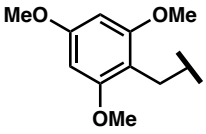
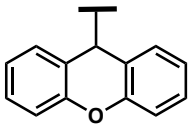
1.4 Chemical Synthesis of Disulfide-Rich Mini-proteins

The *in vitro* studies of protein folding have given information to develop suitable strategies to chemically access disulfide-rich mini proteins. From Anfinsen's *in vitro* studies, it is accepted that an intrinsic folding code in primary amino acid sequence of a protein determines its correct folding pattern.[41] This implies that as long as we can synthesize the correct primary amino acid sequence, generation of the native 3D structure

of protein is possible with suitable folding methods. This concept opened up a huge area of chemical synthesis of proteins and several methods have been developed to access disulfide-rich mini-proteins.

The first chemical synthesis of conotoxins were carried out in early to mid 1980s and since then, at least a hundred of conotoxins have been synthesized.[57,58,59,60] The primary amino acid sequence is made either by Fmoc or Boc chemistry with thiol protected cysteines using several thiol-protecting groups (**Table 1.2**). There are several methods used to synthesize disulfide-rich peptides and these methods can be categorized into three broad sections based on how the linear peptide or reagents are used in the synthetic process; either in solution or on a solid support. They are namely 1) solution-phase oxidation 2) on-resin oxidation and 3) oxidation via solid-supported oxidants. When performing each of these strategies, a special attention needed to be taken to optimize variables in the folding reaction, such as peptide concentration, temperature, folding time, folding additives and solvents to increase the folding efficiency.[61,62,63]

Table 1.2 The most common thiol protecting groups used in peptide synthesis

Protecting Group	Structure	Stability	Removal
4-methylbenzyl (Bzl(4-Me))		TFA, Ag(I), base, RSCl	HF (0 °C),Tl(Ii), $Ph_2SO/MeSiCl_3$
4-methoxybenzyl (Mob)		TFA, base, RSCl, I_2	HF (0 °C), Ag(I), Hg(II), Tl(III), $Ph_2SO/MeSiCl_3$
tert-butyl (*t*Bu)		TFA, HF (0 °C), Ag(I), I_2	HF (0 °C), Hg(II), RSCl, $Ph_2SO/MeSiCl_3$
Acetamidomethyl (Acm)		TFA, HF (0 °C), base	I_2, Ag(I), Hg(II), RSCl, Tl(III), $Ph_2SO/MeSiCl_3$
tert-butylsulfanyl (S*t*Bu)		TFA, HF (partial), base, RSCl	RSH, Bu_3P, $(HOOC\text{-}CH_2\text{-}CH_2)P$
3-nitro-2-pyridylsulfanyl (Npys)		HF	RSH, Bu_3P, $(HOOC\text{-}CH_2\text{-}CH_2)P$
triphenylmethyl, trityl (Trt)		base, nucleophiles	dil.TFA/scavenger, I_2, Ag(I), Hg(II), Tl(III), RSCl
4-methoxytrityl (Mmt)		base, nucleophiles	dil.TFA/scavenger, I_2, Ag(I), Hg(II), Tl(III)
2,4,6-trimethyoxybenzyl (Tmob)		base, nucleophiles	dil.TFA/scavenger, I_2, Tl(III)
9H-xanthen-9-yl (Xan)		base, nucleophiles	dil.TFA/scavenger, I_2, Tl(III)

1.4.1 Solution-Phase Synthesis of Disulfide-Rich Peptides

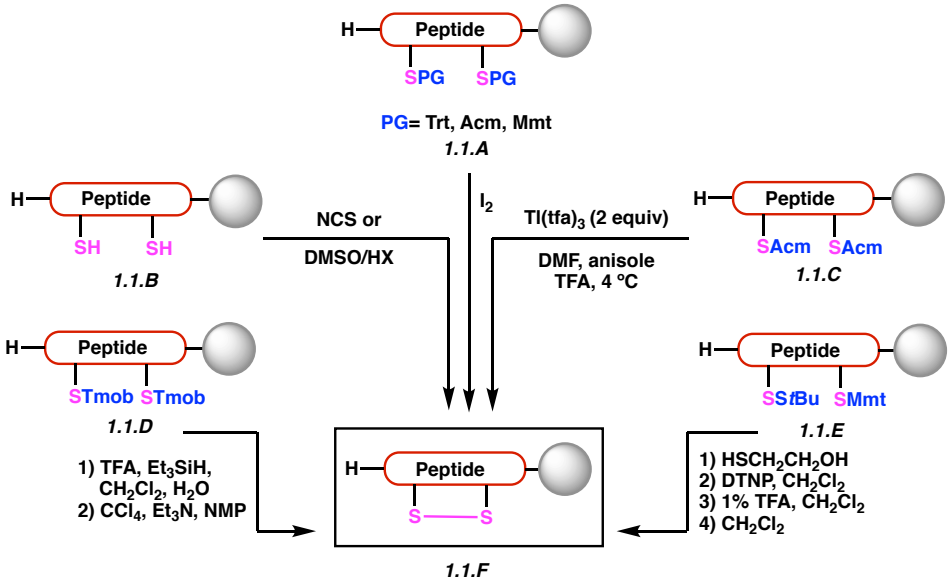
Solution-phase oxidation is the most common method to form intramolecular disulfide bonds in peptides.[51,52,64,65,66] The linear synthesis of the peptide is assembled via solid phase peptide synthesis (SPPS), and the peptide folding is performed after cleavage of the linear peptide from the resin. Solution-phase disulfide bond formation requires highly dilute conditions such as 20 – 50 μM concentrations of peptide solution. In solution, there is a competition between intramolecular disulfide bond formation and intermolecular disulfide bond formation. At higher peptide concentrations, the effective concentration of intermolecular thiol becomes relatively higher, which facilitates dimerization and oligomerization of the peptide and lowers the folding yields.[67] Numerous oxidants have been exployed to perform solution-phase peptide folding.[68] Common examples of such oxidants are, glutathione, cysteine, cystamine, DMSO, *trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC) and molecular oxygen. Glutathione-based redox buffers are common in solution-phase disulfide bond formation methods as they are hypothesized to better reflect oxidation pathways *in vivo*.[69,70] The folding efficiency and extent of oxidation are influenced by the ratio of GS-SG to GSH.[71] Dilute dimethyl sulfoxide with final concentration of 5 – 10% is another common oxidant, often requiring longer folding times.[72] The major drawback of DMSO oxidation is the difficulty of removal of excess DMSO from the folded product. Molecular oxygen is also used to form disulfide bonds in peptides and oxygen is drawn from either air or oxygen atmosphere for the folding process. Oxygen-mediated peptide folding method relies on low levels of oxygen dissolved in solution. Thus, the method is relatively slow.[67] When performing multistep

folding procedures in solution-phase, in addition to the final product, the intermediate products from each step usually need to be purified before the next step. Multiple purification steps can diminish the final yield of the folded peptide.

1.4.2 On-resin Synthesis of Disulfide-Rich Peptides

High dilution conditions in solution-phase folding protocols introduce problems with larger scale and high throughput folding. To avoid this issue, peptide disulfide bond formation can be performed on solid support, requiring less solvent. After the linear synthesis of the target peptide on resin, the folding procedures are carried out while the peptide is still attached to the resin. The reagents are easily filtered out after the synthesis and the resulting folded product can be cleaved form the resin and purified. Therefore, this method does not need time-consuming and wasteful purification steps. When disulfide is formed on a resin with medium to low loading, a phenomenon called pseudo-dilution[73] is generated within the reaction core, which ensure that the molecule of peptide is isolated. This favors intramolecular disulfide bond formation over intermolecular disulfide bond formation, minimizing formation of dimers and oligomers as by products. Several methods have been developed to form disulfide bonds in resin-bound peptides using suitable cysteine protecting groups and reagents, which are compatible with resin-based reaction conditions. Few of them are summarized in **Scheme 1.2**. One of the traditional approaches to access disulfide bonds on resin is the iodine oxidation. On-resin peptides with Trt, Acm or Mmt protected cysteines (**1.1.A**) are treated with excess iodine.[74,75]

Scheme 1.2 Known methods for on-resin disulfide formation



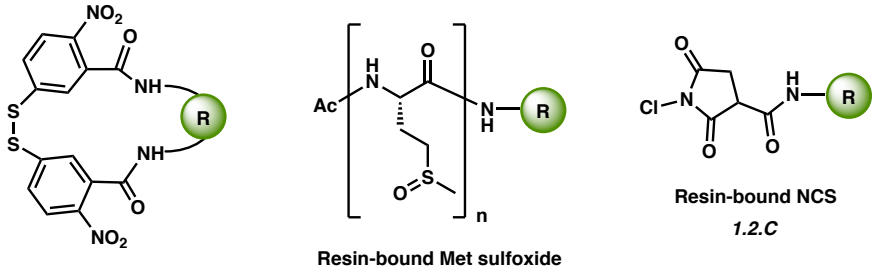
This is a convenient method to form on-resin disulfide linkages, but it cannot always be employed as peptides containing Met or Trp amino acids are prone to oxidation and peptides on TGT and CTC resins can be cleaved under iodine oxidation condition.[76] In addition to iodine oxidation, $Tl(tfa)_3$ can be used on peptides **1.1.C** with Acm protected cysteine to directly yield the product with desired disulfide bond. This approach is not orthogonal to other cysteine protecting groups or acid-labile side chain protecting groups, and it can induce cleavage of peptides from TGT and CTC resins.[77] On-resin oxidation of free thiols, **1.1.B,** can be achieved using either *N*-chlorosuccinimide[78] or acidic DMSO (HX = HCl or HOAc).[79] Cysteines with benzyl derived protecting groups, **1.1.D**, need 2-step process to form disulfide bonds. First, low concentration of TFA is used to the remove benzyl group, and then, the resulting free thiols are oxidized with CCl_4/TFA to form corresponding disulfide bond. This method is not compatible with Cys(Mmt), Cys(Trt) or TGT and CTC resins as TFA can cause cysteine deprotection and/or peptide cleavage. The multistep approach with S*t*Bu and Mmt protected cysteines, **1.1.E**, was developed to

improve the purity of peptides.[80] The disulfide bond formation takes place based on the sequence of two disulfide exchange reactions and no oxidizing agent is needed. First, the on-resin peptide is treated with mercaptoethanol to remove S*t*Bu group by reduction to expose free thiols. Then, the peptide undergoes first disulfide exchange reaction when treated with excess DTNP to activate the free thiol with 5-Npys group. Deprotection of Cys(Mmt) group initiates second disulfide exchange reaction, where the nucleophilic attack of free thiol on the resin-bound 5-Npys-activated hetero disulfide leads to the desired folded product. This approach is very effective and widely used. The reaction can also be carried out under microwave heating to decrease the reaction time.[81] Current limitations in on-resin disulfide bond formation are often poor yield recovery cause by undesired polymerization or disulfide interaction with the resin.[82]

1.4.3 Disulfide Bond Formation via Solid-Supported Oxidants

Another solution for the issues associated with high dilution conditions in solution-phase disulfide bond formation is use of solid-supported oxidants. A variety of solid-supported oxidants have been explored using different types of polymers/resins such as hydrocarbon-based resins,[83] agarose-bound folding chaperones,[84] PEG-PS, Sephadex, controlled pore glass[85] and cross-linked ethoxylate acrylate resin (CLEAR)[86] (**Figure 1.3**). As in on-resin disulfide bond formation, the low loading of the resin takes advantage of the pseudo-dilution effect and favors the intramolecular disulfide bond formation. In addition, the reagent can be easily filtered off upon completion of disulfide bond formation.

Figure 1.3 Soild-supported oxidants for disulfide bond formation in peptides



Resin-bound Ellman's Reagent
1.2.A

Resin-bound Met sulfoxide
1.2.B

Resin-bound NCS
1.2.C

Polymer-bound version of Ellman's reagent (**1.2.A**) can be made by linking the oxidant to a polymer matrix through a lysine residue.[86] When a solution of reduced peptide is treated with solid-supported Ellman's reagent, the reduced peptide is temporarily immobilized on to the solid-supported disulfide, generating a peptide – resin disulfide intermediate that is released again upon intramolecular disulfide-bond formation. The Clear resin preloaded with Ellman's reagent called Clear-OX[69] has been successfully utilized in the synthesis of many conotoxins.[66,87] The drawback of this method is lower yield of recovery due to the covalent trapping of the peptide within the resin.

The solid-supported Met sulfoxide (**1.2.B**), which is known as Oxyfold, can be readily prepared on aminopoly(ethylene glycol)polyacrylamide (PEGA) resin by methionine *N*-carboxyanhydride polymerization initiated by free amine groups on the resin.[88] The disulfide formation of resin-bound Met sulfoxide is analogous to DMSO oxidation, and therefore, it can be used as a substitute of DMSO. As mentioned earlier, the major drawback of DMSO oxidation is the difficulty in removal of excess DMSO from the oxidized product. The resin-bound Met sulfoxide does not have this disadvantage as it can be removed by simple filtration. The efficiency of the resin-bound Met sulfoxide can be increased by generating longer Met sulfoxide chains, which increases the number of

Met sulfoxide groups per gram of resin.[73] Besides the longer reaction time, the resin-bound Met sulfoxide oxidation shows quantitative formation of the disulfide bonds with no dimer formation, and hence, it can be used to get high yields for more complex or valuable peptide sequences.[89]

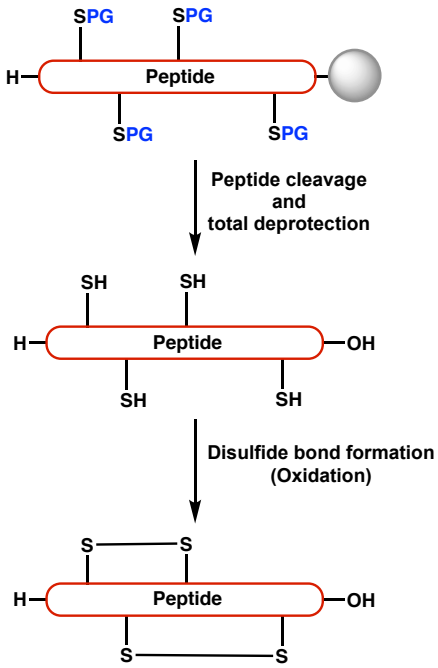
N-chlorosuccinimide can also be immobilized on the versatile poly(ethylene glycol)-based ChemMatrix resin (**1.2.C**). The resin-bound NCS can be used in either aqueous or organic media and have shown efficient reactivity to form disulfide bonds on peptides.[90]

1.4.4 Thermodynamically controlled Folding Strategy for Disulfide Bond Formation in Peptides

The classic work of Anfinsen and co-workers towards *in vitro* protein folding[41] inspired the development of a thermodynamically controlled oxidative folding strategy to access disulfide-rich bioactive mini-proteins. This approach involves random oxidation, reduction and reshuffling of the fully deprotected peptide to the native isomer (**scheme 1.3**). Disulfide bond formation is performed mainly in solution phase and all the cysteine groups are usually protected with a single protecting group during the SPPS. Common folding conditions include slightly alkaline aqueous or aqueous/organic buffers, such as GSH/GSSG buffer. In addition, DMSO-promoted oxidation is done frequently. Most recently, Clear-OX was successfully used in the oxidative folding of three disulfide-bridged conotoxins such as μ-SIIIA, μ-KIIIA, ω-GVIA.[72] As the name implies, disulfide bond connectivity in this method is not controlled by reagents. Therefore, the final yield and disulfide connectivity is highly dependent on the encoded structure information built-

in the primary amino acid sequence and thermodynamic stability of the native conformation.[91,92,93]

Scheme 1.3 Thermodynamically controlled disulfide bond formation



This can result in many non-native or misfolded products in addition to the native product. Although the method requires only one purification step, separation of the native product from all the non-native products is very challenging. Despite its disadvantages, this is still the most commonly use approach to access peptides, especially when the native configuration is substantially thermodynamically favored.

1.4.5 Regioselective Folding Strategy for Disulfide Bond Formation in Peptides

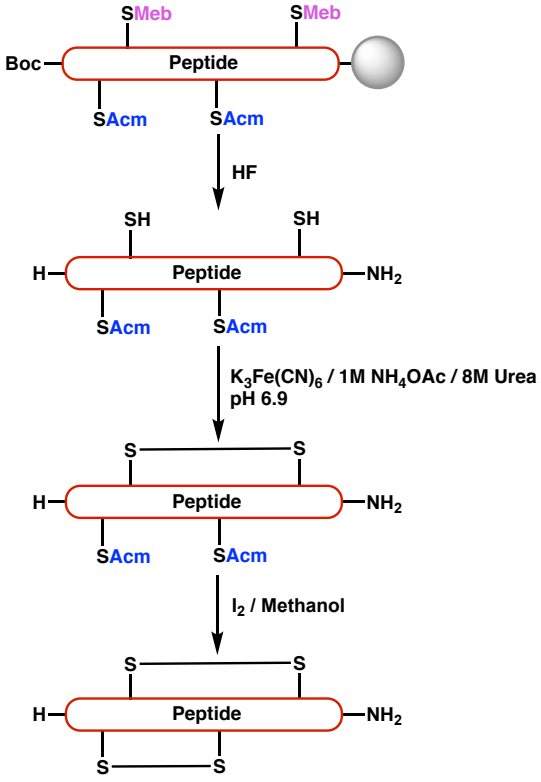
The regioselective folding approach is a step-by-step process to form multiple disulfide bonds in desired pattern. The strategy is based on selective deprotection and oxidation of each cysteine pair in the peptide. As this is a step-wise process, reshuffling of disulfide bonds can take place by the breakage of disulfide bonds that already formed. Hence, exposure to alkaline conditions, thiols and other nucleophiles and lengthy reaction

times should be avoided to prevent competitive disulfide-bond disproportionation, which can lower the product recovery. Most importantly, regioselective folding strategies require careful planning and designing of the synthetic route by analyzing the orthogonal behavior of cysteine protecting groups. Regioselective disulfide bond formation can be conducted either in solution phase, after the peptide been cleaved from the resin, or solid phase, while the peptide is still attached to the resin.

1.4.5.1 In-Solution Regioselective Disulfide Bond Formation in Peptides

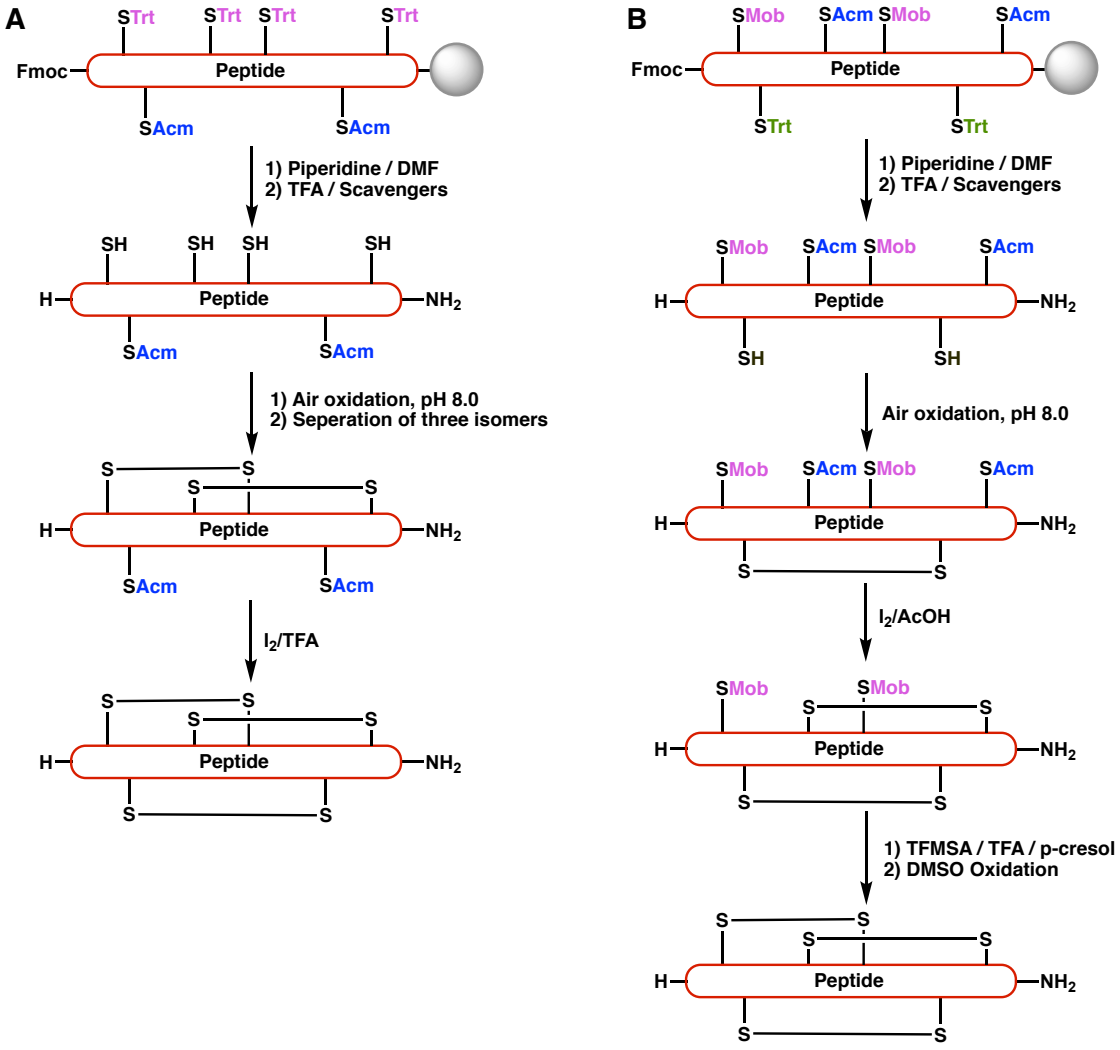
Nishiuchi and Sakakibara demonstrated the use of Acm protected cysteine in combination with Trt protected cysteine to form disulfide bonds in α-conotoxin GI using Boc chemistry (**Scheme 1. 4**).[94] Since then, Acm group has been widely used in the synthesis of disulfide bonds in peptides. As Acm group is resistant to TFA or HF cleavage, the linear peptide can be cleaved form the resin together with all other protecting groups without affecting the Acm group. In the synthesis of α-conotoxin GI, the resulting free thiols from HF cleavage were oxidized by mild oxidant, ferricyanide, to form the first disulfide bond. The second disulfide bond formation was then achieved after Acm group removal and oxidation by iodine oxidation.

Scheme 1.4 Regioselective folding strategy for synthesis of α-GI using Cys(Acm) and Cys(Meb)



The utility of S-Acm protection strategy to synthesize conotoxins containing three disulfide bonds has also been explored. A semi-directed method was used to synthesize ω-conotoxin MVIID using Acm group in combination with Trt group to protect pairs of cysteine residues (**Scheme 1.5.A**).[95] During the resin cleavage all the protecting groups get cleaved except for the Acm group, forming two pairs of reduced cysteine residues. Upon treatment with air, the reduced peptide undergoes a random oxidation to form first two disulfide bonds. This step gave predominantly a single isomer which reflects the efficiency of the synthetic design. The second disulfide linkage was formed by simultaneous Acm group removal and oxidation using iodine-mediated oxidation condition.

Scheme 1.5 **A.** Semi-directed folding strategy for synthesis of ω-MVIID using Cys(Acm) and Cys(Trt) **B.** Regioselective folding strategy for synthesis of ω-MVIIA using Cys(Acm), Cys(Mob) and Cys(Trt)

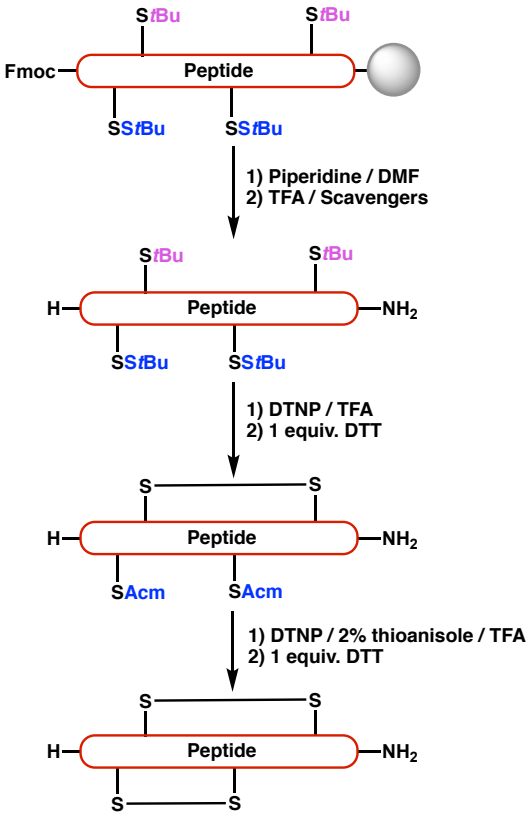


The synthesis of ω-conotoxin MVIIA, which is a three disulfide bond containing peptide, has been done in a more selective approach using Acm, Mob and Trt protected cysteines.[96] It allows a step-by-step oxidation by selective deprotection and oxidation of each pair of cysteine groups. First, selective Cys(Trt) deprotection was achieved together with total deprotection by TFA treatment. Then, air oxidation was performed to oxidize the free thiols to access the first disulfide bond. Iodine oxidation ensures selective cleavage of Acm and oxidation to introduce the second disulfide bond in the peptide. The fully

folded peptide was completed by deprotection of Cys(Mob) to get the reduced cysteines followed by DMSO oxidation of free thiols.

A method was reported in which DTNP was used to remove several commercially available protecting groups.[97] In the regioselective synthesis of apamin, no Acm protection was applied, S*t*Bu and *t*Bu groups were used to protect cysteine residues (**Scheme 1.6**). DTNP was used to deprotect both S*t*Bu and *t*Bu groups, and the selectivity of one group removal over the other was achieved by the presence or absence of thioanisole in the reaction solution. As S*t*Bu group was found to be stable in DTNP reaction condition without thioanisole, Cys(*t*Bu) deprotection was done first using DTNP in absence of thioanisol.

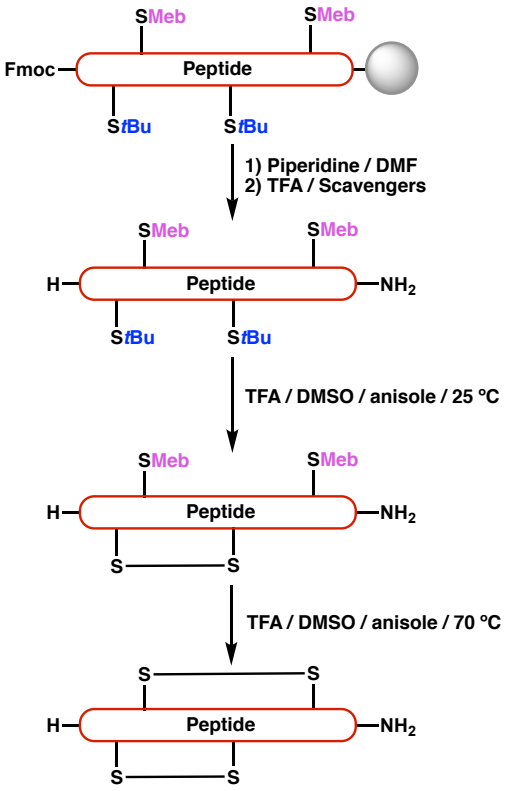
Scheme 1.6 Regioselective DTNP-mediated strategy for synthesis of apamin using Cys(*t*Bu) and Cys(S*t*Bu)



A bis-Npys-protected peptide was made upon removal of *t*Bu group with DTNP, addition of DTT facilitated the first disulfide bond formation instantaneously. After purifying the mono-cyclic peptide, it was subjected to the same reaction condition, but with thioanisole, to deprotect Cys(S*t*Bu) and form second disulfide bond in the target peptide.

Although most of the regioselective folding approaches have multiple disulfide formation steps with a distinct set of reagents for each step, one-pot regioselective method has been developed to synthesize α-conotoxin SI.[98] (**Scheme 1.7**). In this one-pot regioselective approach, combination of Meb and *t*Bu protected cysteines were used, and synthesis was designed based on the difference temperature dependence of these groups towards DMSO / TFA / anisole oxidation.

Scheme 1.7 Regioselective in-solution folding strategy for synthesis of α-SI using Cys(Meb) and Cys(*t*Bu)

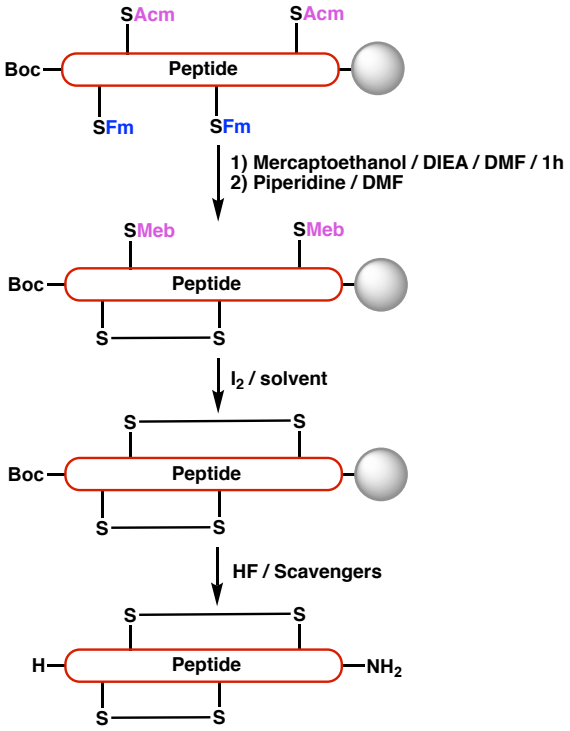


At room temperature, *t*Bu protected cysteines rapidly get converted to the corresponding disulfide bond without affecting Meb group removal. Subsequent heating of the same reaction solution facilitates the deprotection and oxidation of Cys(Meb) groups to form the second disulfide bond.

1.4.5.2 On-resin Regioselective Disulfide Bond Formation in Peptides

As an on-resin strategy, the regioselective disulfide formation is done while the peptide is still attached to the resin. The specific methods of on-resin disulfide formation (see Section 1.4.2) can be combined carefully, based on their orthogonality towards other excising cysteine protecting groups, to control formation of each disulfide bond in the peptide. One of the examples is use of Cys(Fm) in combination with Cys(Acm) to synthesize α-conotoxin GI using Boc chemistry **(Scheme 1.8)**.[99]

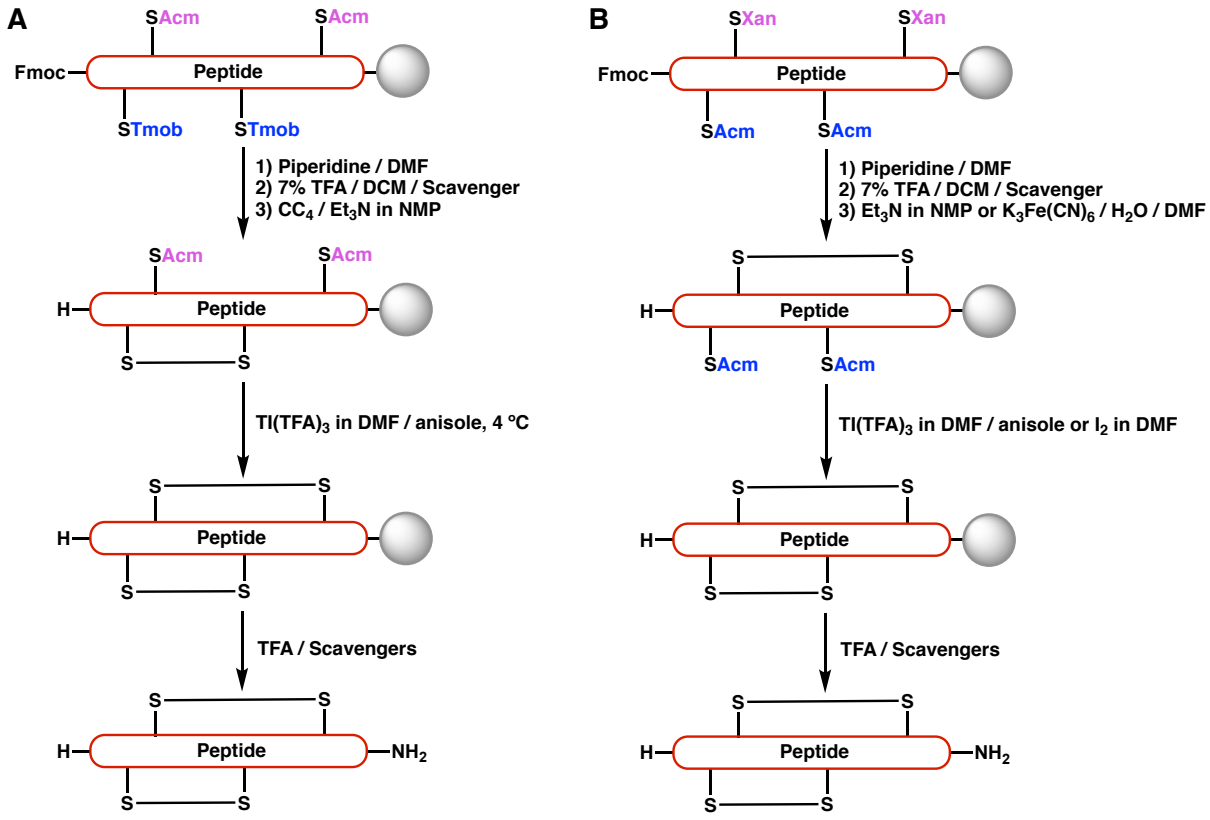
Scheme 1.8 Regioselective on-resin folding strategy for synthesis of α-GI using Cys(Acm) and Cys(Fm)



In this synthetic route, piperidine in DMF was used to base-labile Fm group and iodine-mediated oxidation was used for simultaneous Acm removal and oxidation. Final peptide cleavage and side group deprotection has to be done using HF condition. This step is challenging as disulfide bonds are found to be unstable under highly acidic condition. Therefore, careful selection of scavengers is critical for isolation of the native isomer in good yields.

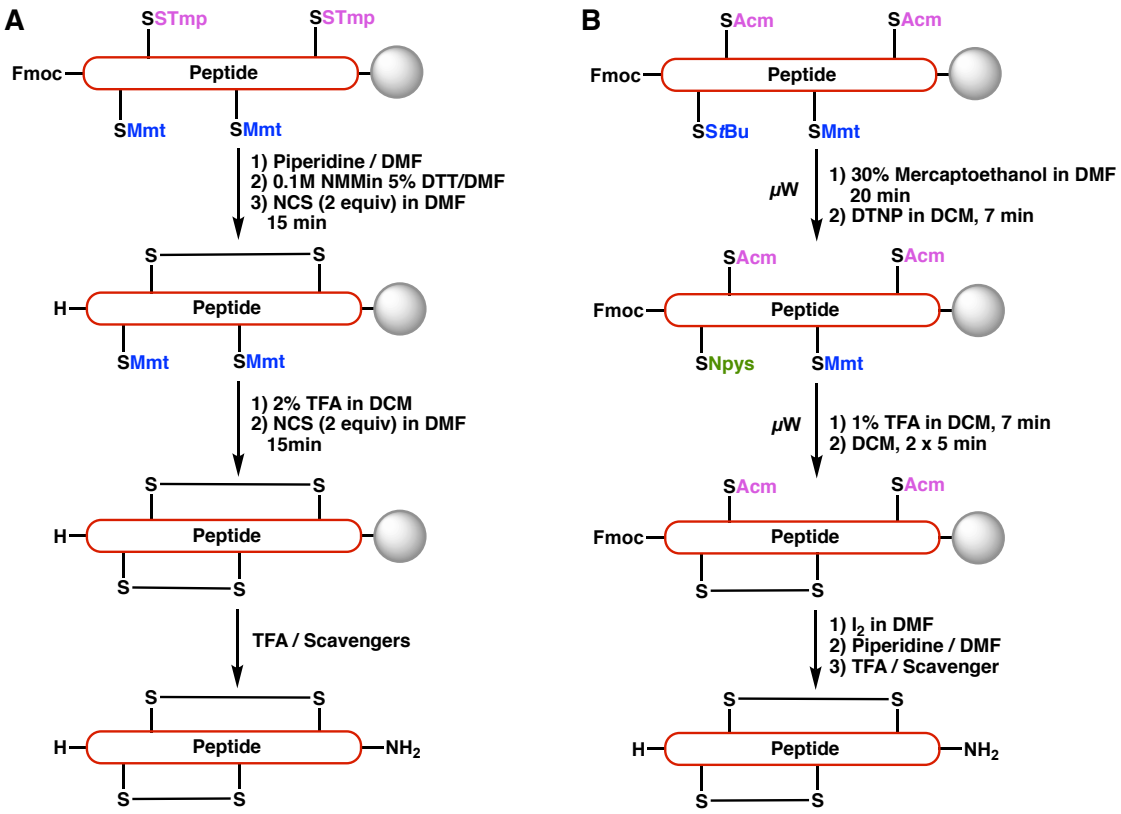
Fmoc chemistry can be carried out as an alternative method to avoid HF conditions in the synthesis of disulfide bonds. Two different approaches have been reported to synthesize α-conotoxin GI by Fmoc chemistry (**Scheme 1.9**). The first approach (**Scheme 1.9.A**) was done using Tmob group in combination with Acm group for cysteine protection.[100] The challenging step in this protocol is deprotection of Cys(Tmob) residue, as it requires TFA conditions, which can affect the peptide cleavage from resin. Therefore, this step needs to be run carefully to avoid peptide loss. The resulting free thiols can be oxidized with CCl_4-Et_3N in NMP to form the first disulfide bond without Acm group removal. Second disulfide bond formation can be done by oxidation of Acm group with $Tl(TFA)_3$ in DMF in presence of anisole as the scavenger. In the second approach (**Scheme 1.9.B)**, the orthogonal combination of Acm and Xan group was used to obtain a selective disulfide bond formation.[101] The deprotection and selective disulfide bond formation conditions were similar to that of approach **A** in Scheme 1.9. These methods illustrate the utility of orthogonal combination of Acm group with various other cysteine protecting groups to access multiple disulfide bonds on resin.

Scheme 1.9 Regioselective on-resin folding strategy for synthesis of α-SI using; **A.** Cys(Acm) and Cys(Fm) **B.** Cys(Acm) and Cys(Xan)



N-chlorosuccinimide-mediated oxidation can also be used in on-resin regioselective disulfide bond formation.[79] Regioselective synthesis of α-conotoxin SI is reported utilizing NCS as only oxidant to form disulfide bonds (**Scheme 1.10.A**). Orthogonal combination of STmp and Mmt groups were chosen as cysteine protecting group for the synthesis, which is an example for an on-resin regioselective synthesis without Acm group protection. A solution of 2 equivalents NCS in DMF was needed for each oxidation step and each disulfide formation was completed in 15 min.

Scheme 1.10 A. Regioselective on-resin folding strategy for synthesis of α-SI using Cys(STmp) and Cys(Mmt) **B.** Regioselective on-resin folding strategy for synthesis of α-MII using Cys(S*t*Bu), Cys(Mmt) and Cys(Acm)



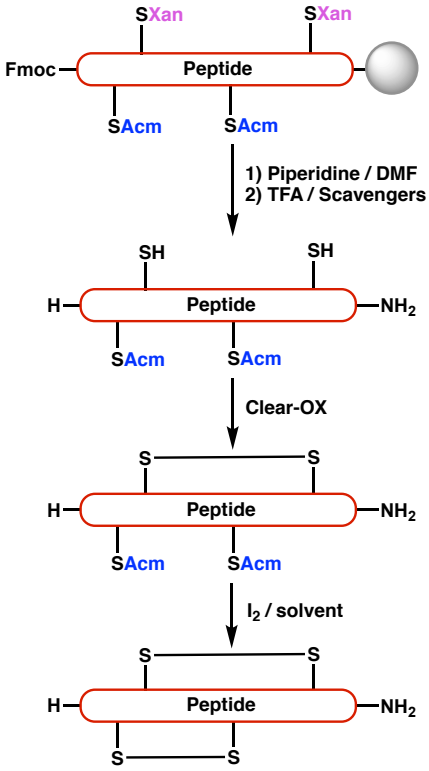
A microwave-assisted on-resin regioselective disulfide formation method was reported for the synthesis of α-conotoxin MII[102] (**Scheme 1.10.B**). This is a displacement method that can be used to form the first intramolecular disulfide bond in a regioselective scheme to access peptides with two disulfide bonds. For this method, three cysteine protecting groups were used, S*t*Bu, Mmt and Acm, and two of them were facilitated the displacement process to access first disulfide bond. 30% Mercaptoethanol in DMF was used to remove the S*t*Bu group under microwave heating. Upon addition of excess DTNP to the resulting on-resin peptide, reaction between the free thiol from S*t*Bu removal and DTNP forms an activated 5-Npys cysteine group. Removal of highly acid sensitive Mmt group under microwave heating facilitates the nucleophilic attack of free thiol on to the activated

cysteine to form the first disulfide bond by releasing one molecule of 5-nitropyridine-2-thione. Neither the deprotection conditions nor activation process in this displacement step affects the Cys(Acm) group in the peptide. The second disulfide formation was achieved by traditional on-resin Acm removal and oxidation via iodine oxidation. The significance of this method is reduction in the time required for disulfide bond formation, which highlights the power of microwave heating in combination with a displacement method.

1.4.5.3 On-Resin Oxidants for Regioselective Disulfide Bond Formation in Peptides

As described in section 1.4.3, there a several on-resin oxidants available for formation of disulfide bonds in peptides. Commercially available Clear-OX resin has been successfully applied in regioselective two-step disulfide formation procedure to access a-conotoxin GI (**Scheme 1.11**).[84] Both Xan and Acm groups were used to protect cysteine residues selectively and standard free thiol oxidation methods have been replaced by Clear-OX oxidation to form the first disulfide bond.

Scheme 1.11 Regioselective on-resin folding strategy for synthesis of α-GI using solid supported oxidant (Clear-OX)



1.5 Objective of the Project

Cysteine-rich peptides, including plant derived protease inhibitors, defensins from both vertebrates and invertebrates and neurotoxins from spiders, scorpions and mollusks, demonstrate high potency and good target specificity, making them useful tool compounds in biomedical and pharmaceutical research. The importance of these peptides is not limited to exclusively to therapeutic peptides. They can also be used in other applications such as bioconjugation,[103] β-sheet stabilization,[104] siRNA delivery,[105] increasing *in vivo* stability,[106] stabilization of peptide-based nanocarriers, and peptide backbone linkers.[107,108] Due to their small size, chemical synthesis has been the method of choice to access these bioactive targets in large quantities. Although several strategies

have been developed to synthesize cysteine-rich mini-proteins, a convenient and straightforward preparation of the native disulfide bond connectivity in these peptides still needed to be investigated. The goal of this dissertation is to further explore cysteine protecting groups with effective deprotection conditions and develop efficient methods for on-resin regioselective disulfide bond formation to access disulfide-rich bioactive peptides.

CHAPTER 2: DIRECT Pd-MEDIATED ON-RESIN DISULFIDE BOND FORMATION FROM ALLOCAM PROTECTED PEPTIDES

2.1 Introduction

The preparation of disulfide bonds in bioactive peptide is currently limited by the lengthy nature of solution-phase folding procedures. On-resin approach for disulfide bond formation is advantageous as it avoids multiple purification steps and reaction manipulations associated with solution-phase protocols. In addition, it favors intramolecular disulfide bond formation.[74] Much effort has been devoted to the development of new cysteine protecting groups to improve the available toolbox to access disulfide-rich bioactive peptides. Although some of these groups are selective in their cleavage, several have practical limitations preventing their widespread use. In this chapter, we are reintroducing Allocam group as cysteine protecting group with novel Pd-mediated deprotection-oxidation method to form on-resin disulfide bonds in peptides.

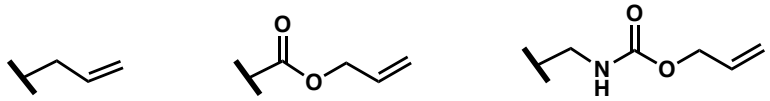
2.2 Allyloxycarbonylaminomethyl as Cysteine Protecting Group

Kunz and coworkers are the pioneers who introduced the allyl (All) and Allyloxycarbonyl (Alloc) protecting groups (**Figure 1.1**) in to the fields of peptide synthesis.[109,110] The groups became increasingly popular due to their orthogonality with the widely used Boc chemistry and Fmoc chemistry in peptide synthesis.[111] They can be selectively cleaved under mild condition by catalytic π-allyl palladium chemistry which involves combination of a palladium catalyst and a nucleophilic species as an allyl group scavenger.[112] These groups have been used for α-amino and α-carboxy groups and various side chain reactive functionalities such as carboxylic groups of aspartic acid and

glutamic acid,[113,114] phenolic groups of tyrosine,[115] nitrogen functions of lysine[116] or indole.[117] However, neither the allyl nor the Alloc groups are suitable for thiol group protection in cysteine residues. The allyl thioesters are not cleaved by palladium and can undergo intermolecular nucleophilic attack by neighboring α-amino function under basic conditions of Fmoc removal or even during coupling processes.[114]

As an alternative group for allyl-based cysteine protection, the allyloxycarbonylaminomethyl (Allocam, **Figure 1.1**) group was introduced by Loffet and co-workers.[118,119] It is stable in the basic conditions of Fmoc deprotection but only marginally stable under acidic conditions.

Figure 2.1 Structure of Allyl, Alloc and Allocam groups

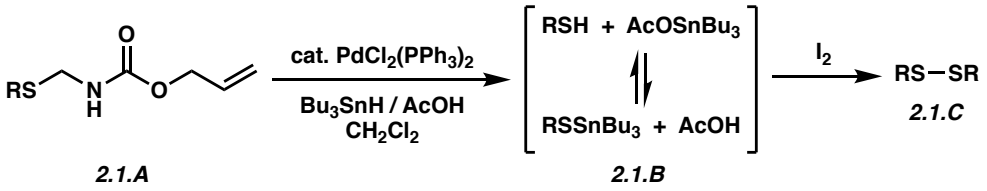


Allyl **Alloc** **Allocam**

Allocam protected thiols can be cleaved by palladium-catalyzed hydrostannolysis with Bu_3SnH. A nucleophilic species should be in a reaction medium to trap the π-allyl entity before any rearrangements take place. Examples of such scavengers are *N,N'*-dimethybarbituric acid (NDMBA),[120] *N*-trimethylsilylamines[121] and phenylsilane.[122] In early studies of Allocam deprotection, it was found that the deprotection reaction stops at initial stage as a result of catalyst poisoning. The system of $PdCl_2(PPh_3)_2/Bu_3SnH$ /AcOH was found as the best deprotection conditions to get rapid and complete removal of Allocam. The success of the procedure may be due to the combination of very high rate of the palladium catalyzed hydrostannolytic cleavage and presence of acetic acid, which prevent the catalytic poisoning.[120] However, 5-10% of allyl thioether formation was

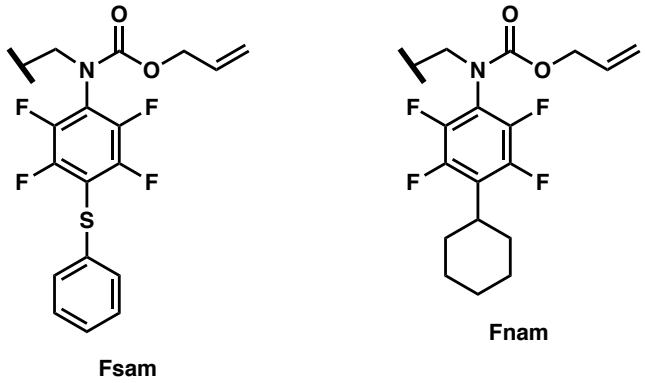
observed during reaction. In addition, the hydrostannolytic procedure give a mixture of the thiol, its tributylstannyl salt and minor amount of disulfide (**Scheme 2.1).**

Scheme 2.1 Reported cleavage and oxidation condition for Allocam



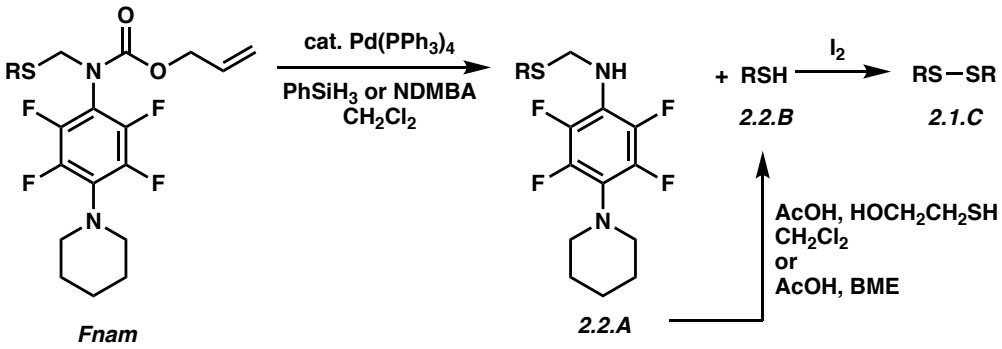
Therefore, isolation of the deprotected product is difficult. In order to characterize the product, the solvent and most of the acetic acid were evaporated and the crude reaction mixture was treated with iodine until persistence of I_2 coloration. Reaction with iodine convert all thiols and their tin salts into disulfide. Theses disulfides were then purified from the side-products (catalyst and tin compounds) by appropriate extractive procedures followed by chromatographic purification.[120] The acid lability of Allocam group, use of Bu_3SnH as an allyl scavenger, and the observation of significant amounts of *S*-allylated side products in the deprotection process have limited the general use of Allocam group. To improve the acid stability of Allocam group, two new protecting group, Fsam and Fnam, were introduced by substitution of an electron-withdrawing group on the carbonate nitrogen (**Figure 2.2**).[123,124] Both Fnam and Fsam are completely stable to basic and acidic conditions used in both Boc and Fmoc based peptide synthesis and can be selectively cleaved by palladium-catalyzed allylic cleavage in the presence of nucleophiles (**Scheme 2.2**).[124,125]

Figure 2.2 Structure of Fsam and Fnam groups



However, in the deprotection process, they require additional acidic treatment to convert intermediate **2.2.A** to the free thiol **2.2.B**.

Scheme 2.2 Reported cleavage and oxidation condition for Fnam



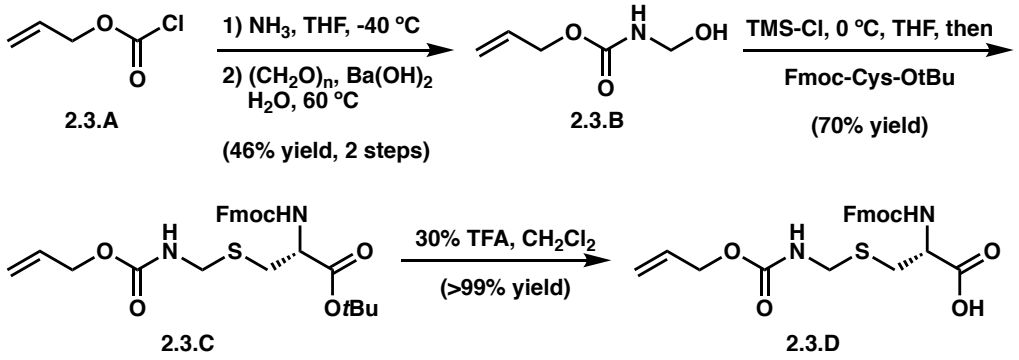
The reported deprotection methods for Allocam, Fnam and Fsam groups result the reduced cysteine and a separate oxidation step need to be performed to access disulfide bond **2.1.C**.

2.3 Synthesis of Fmoc-Cys(Allocam)-OH

Loffet and co-workers have reported synthesis of Allocam protected thiols.[120] The reported synthesis cannot be reproduced and hence, we developed an alternative approach starting from commercially available allyl chloroformate (**Scheme 2.3**). First, ammonia was bubbled through a solution of allyl chloroformate (**2.3.A**) to access the intermediate carbamate in 95% yield. Then, it was treated with paraformaldehyde and

$Ba(OH)_2$, which generated hydroxymethyl carbamate (**2.3.B**) in 48% yield. Next, the hydroxymethyl group was activated with trimethylsilyl chloride and addition of Fmoc-Cys(H)-O**t**Bu produced fully protected cysteine (**2.3.C**) in 70% yield. Finally, the **t**-Bu ester was removed with 30% TFA to produce Fmoc-Cys(Allocam)-OH (**2.3.D**) in 99% yield.

Scheme 2.3 Synthesis of Fmoc-Cys(Allocam)-OH



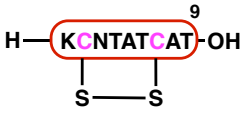
This approach can be used to synthesize Fmoc-Cys(Allocam)-OH in larger scale with good yields.

2.4 Optimization of Solid Phase Peptide Synthesis (SPPS) using Fmoc-Cys(Allocam)-OH

With a convenient protocol to synthesize Fmoc-Cys(Allocam)-OH in hand, we wanted to develop new deprotection conditions and reintroduce Allocam group for cysteine protection in peptide synthesis. First, compatibility of Allocam as cysteine protecting group in Fmoc-SPPS process was evaluated. For the studies, a model peptide derived from the first 9 amino acid residues of islet amylod polypeptide was selected ($IAPP_{1-9}$, **Figure 2.3**). The main reason for the selection of $IAPP_{1-9}$ as the model peptide was its higher potential in formation of a disulfide linkage between the two cysteine residues without any secondary structures.[125,126] In addition, linear synthesis of a peptide with 9 amino acid residues can be done relatively shorter period of time.

Figure 2.3 Structure of Islet amyloid polypeptide and the model peptide

Islet amyloid polypeptide (IAPP)



Model Peptide ($IAPP_{1-9}$)

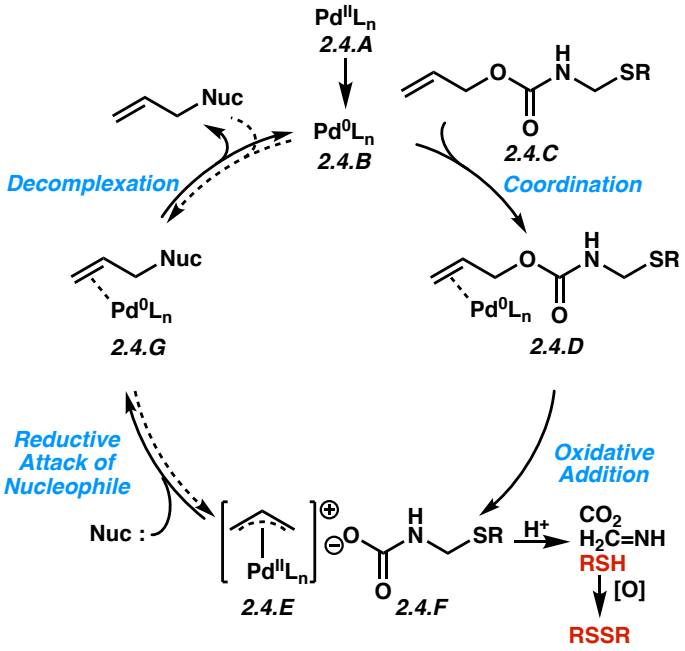
Initial SPPS was done on TGT resin using HATU as the coupling agent and DIPEA as the base under microwave heating. Single coupling step was sufficient enough to incorporate Fmoc-Cys(Allocam)-OH successfully into the linear $IAPP_{1-9}$ sequence. Allocam group is stable to the TGT resin cleavage condition, TFE:AcOH:DCM (1:1:3). Moreover, Fmoc-Cys(Allocam)-OH is compatible with SPPS on CTC, Wang and Rink amide resins. When cleaving peptides from Wang and Rink amide resins, the Allocam group get cleaved with 95% TFA condition. In order to cleave peptides from Wang and Rick amide resins, without affecting Allocam removal, a cleavage solution of 30% TFA in DCM in presence of a scavenger can be used.

2.5 Palladium-Mediated On-Resin Disulfide Formation in Peptides using Allocam Protected cysteines

As mentioned in section 2.2, removal of Allocam group can be done selectively via π-allyl palladium chemistry. We envisioned a combination of palladium mediated Allocam cleavage and *in situ* oxidation to access disulfide bonds in a single step (**Scheme 2.4**). If a Pd(II) pre-catalyst **2.4.A** is employed to the process, first, in situ reduction would convert

Pd(II) to Pd(0). Then, Pd(0) complex **2.4.B** coordinates to the π-allyl system in Allocam protected thiol **2.4.C** and generate the system **2.4.D**.

Scheme 2.4. Postulated catalytic cycle for deprotection of Allocam



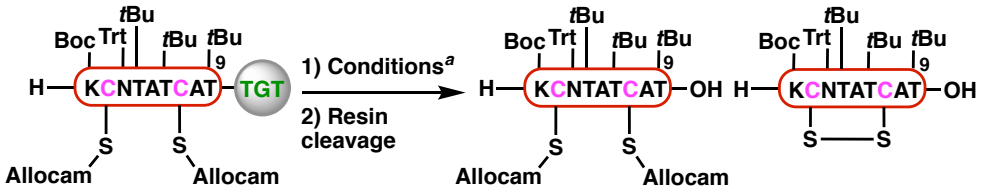
Next, oxidative addition of Pd(0) complex leads to cationic π-allyl Pd(II) species **2.4.E** and intermediate **2.4.F**. The intermediate **2.4.F** can undergo decarboxylation to release an equivalent of formimine, and liberates the thiol. The π-allyl Pd(II) species **2.4.E** undergoes nucleophilic attack to turn over the catalyst (**2.4.G** then **2.4.B**) and release the allylated nucleophile. In presence of an oxidant, the thiol can undergo *in situ* oxidation to form a disulfide bond.

In Albericio and co-workers' study of cleavage conditions for an allyl aspartate, they observed undesired disulfide bond formation when a mixture of DMSO, AcOH, and NMM was used in combination with $Pd(PPh_3)_3$ complex.[127] These conditions were taken as the starting point for our investigation and the model peptide $IAPP_{1-9}$ was subjected to different Pd complexes with various solvent systems and additives (**Table 2.1**). First, 5

equivalents of $Pd(PPh_3)_4$ in a DMSO/THF solvent mixture was used in presence of 3% NMM and 5% AcOH (entry 1). A mixture of unreacted starting peptide (**2.5.B**), desired disulfide (**2.5.C**), and the mono-Allocam version of the starting peptide was observed after 2 hours. When the amount of NMM was increased, an increment in the conversion of disulfide was observed. In addition, ~1% mono-S-allylated product was observed along with the mono-Allocam side product (entry 2). With 7 equivalent $Pd(PPh_3)_4$, the desired disulfide was observed as the major product (80% conversion) in addition to the minor amounts of mono-Allocam, reduced, and mono- and bis-allylation products (entry 3). Next, the Pd(0) source was switched to Pd(II) complexes due to the practical difficulties in handling air sensitive Pd(0) complexes. When $Pd(dppf)Cl_2{\cdot}CH_2Cl_2$ was used in presence of regular DMSO-AcOH-NMM solvent system, the reaction was completely shut down and no disulfide bond formation was observed (entry 4). However, repeating this reaction in the presence of 20 equivalent of phenylsilane resulted in nearly complete conversion to the desired disulfide bond, and most importantly, with no S-allylation by products (entry 5). Beside $Pd(dppf)Cl_2{\cdot}CH_2Cl_2$, $Pd(OAc)_2$ was used as the Pd(II) complex for this reaction. In the presence of 7 equivalent of $Pd(OAc)_2$, 10% NMM, and 5% AcOH in DMSO, about <5% starting material was observed within 2 hours as well as a small amount of mono-Allocam peptide (entry 6). Lowering the loading to 5 equivalent and increasing the reaction time to 4 hours led to complete disulfide formation with no side products (entry 7). However, further reduction in the loading of $Pd(OAc)_2$ to only 1 equivalent resulted insignificantly reduced conversion and increased side product formation (entry 8). Therefore, the loading of $Pd(OAc)_2$ was slightly increased to 1.5

equivalent (entry 9), and a nearly complete conversion was observed. When less NMM was used the reaction can be pushed towards a 100% conversion of the desired product (entry 10).

Table 2.1 On-resin deprotection/oxidation optimization for model peptide ($IAPP_{1-9}$)



2.5.A *2.5.B* *2.5.C*

entry	catalyst	equiv	additive (%v/v) NMM	additive (%v/v) AcOH	solvent	time (h)	*2.5.B* : 2.5.*C* : *other*[e]		
1.[b]	$Pd(PPh_3)_4$	5.0	3%	5%	DMSO:THF (1:1)	2	57	34	9[f]
2.[b]	$Pd(PPh_3)_4$	5.0	10%	5%	DMSO:THF (1:1)	2	11	71	18[f,h]
3.[b]	$Pd(PPh_3)_4$	7.0	10%	5%	DMSO:THF (1:1)	2	1	80	19[f-i]
4.[b]	$Pd(dppf)Cl_2$	7.0	10%	5%	DMSO	2	100	0	0
5.[b,d]	$Pd(dppf)Cl_2$	7.0	10%	5%	DMSO	2	2	98	0
6.[b]	$Pd(OAc)_2$	7.0	10%	5%	DMSO	2	4	93	3[f]
7.[b]	$Pd(OAc)_2$	5.0	10%	5%	DMSO	4	0	100	0
8.[b]	$Pd(OAc)_2$	1.0	10%	5%	DMSO	4	10	63	27[f,g]
9.[b]	$Pd(OAc)_2$	1.5	10%	5%	DMSO	4	4	96	0
10.[b]	$Pd(OAc)_2$	1.5	3%	5%	DMSO	4	0	100	0
11.[c]	$Pd(OAc)_2$	0.75	3%	5%	DMSO	4	89	11	0
12.[c]	$Pd(OAc)_2$	1.0	3%	5%	DMSO	4	50	50	0
13.[c]	$Pd(OAc)_2$	1.5	3%	5%	DMSO	2	0	100	0

[a]Unless otherwise noted, reaction was performed using 10 mg of TGT resin (loading 0.2 mmol/g) and 250 μL total volume (all liquids). LCMS analysis is performed directly on crude peptide following removal of solvents from resin cleavage. [b] NovaSyn PEG-PS copolymer resin with TGT linker, preloaded with Fmoc-Thr(OtBu). [c] Aminomethyl ChemMatrix Resin manually functionalized with TGT linker. [d] 20 equiv PhSiH3 added. [e] Ratio determined by integration of relevant peaks in mass spectra. [f] 1 Allocam remaining. [g] 2 Free thiols. [h] 1 S-Allyl. [i] 2 S-Allyls.

These were taken as the optimized conditions for the NovaSynTGT resin. As PEG resins have superior swelling properties relative to polystyrene resins,[128,129,130] we wanted to evaluate the reaction conditions on ChemMatrix resin. After linear synthesis of the model peptide $IAPP_{1-9}$ on ChemMatrix resin, the reaction was performed with lower loadings of

$Pd(OAc)_2$ (entries 11 and 12). Upon exposure to 0.75 or 1.0 equiv $Pd(OAc)_2$, only 11 or 50% conversion was observed after 4 h. However, under the previously optimized conditions, complete conversion was observed after 2 h instead of 4 h (entry 13). Overall, we were delighted with the low loadings of palladium needed for the transformation.[131] Addition of a Pd scavenger to the rinsing procedure prevents any detectable contamination of the cleaved products. Most importantly, neither partially deprotected nor S-allylated products were observed with the optimized Allocam deprotection-oxidation conditions.

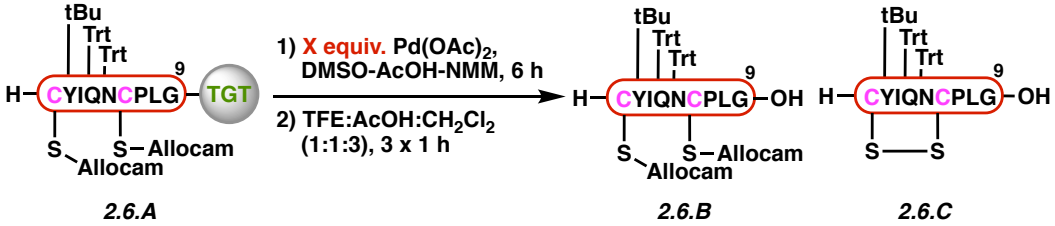
Moreover, we were interested in understanding the mechanism of Pd-DMSO mediated simultaneous Allocam removal and oxidation. To evaluate the function of each component in the reaction system and to understand how each component affects reactivity of the reaction, a set optimization were done. In the optimization process reactions were done by removing each component from the optimized condition at a time to evaluate the role of the component in deprotection-oxidation process. In addition, components such as NMM and AcOH were replaced by ionic NH_4OAc to understand the mechanism of action. Further details are needed to conclude the mechanism of Pd-DMSO mediated simultaneous Allocam cleavage-oxidation and the investigations are still ongoing in our lab

2.6 Synthesis of Oxytocin using Allocam Protected Cysteine

With optimized conditions in hand, we wanted to utilize this direct, on-resin disulfide bond formation method to form disulfide bond in oxytocin.[132,133,134,135] Oxytocin is a neurotransmitter which has 9 amino acid residues and one disulfide bond. Carboxy-

oxytocin was synthesized in order to facilitate analysis of the disulfide bond forming reaction. Linear synthesis of the peptide **2.6.A** was done on a TGT-linked ChemMatrix resin via Fmoc-SPPS. The resulting fully protected peptide was subjected to Pd-DMSO mediated simultaneous Allocam cleavage and oxidation conditions (**Table 2.2**). First, the optimized conditions for $IAPP_{1-9}$ was used and only ~80% conversion of the product 2.6.C was observed after 6 hours (Entry 1). Then, $Pd(OAc)_2$ loading was increased to 3.0 equivalent and it facilitated a complete conversion of folded peptide **2.X.B** (Entry 2). The resulting folded peptide was cleaved from the resin followed by side chain protecting groups deprotection.

Table 2.2 Reaction optimization for on-resin folding of caboxy-oxytocin



2.6.A *2.6.B* *2.6.C*

Entry	X equiv	*2.6.B* :	*2.6.C*
1	1.5	20	80
2	3.0	00	100

Fully deprotected carboxy-oxytocin was purified via RP-HPLC and 32% isolated yield was obtained.

2.7 Summary

In this study, Allocam group was reintroduced to the field of peptide synthesis as a cysteine protecting group with novel Pd-mediated deprotection-oxidation method to form disulfide bonds on resin. This is a one-pot approach, which avoids solution-phase

manipulations of the peptide and minimizes the number of time consuming purification steps. No special equipment is needed and the reaction can be done with bench-stable reagents. Reaction conditions were optimized for no trace of Pd or *S*-allylated byproducts. The utility of the method was demonstrated by solid phase synthesis and folding of the carboxy-oxytocin, which consist of single disulfide bond. This method can be broadly applied in the synthesis of more complex targets with multi-disulfide bonds. Detailed investigations into the mechanism of Pd-DMSO mediated simultaneous Allocam removal and oxidation are still ongoing.

2.8 Materials and Methods

2.8.1 General Information

Unless otherwise specified, all commercially available reagents were purchased from Sigma-Aldrich and used without further purification. Anhydrous Et_2O, MeCN, DMF, DMSO, CH_2Cl_2 were purchased from Fisher. These were passed through a commercial solvent purification system (2 columns of alumina) and used without further drying. Hünig's base was distilled over CaH_2 immediately prior to use. All amino acids were purchased from Chem-Impex Int'l. Inc. unless otherwise noted. HATU were purchased from Chem-Impex Int'l. Inc. Unless otherwise noted, all reactions were performed in 2 mL, 5 mL and 10 mL Biotage reactor vials with PTFE frit (depend on reaction volume) at room temperature. All yields refer to chromatographically and spectroscopically pure products. All HPLC analyses and purifications were performed on a Custom Reverse Phase Shimadzu Liquid Chromatograph Mass Spectrometer (LCMS-2020), which can toggle between analytical and semi-preparative columns. This instrument has a photodiode

array (PDA) detector (D2 & W lamp), which collects a range of wavelengths, in place of a traditional single channel UV detector. RP-HPLC-MS mobile phases (MeCN and H_2O) contained 0.1% Formic Acid. Analytical HPLC was performed on a Phenomenex Kinetex C18 column (5 μm, 250 x 4.6 mm) and a Thermo Scientific Hypersil Gold C8 column (5 μm, 250 x 4.6 mm). Semi-Preparative HPLC was conducted using a Thermo Scientific Hypersil Gold C8 column (5 μm, 150 x 10 mm). All peptide yields are calculated based on the final loading.

Fmoc-SPPS General Information. Solid-phase peptide synthesis was executed on a Biotage Isolera+ semi-automated synthesizer with microwave heating.

- *Reactor Vials [Vial size (Volume range allowed)]:* 2 mL reactor vial (0.8-1.1 mL), 5 mL reactor vial (1.6-3.2 mL), and 10 mL reactor vial (3.2-6.4 mL)
- *Swelling + Heat:* DMF was added and vortexed at 1200 RPM for 20 m at 70 ºC. The solvent was then removed over 1 m followed by two DMF washes (DMF was added and the suspension was vortexed at 600 RPM for 45 s, followed by the removal of solvent (over 2 m)).
- *Coupling:* A solution of Fmoc-aa-OH (3 equiv), HATU (3 equiv), and DIPEA (6 equiv) in DMF was made immediately prior to addition to the reaction vial containing the resin. Once the solution was added, the suspension was heated to 75 ºC (except for Fmoc-Cys-OH which was heated to 50 ºC) for 5 minutes with a vortex rate of 1200 RPM. After the reaction, the solution was removed (over 2 m) and the resin was rinsed with DMF 4 times (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).

- *Fmoc Removal (Deprotection):* The reactor vial was filled with 20% piperidine in DMF. The suspension was vortexed at 1200 RPM for 3 m at RT. The solvent is removed followed by addition of 20% piperidine in DMF. The suspension is vortexed again at 1200 RPM for 10 m at RT. The solvent was removed over 2 m, followed by 4 DMF washes (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).
- *Wash*: DMF was added to the reaction vial and agitated at a vortex rate of 1200 RPM for 1 m. The solvent was removed over 1 m and repeated for a total of 4 times.
- *Final Wash:* Resin was rinsed with CH_2Cl_2 (3 x 1 mL) and MeOH (3 x 1 mL).
- *Drying for Storage/Weighing:* After the final wash, the resin was placed on the lyophilizer overnight for drying.

General Procedure for Capping - First, 25% acetic anhydride in DMF (3 mL) was added and agitated for 5 min. Then,1.5 eqiuv. of DIPEA was added to the reaction and agitated for 30 min. Finally, the reaction solution was filtered and the resin was washed with DMF (5 x 3 mL).

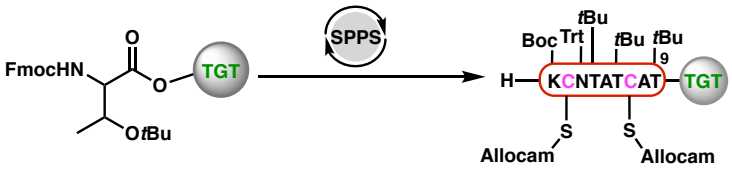
Procedure for Resin Loading Analysis. Initial and final loading of resin was analyzed based on amount of Fmoc group.

Fmoc Deprotection – 10 mg of the resin was weighed out and swelled in DMF in the peptide synthesizer. Then, 1.00 ml of 20% piperidine in DMF was added and it was allowed to vortex in the peptide synthesizer at room temperature for 3 min. After 3 min, the filtrate was collected in to a separate tube, another 1.00 ml of 20% piperidine in DMF was added to it and allowed to vortex in the peptide synthesizer at room temperature for

10 min. The resulting filtrate was collected to the same tube and the deprotected resin was washed with DMF (4 x 1.75 ml).

Sample preparation for UV analysis – From the collected "deprotection solution" above, 100 μL was transferred in to an eppendorf tube and it was diluted with 900 μl of DMF. The resulting diluted sample was used to get the absorbance at 301 nm versus a DMF blank using a UV spectrometer with 1 cm cuvette.

2.8.2 Experimental Procedures and Spectroscopic Data



2.5.A' *2.5.A*

Synthesis of IAPP$_{1\text{-}9}$ (2.5.A). The standard Fmoc-SPPS protocol described was used to synthesize IAPP$_{1\text{-}9}$ on preloaded TGT resin **2.5.A'** using a semi-automated peptide synthesizer, in a 10-mL reaction vial. First, the resin was swelled followed by deprotection. Then, each amino acid was added accordingly followed by coupling and deprotection steps. Finally, the resin + peptide was washed with DCM and Methanol. To analyze the peptide, 10 mg of the resin was subjected to 300 μL of the cleavage mixture (1:1:3 TFE:AcOH:DCM) for 30 min. The filtrates were collected, concentrated and analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 40-80% MeCN/H_2O gradient for 60 minutes. *m/z* ES calc'd for Allocam IAPP (**2.5.A**) $[(C_{80}H_{121}N_{13}O_{20}S_2)+1]^+$: 1648.83; observed: 1649.60; m/z ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 824.92; observed: 825.30.

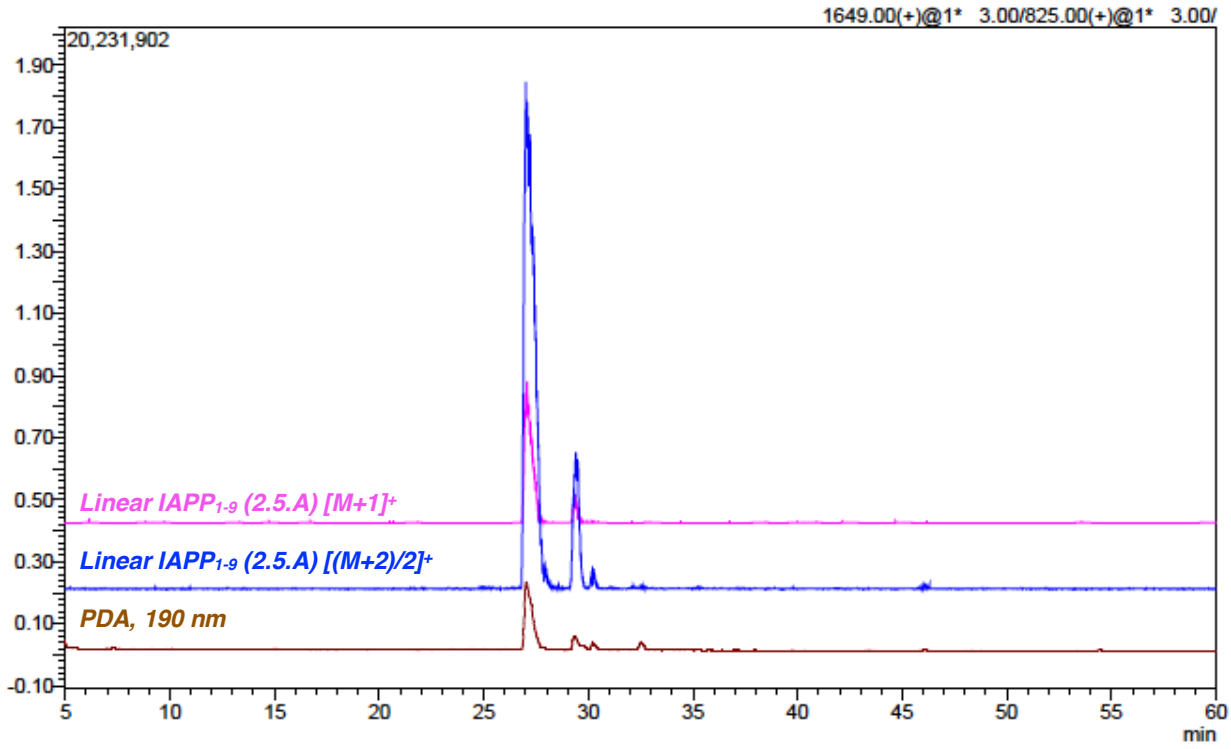


Figure 2.4 Analytical Column PDA for linear $IAPP_{1-9}$ 2.5.A; Gradient: 40-80% MeCN/H_2O + 1% HCOOH over 60 min. Retention time 27.26 min

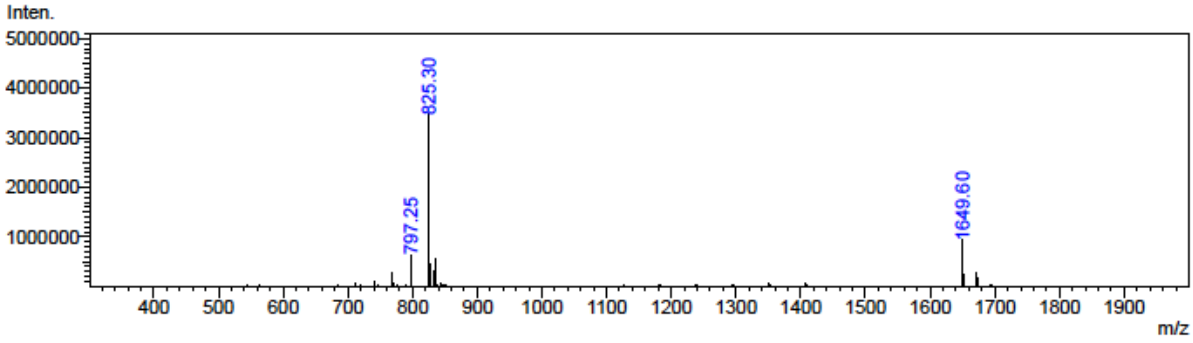
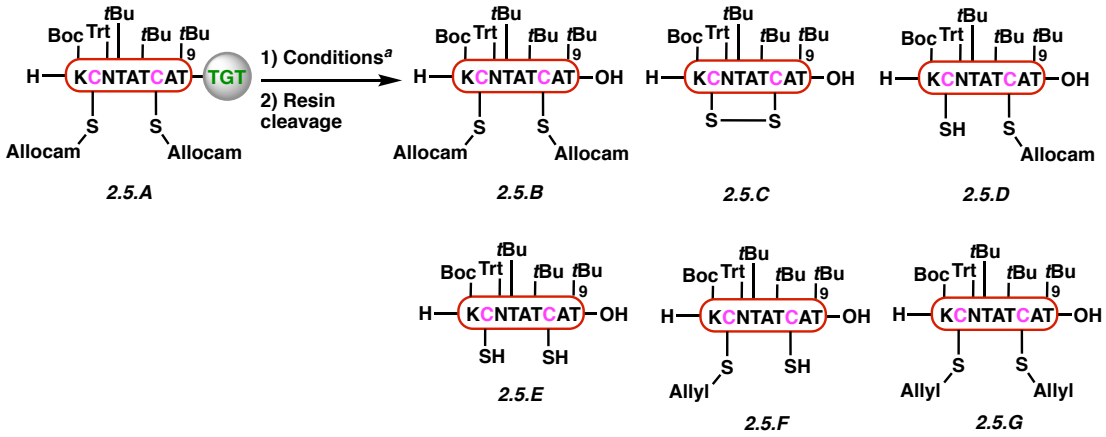
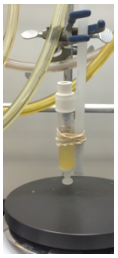


Figure 2.5 ES-MS spectrum for linear $IAPP_{1-9}$ 2.5.A



General Procedure for Allocam Cleavage/Oxidation for IAPP$_{1-9}$ (Table 2.1). IAPP$_{1-9}$ on the stated resin (10 mg, 2.0 μmol) was swelled in DMF (800 μL) on the Biotage Isolera+ synthesizer. After removing excess DMF, the cartridge was removed from the synthesizer and capped on the bottom. A solution of the stated catalyst in 5% AcOH/DMSO solution (250 μL) was added, and then the stated amount of $PhSiH_3$ (if relevant) and NMM were added. The cartridge was capped on the top, attached to a magnetic stirbar retriever using a rubber band (see image), and agitated for the stated amount of time under an atmosphere of ambient air. The reaction was filtered then transferred back to the synthesizer, where it was washed successively: 1) 750 μL of DMF was added and vortexed 2 min, then filtered (3 total times), 2) 750 μL of CH_2Cl_2 was added and vortexed 2 min, then filtered (3 total times), 3) 800 μL of 0.2 M sodium diethyldithiocarbamate in DMF was added and vortexed 15 min, then filtered (3 total times). *Note: resin changes from dark orange/brownish to yellowish/tan. 4) 750 μL of DMF was added and vortexed 2 min, then filtered (5 total times), 5) 750 μL of CH_2Cl_2 was added and vortexed 2 min. The resin was then subjected to a cleavage mixture of TFE:AcOH:DCM (1:1:3, 300 μ□) for 3 x 15 min (not on synthesizer). The filtrates were



collected and concentrated with a stream of nitrogen gas. The concentrated solution was analyzed directly (with no lyophilization) by analytical HPLC-MS using a Hypersil GOLD column (5 μL, 150 x 4.6 mm) at a flow rate of 1 mL/min with 20–80% MeCN/H_2O gradient for 60 min. The possible expected masses are:

m/z ES calc'd for Allocam IAPP (**2.5.A**) $[(C_{80}H_{121}N_{13}O_{20}S_2)+1]^+$: 1648.83; observed: 1649.60; m/z ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 824.92; observed: 825.30.

m/z ES calc'd for IAPP Disulfide (**2.5.B**) $[(C_{70}H_{105}N_{11}O_{16}S_2)+1]^+$: 1420.72; observed: 1421.05; *m/z* ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 710.86; observed: 711.100.

m/z ES calc'd for 1-Allocam (**2.5.C**) $[(C_{70}H_{105}N_{11}O_{16}S_2)+1]^+$: 1535.78; observed: 1535.00; *m/z* ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 768.39; observed: 767.85.

m/z ES calc'd for free thiols (**2.5.D**) $[(C_{70}H_{105}N_{11}O_{16}S_2)+1]^+$: 1422.73; observed: 1422.25; *m/z* ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 711.86; observed: 712.10.

m/z ES calc'd for 1 allyl (**2.5.E**) $[(C_{70}H_{105}N_{11}O_{16}S_2)+1]^+$: 1462.77; observed: 1463.15; *m/z* ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 731.88; observed: 732.20.

m/z ES calc'd for 2 allyls (**2.5.F**) $[(C_{70}H_{105}N_{11}O_{16}S_2)+1]^+$: 1502.80; observed: 1502.25; *m/z* ES calc'd for $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 751.90; observed: 752.15.

Table 2.1, entry 1. The general procedure was followed. The relevant quantities are: $Pd(PPh_3)_4$ (11.6 mg, 10 μmol), NMM (6.3 μL, 58 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

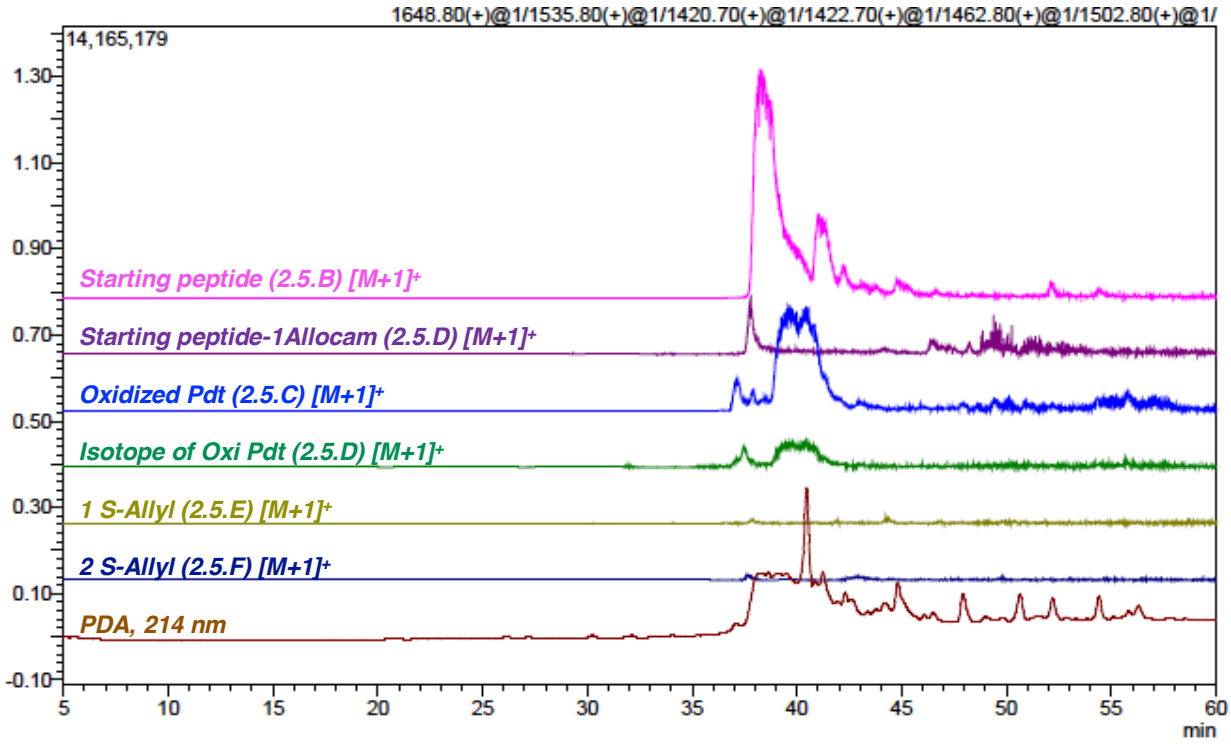


Figure 2.6 Analytical Column PDA for determination of conversion; Table 2.1. Entry 1. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 2. The general procedure was followed. The relevant quantities are: $Pd(PPh_3)_4$ (11.6 mg, 10 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

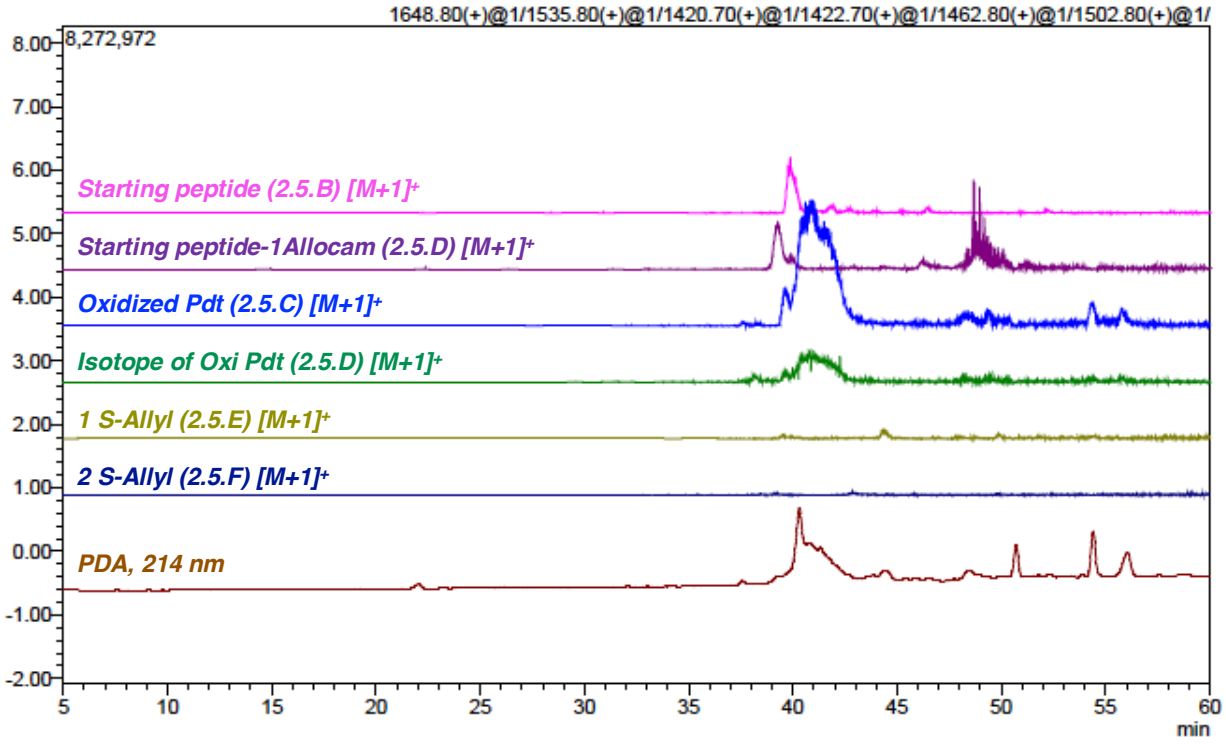


Figure 2.7 Analytical Column PDA for determination of conversion; Table 2.1. Entry 2. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 3. The general procedure was followed. The relevant quantities are: $Pd(PPh_3)_4$ (16.2 mg, 14 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

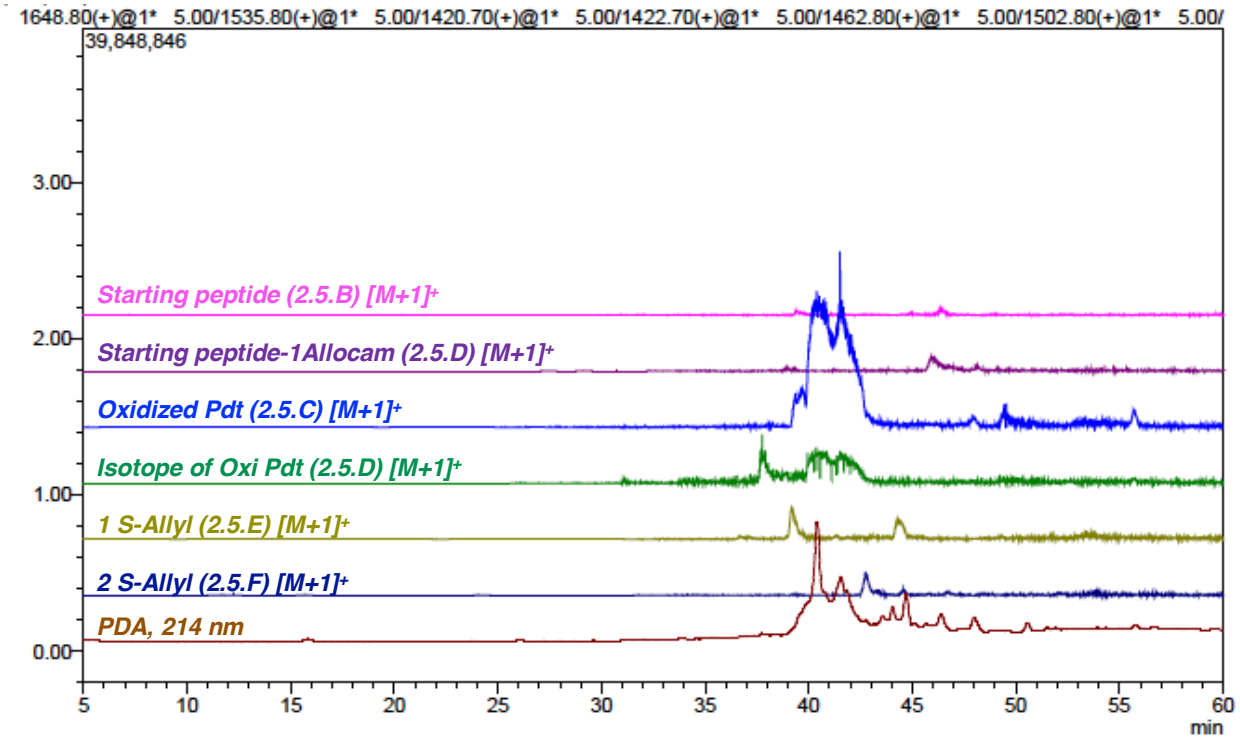


Figure 2.8 Analytical Column PDA for determination of conversion; Table 2.1. Entry 3. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 4. The general procedure was followed. The relevant quantities are: $Pd(dppf)Cl_2$ (11.4 mg, 14 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

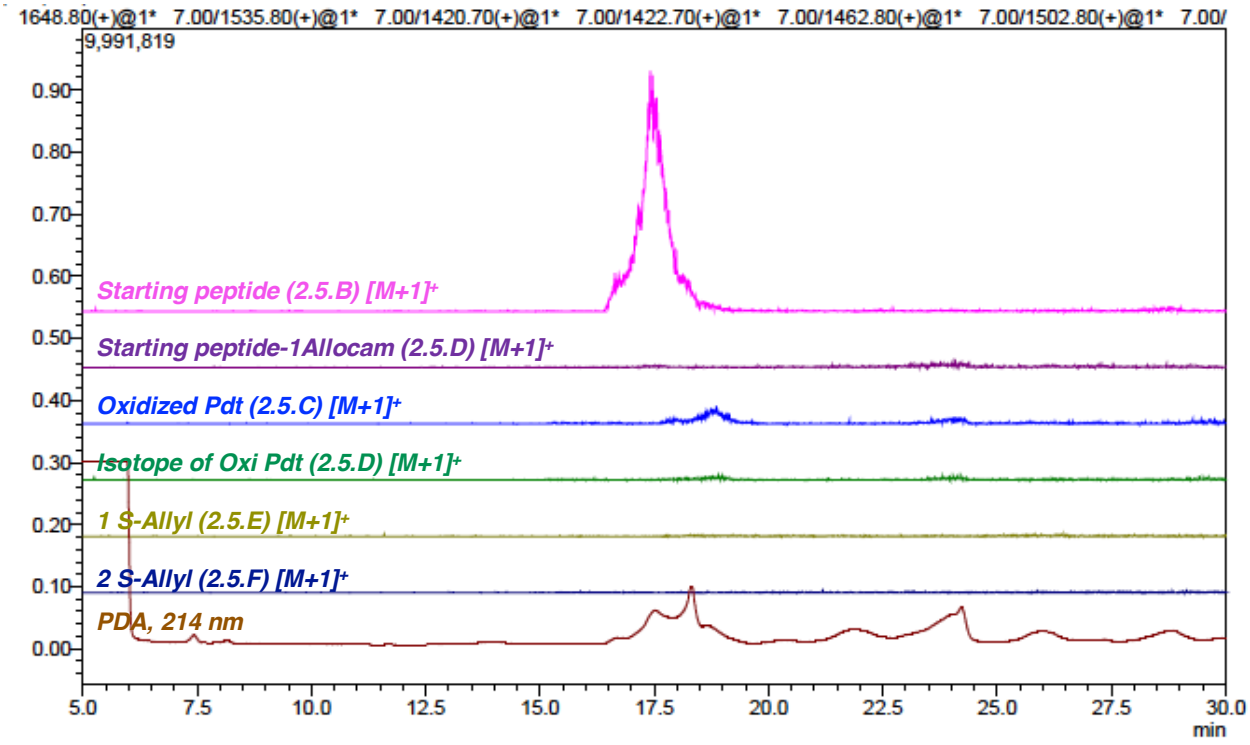


Figure 2.9 Analytical Column PDA for determination of conversion; Table 2.1. Entry 4. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 5. The general procedure was followed. The relevant quantities are: $Pd(PPh_3)_4$ (11.4 mg, 14 μmol), $PhSiH_3$ (5 μL, 40 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

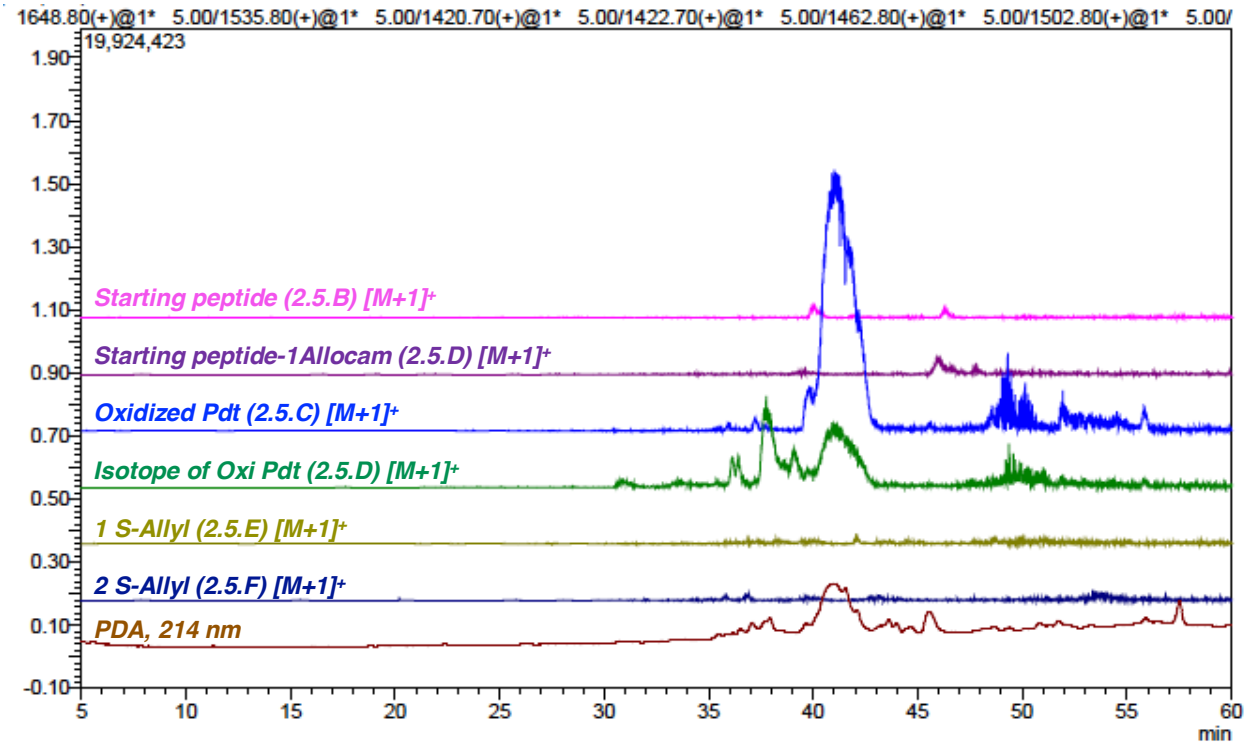


Figure 2.10 Analytical Column PDA for determination of conversion; Table 2.1. Entry 5. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 6. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (3.1 mg, 14 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

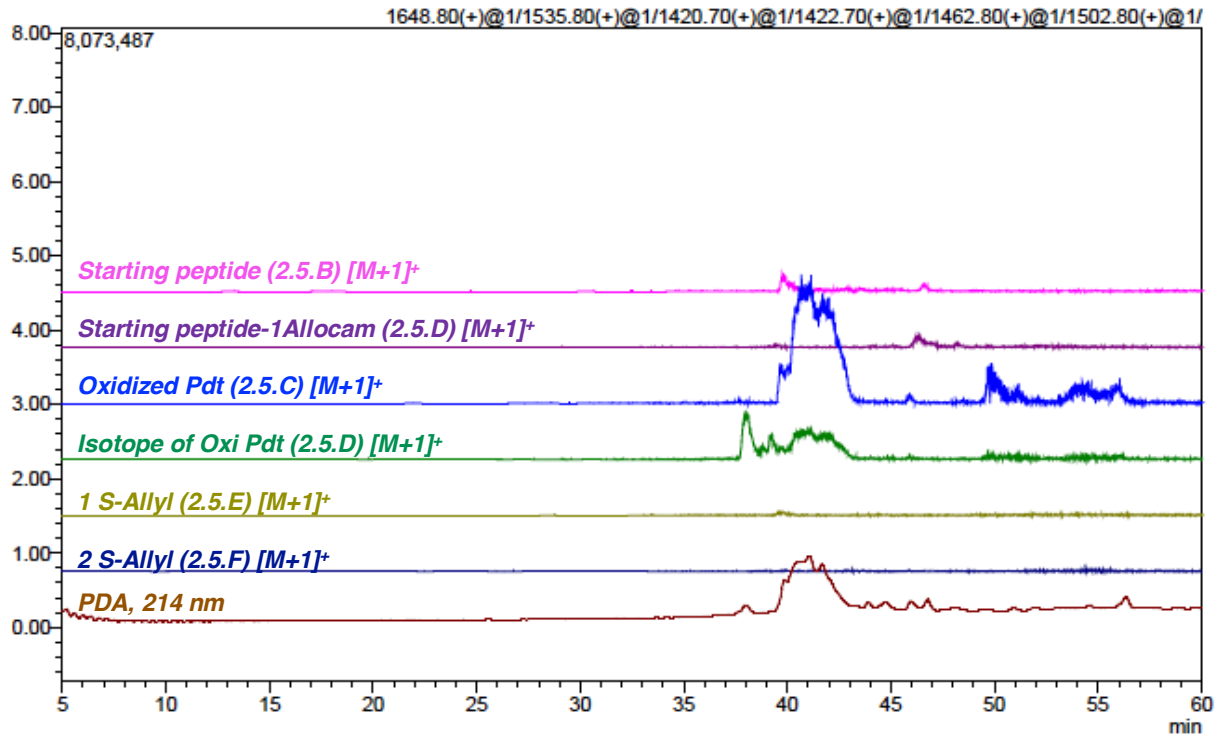


Figure 2.11 Analytical Column PDA for determination of conversion; Table 2.1. Entry 6. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 7. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (2.2 mg, 10 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 4 hours.

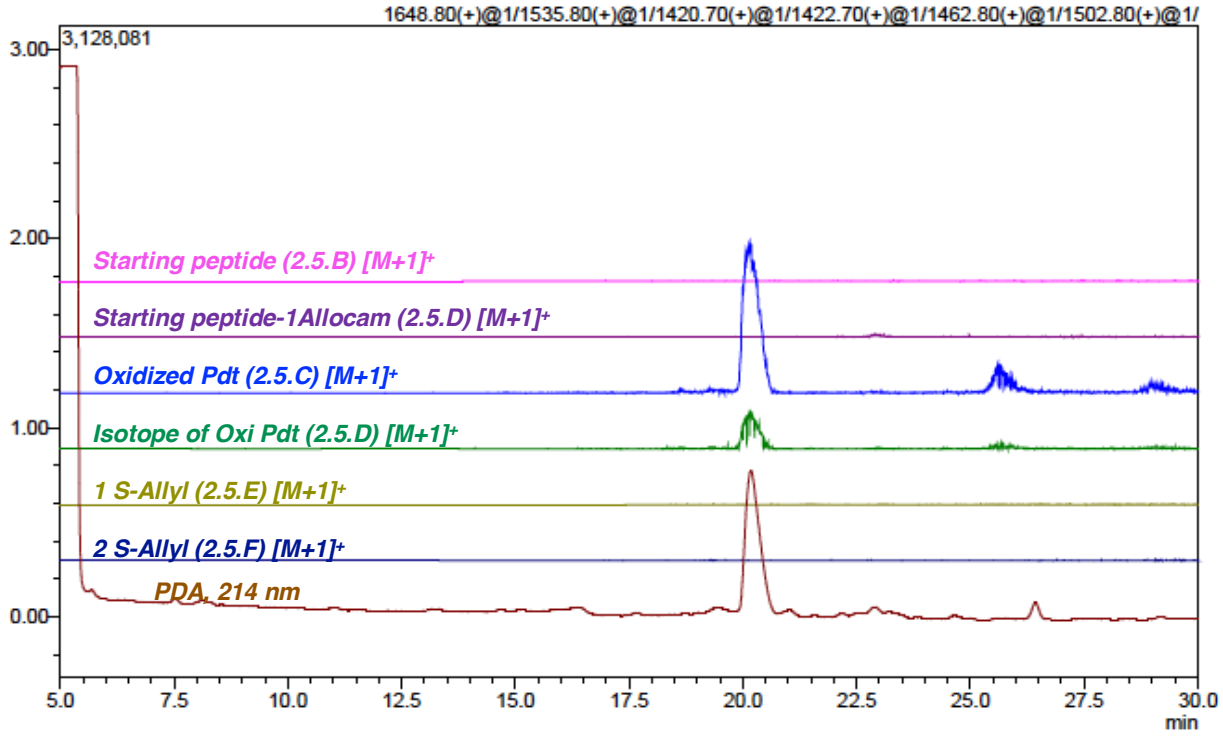


Figure 2.12 Analytical Column PDA for determination of conversion; Table 2.1. Entry 7. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 8. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (0.45 mg, 2 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 4 hours.

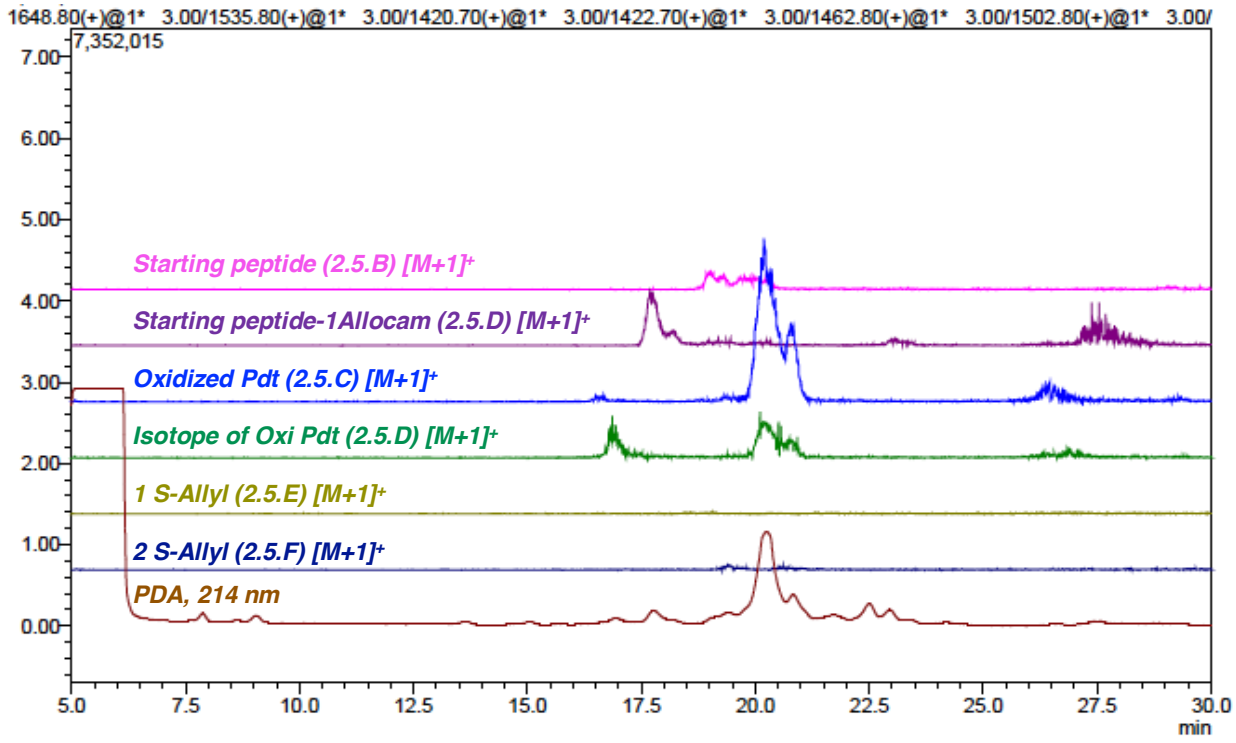


Figure 2.13 Analytical Column PDA for determination of conversion; Table 2.1. Entry 8. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 9. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (25 μL, 227 μmol). The reaction mixture was agitated in a closed vial at room temperature for 4 hours.

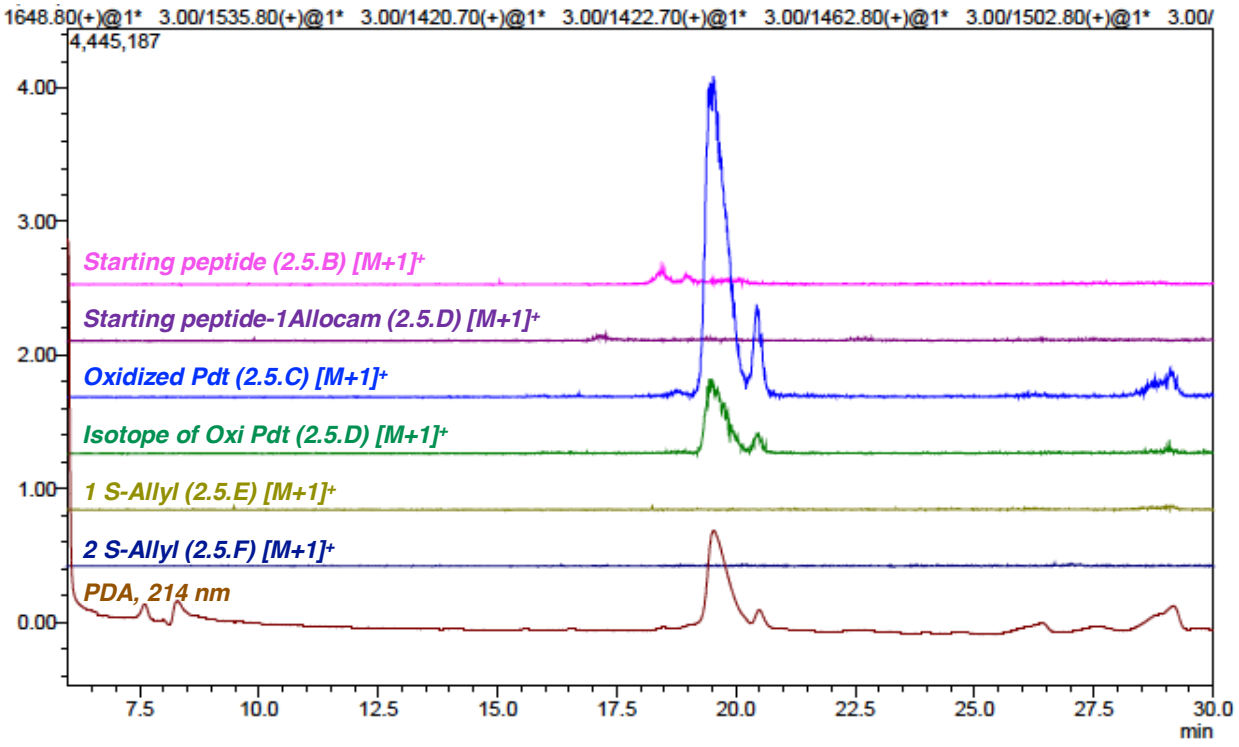


Figure 2.14 Analytical Column PDA for determination of conversion; Table 2.1. Entry 9. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 10. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (7.5 μL, 68.4 μmol). The reaction mixture was agitated in a closed vial at room temperature for 4 hours.

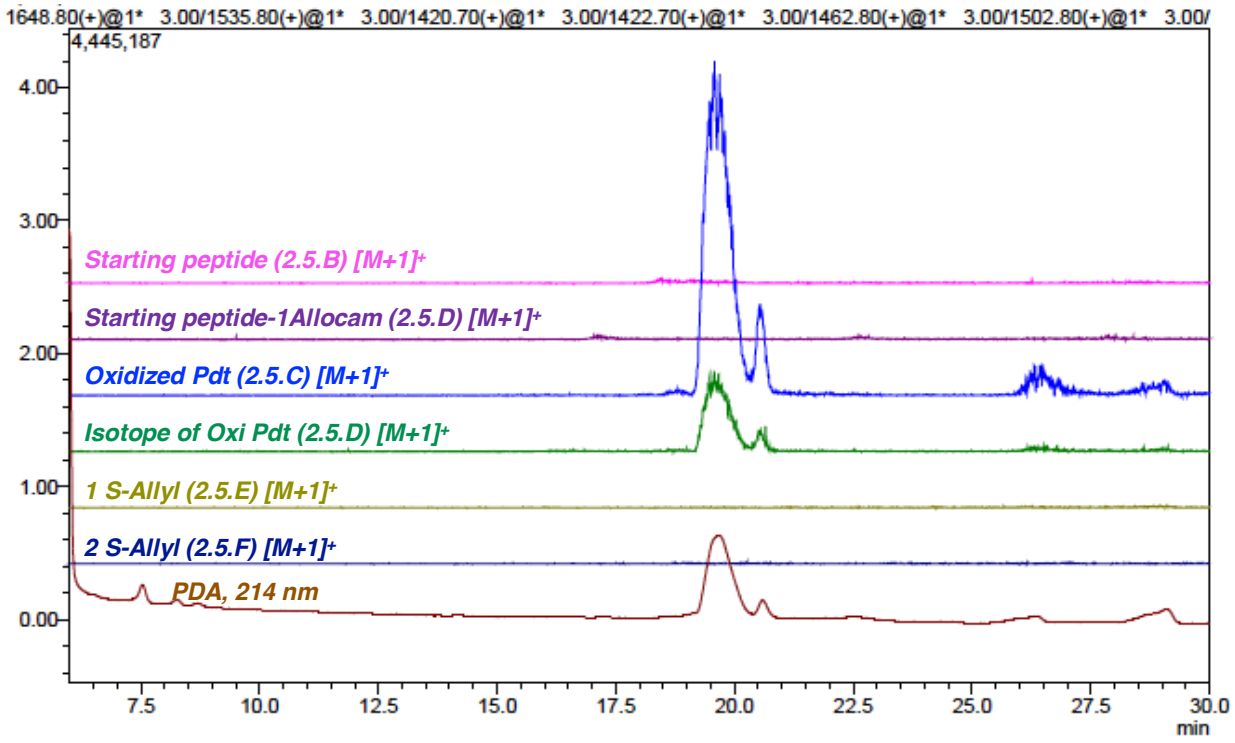


Figure 2.15 Analytical Column PDA for determination of conversion; Table 2.1. Entry 10. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 11. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (0.34 mg, 1.5 μmol), NMM (7.5 μL, 68.4 μmol). The reaction mixture was agitated in a closed vial at room temperature for 4 hours.

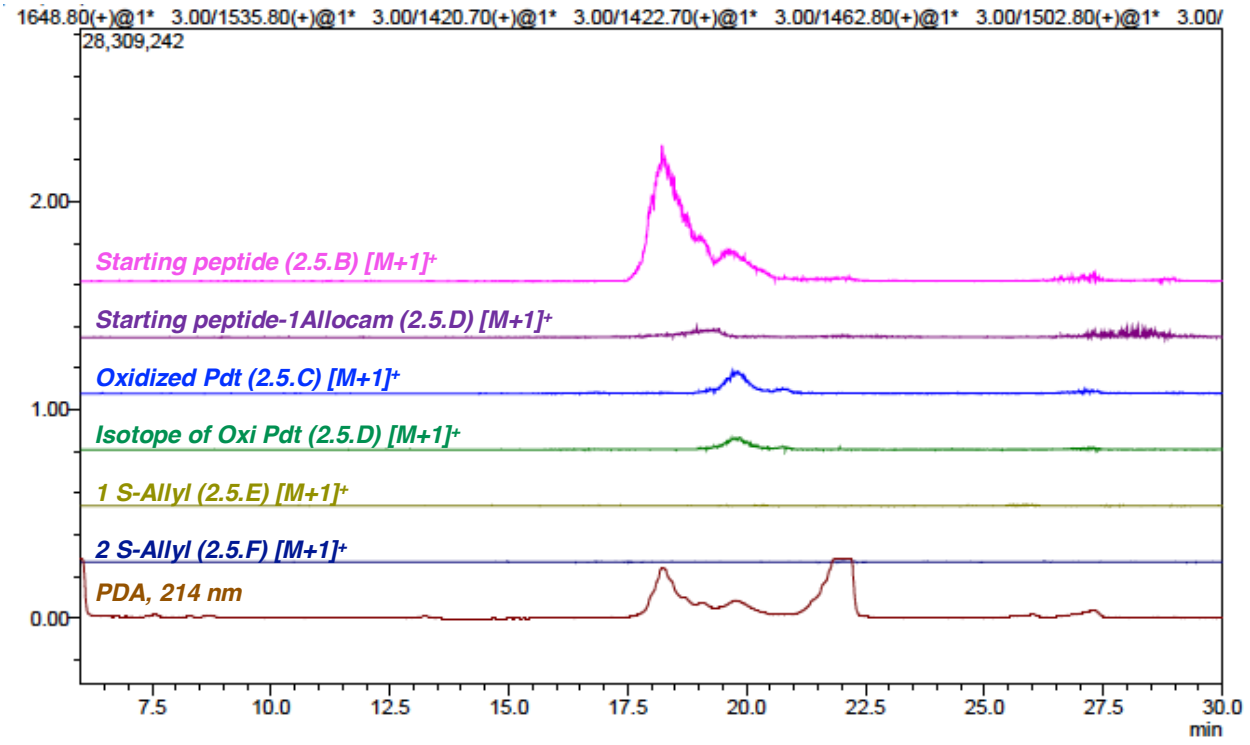


Figure 2.16 Analytical Column PDA for determination of conversion; Table 2.1. Entry 11. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 12. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (0.45 mg, 2 μmol), NMM (7.5 μL, 68.4 μmol). The reaction mixture was agitated in a closed vial at room temperature for 4 hours.

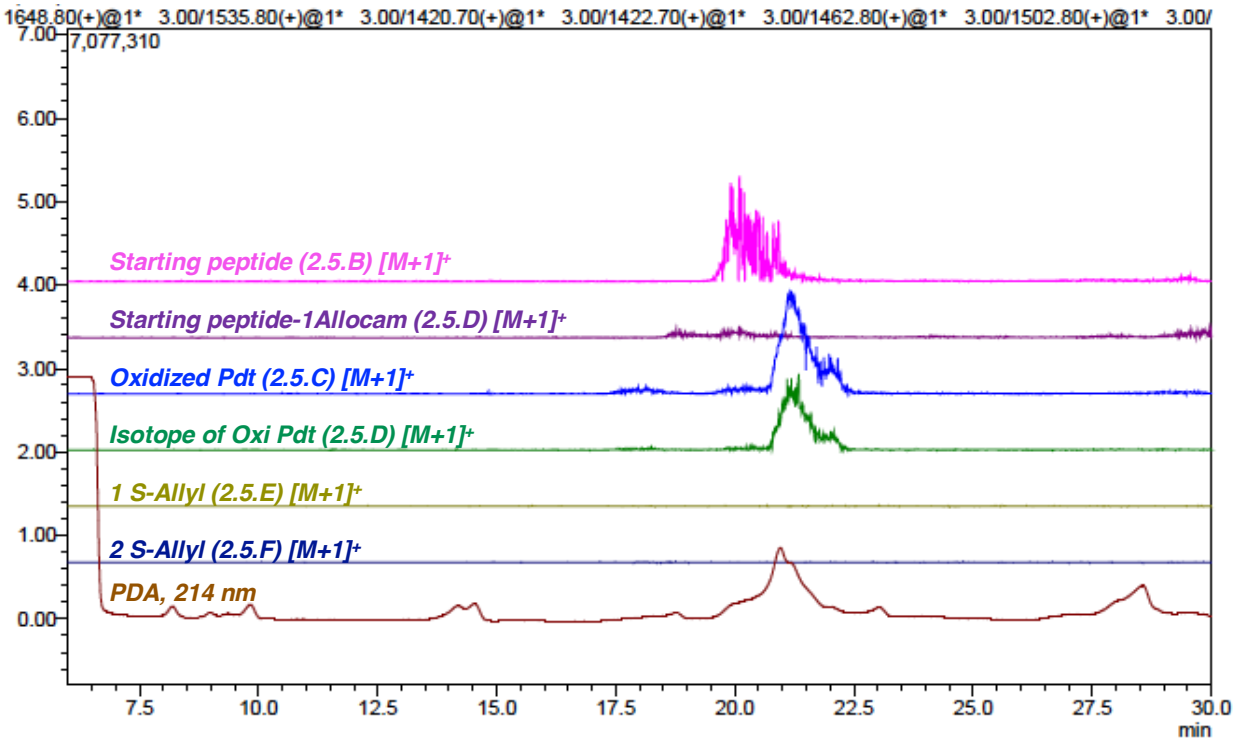


Figure 2.17 Analytical Column PDA for determination of conversion; Table 2.1. Entry 12. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.

Table 2.1, entry 13. The general procedure was followed. The relevant quantities are: $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (7.5 μL, 68.4 μmol). The reaction mixture was agitated in a closed vial at room temperature for 2 hours.

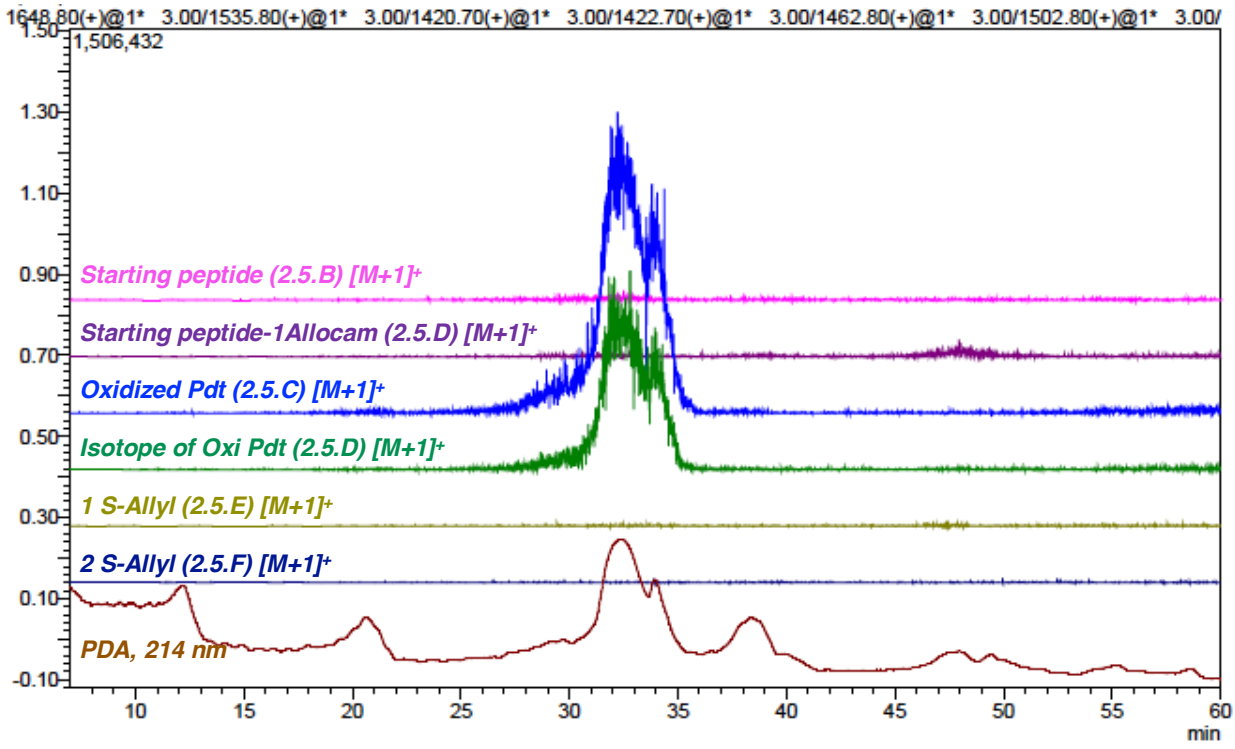
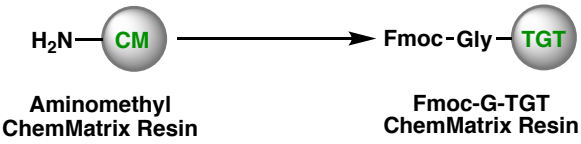
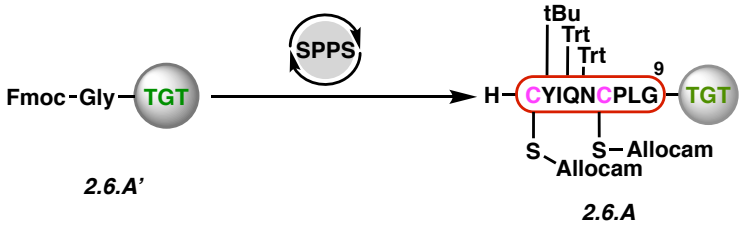


Figure 2.18 Analytical Column PDA for determination of conversion; Table 2.1. Entry 13. Gradient: 20-80% MeCN/H_2O + 1% HCOOH over 60 min.



TGT-ChemMatrix Resin. The coupling of 4-(diphenylhydroxy methyl)benzoic acid on to the Aminomethyl ChemMatrix resin was done using the semi-automated peptide synthesizer, in a 10-mL reaction vial. The general procedure was followed. The relevant quantities are: Aminomethyl ChemMatrix resin (200 mg, 0.112 mmol), 4-(diphenylhydroxy methyl)benzoic acid (170.4 mg, 0.56 mmol), HATU (212.9 mg, 0.56 mmol), and DIPEA (195 μl, 1.12 mmol). After the coupling, the reaction vial with the resin was taken out from the synthesizer and 25% acetic anhydride in DMF (3 mL) was added. It was agitated for 5 min and then DIPEA (30 μl, 0.168 mmol) was added and agitated for another 30 min. After the reaction, the solution was removed and the resin was rinsed with DMF 4 times. Next, 1M NaOH (3 mL) was added to the resin and agitated for 12 h. At the end of the reaction, the solution was removed followed by 4 DMF washes. To the resulting resin in a close-sealed vial, 2% $SOCl_2$ in DCM (3 mL) was added and agitated for 12 h. Finally, the solution was removed and the resin was rinsed with DCM 5 times and 2% DIPEA in DCM 3 times.

Fmoc-Gly-TGT-ChemMatrix. The resin was swelled in DMF and a solution of the desired Fmoc-Gly-OH (5 equiv) with DIPEA (6 equiv) in DMF was added. Then the resulting reaction vial was agitated at RT for 12 h. After the reaction, the solution was drained out and the resin was rinsed with DMF, DCM and MeOH (3 times each). Finally, the resin was dried in the lyophilizer for 12 h.



Synthesis of Linear Oxytocin (2.6.A). The standard Fmoc-SPPS protocol described was used to synthesize linear peptide **2.6.A** on Fmoc-Gly-TGT-Chemmatric resin **2.6.A'** using a semi-automated peptide synthesizer, in a 10-mL reaction vial. First, the resin was swelled followed by deprotection. Then, each amino acid was added accordingly followed by coupling and deprotection steps. Finally, the resin + peptide was washed with DCM and Methanol. To analyze the peptide, 10 mg of the resin was subjected to 300 μL of the cleavage mixture (1:1:3 TFE:AcOH:DCM) for 30 min. The filtrates were collected, concentrated and analyzed by RP-HPLC-MS using an analytical Phenomenex column at a flow rate of 1 mL/min with 5-95% MeCN/H_2O gradient for 30 minutes. Final loading: 0.13 mmol/g. *m/z* ES calc'd for linear peptide **2.6.A** $[(C_{95}H_{117}N_{13}O_{17}S_2)+1]^+$: 1777.18; observed: 1776.95.

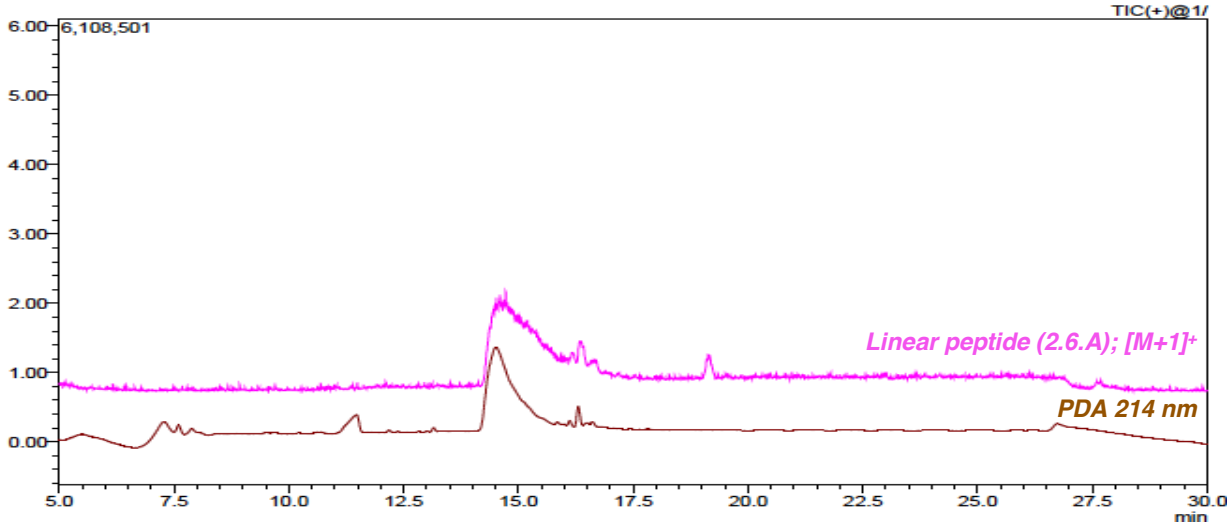


Figure 2.19 Analytical Column PDA for linear carboxy-oxytocin **2.6.A**; Gradient: 2-95% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 14.92 min

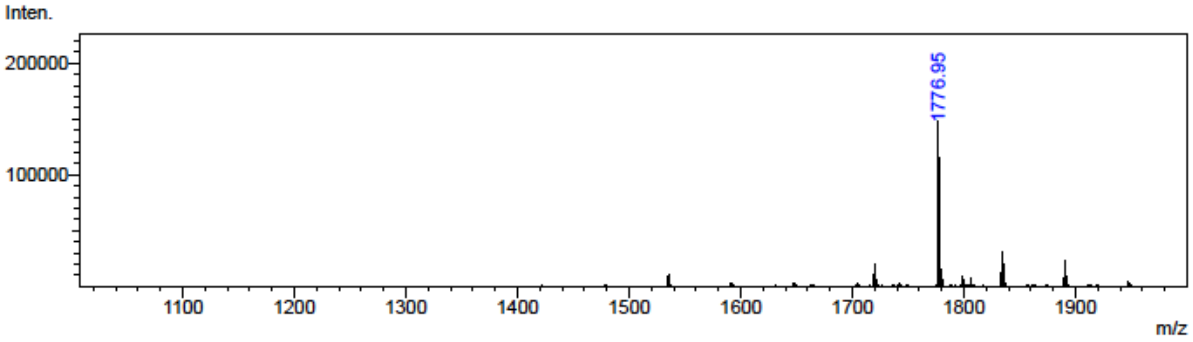
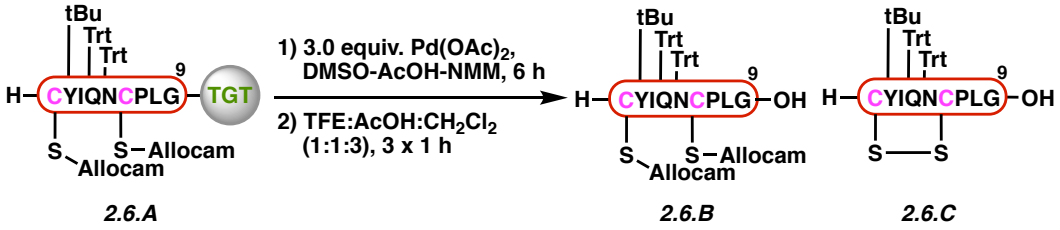


Figure 2.20 ES-MS spectrum for linear carboxy-oxytocin **2.6.A** at retention time 14.92 min



Optimized Conditions for On-Resin Folding of Oxytocin-OH (2.6.A → 2.6.C). The IAPP standard protocol was followed. The relevant quantities are: $Pd(OAc)_2$ (1.34 mg, 6 μmol, 3 equiv), NMM (7.5 μL, 68.4 μmol). The reaction mixture was agitated in a closed vial at room temperature for 6 hours. The resin was then subjected to a cleavage mixture of TFE:AcOH:DCM (1:1:3, 300 μ□) for 3 x 1 h. The resin was then subjected to a cleavage mixture of TFE:AcOH:DCM (1:1:3, 300 μ□) for 3 x 1 h. The filtrates were collected and analyzed directly (with no lyophilization) by analytical HPLC-MS using a Phenomenex column (5 μL, 250 x 4.6 mm) at a flow rate of 1 mL/min with 50–90% MeCN/H_2O gradient for 30 min. The side chain protecting groups were then cleaved with 95:2.5:2.5 TFA:TIPS:H_2O,, the peptide was crashed out with cold ether, redissolved in 1:1 MeCN/H-

$_2$O, and lyophilized. The resulting white solid was purified on a Hypersil GOLD column (5 μL, 150 x 10 mm) at a flow rate 5 mL/min with 10–50% MeCN/H_2O gradient for 30 min. The purified product was analyzed by analytical HPLC-MS using a Phenomenex column (5 μL, 250 x 4.6 mm) at a flow rate of 1 mL/min with 10–50% MeCN/H_2O gradient for 30 min. *m/z* ES calc'd for carboxy-oxytocin $[(C_{43}H_{66}N_{12}O_{12}S_2)+1]^+$: 1007.19; observed: 1008.25.

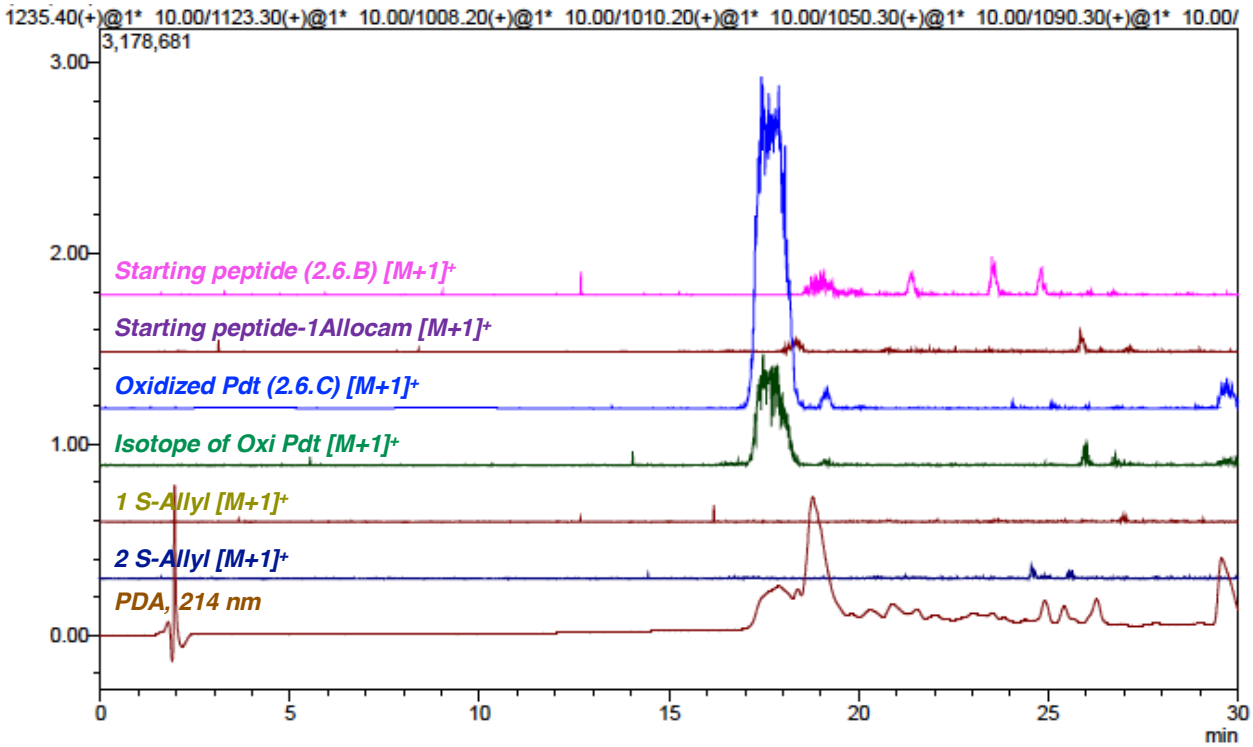


Figure 2.21 Analytical Column PDA for crude carboxy-oxytocin **2.6.C**. Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 17.67 min

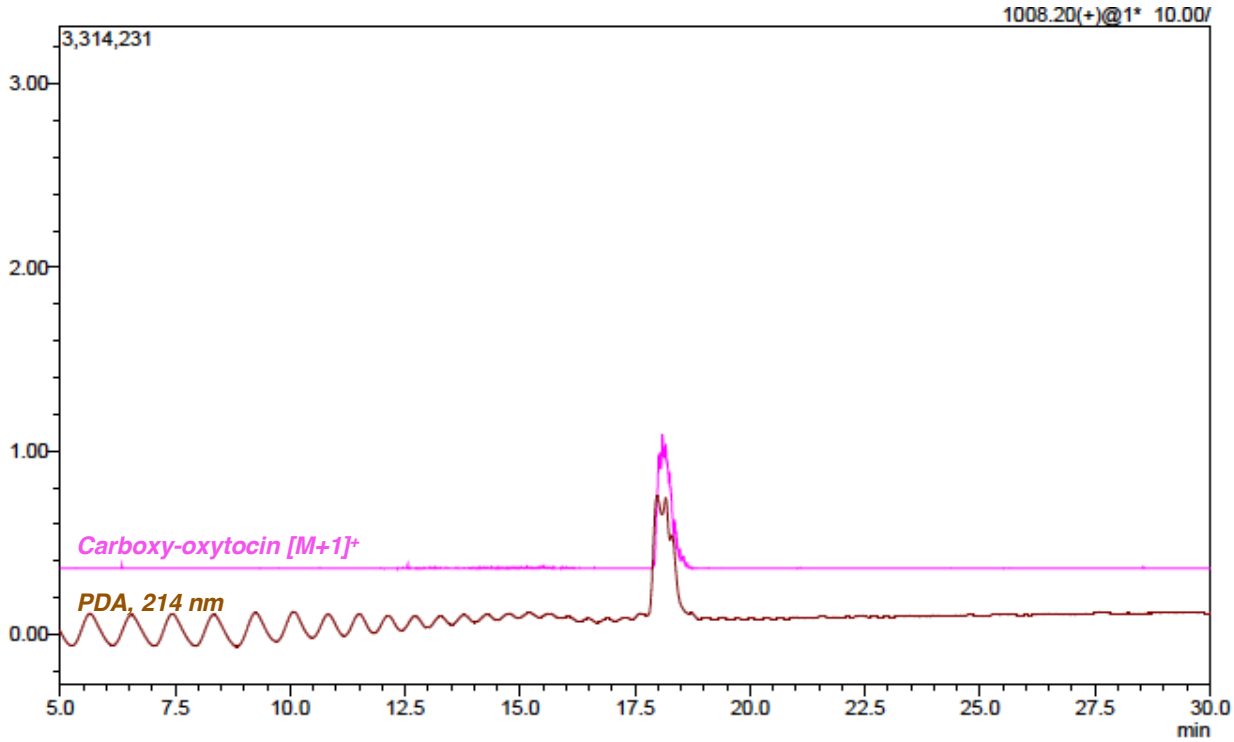


Figure 2.22 Analytical Column PDA for pure carboxy-oxytocin **2.6.C**. Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 17.67 min

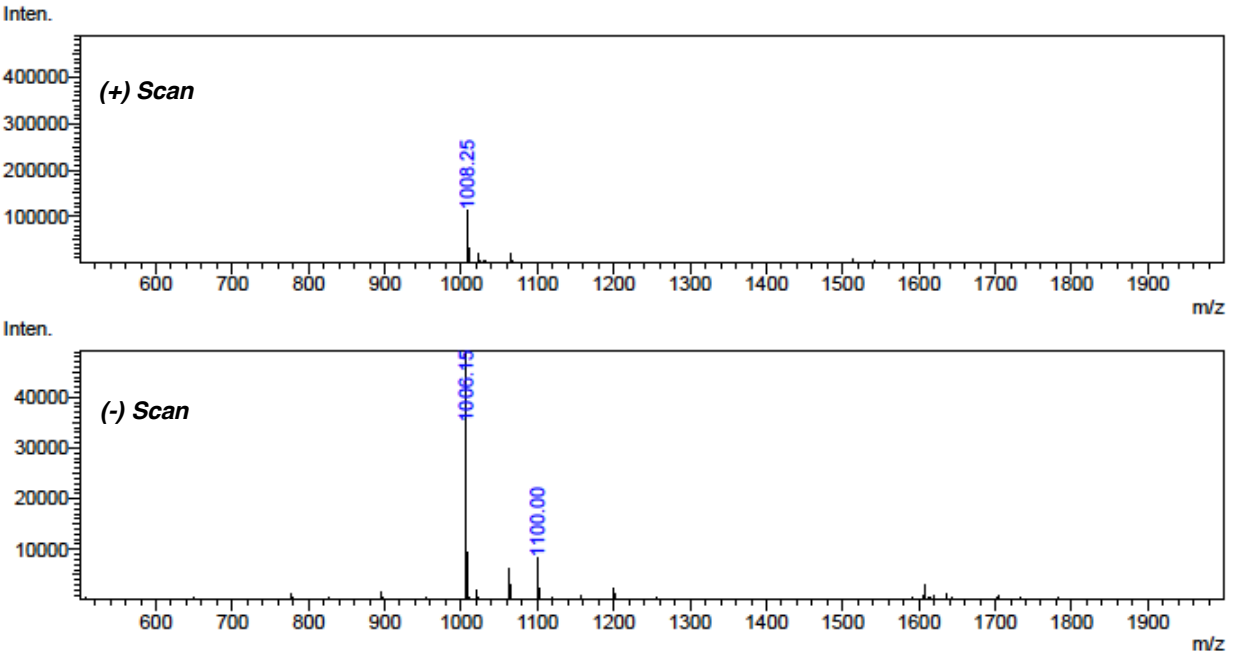


Figure 2.23 ES-MS spectrum for pure carboxy-oxytocin **2.6.C** at retention time 17.67 min

CHAPTER 3: ASSESSING ALLOCAM: ON-RESIN SYNTHESIS AND CONTROLLED FOLDING OF α-CONOTOXIN LvIA

3.1 Introduction

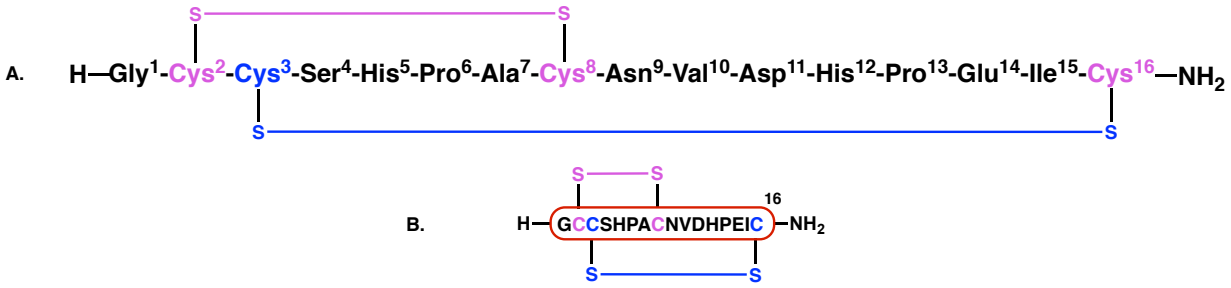
Conotoxins are a group of neuroactive peptides with multiple disulfide linkages and have considerable potential as both therapeutics and biochemical probes.[136,137] The synthesis of conotoxins would enable both biological studies and evaluation of both the ion-gated and ligand-gated structures as lead target for therapeutics.[138,139] The success in synthesis of oxytocin using Allocam-protected cysteine led us to discover the potential of our $Pd^{0/II}$ - DMSO based Allocam cleavage-oxidation strategy to generate two disulfide linkages in peptides with their desired pattern. This chapter details two on-resin synthetic approaches to access α4/7-conotoxin LvIA using Allocam in combination with other cysteine protecting groups.

3.2 α4/7-Conotoxin LvIA

3.2.1 α4/7-Conotoxin LvIA: Structure and Bioactivity

α4/7-conotoxin LvIA is a C-terminally amidated mini-peptide from *C. lividus* with 16 amino acid residues. It is the first α-conotoxin isolated from *C. lividus* and has 4 cysteine residues, resulting two disulfide bridges with constrained conformation.[140] These disulfide bridges lie between Cys^2-Cys^8 and Cys^3-Cys^{16} (**Figure 3.1**). The first loop or bridge contains 4 amino acid residues, and the second loop consists of 7 amino acid residues.

Figure 3.1 A. Structure of α4/7-conotoxin LvIA **B.** Graphical representation of α4/7-conotoxin LvIA structure

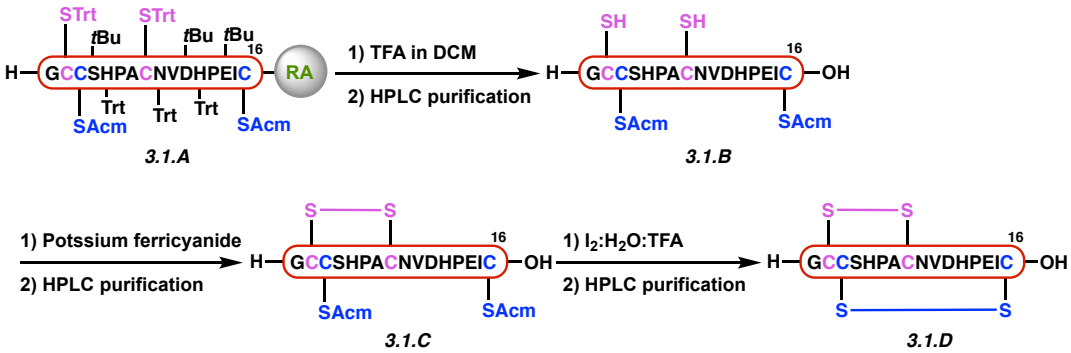


Being an α-conotoxin, α4/7-conotoxin LvIA has unique selectivity on nicotinic acetylcholine receptors (nAChRs) subtypes and mainly block mammalian neuronal nAChRs.[141] Neuronal nAChRs consists of combinations of α (α2-α10) and β (β2-β4) subunits and exit as a complex family of heteromeric and homooligomeric pentameric proteins.[142] These subtypes are important in normal physiology and in a wide range of disease states such as pain, addiction, Alzheimer's disease, myasthenia gravis, schizophrenia, epilepsy, and breast and lung carcinoma.[143,144] α-conotoxins are valuable tools for identifying and probing the composition and roles of nAChR subtypes.[145] Interestingly, α4/7-conotoxins have the ability to discriminate between diverse neuronal α–β nAChR subunit combinations. α4/7-conotoxin LvIA is selective for the α3β2 nAChR, and it is the first α-conotoxin that blocks human α3β2 with high selectivity over α6β2* nAChRs.[146] The receptor residues involved in its binding have been identified, and an analog has been generated that selectively targets α3β2 and α3β6 nAChRs. This analog also facilitated the identification of α3β4* receptors as the major subtype present in human adrenal chromafinn cells.[147]

3.2.2 α4/7-Conotoxin LvIA: Published Chemical Synthesis

Walker and co-workers have reported a solution phase chemical synthesis of α4/7-Conotoxin LvIA (**Scheme 3.1**).[148] In their synthesis protocol, they used Trt and Acm groups to protect cysteine thiols. After linear synthesis of the peptide **3.1.A** using Fmoc-SPPS on rink amide resin, the peptide was removed from the resin and purified. The first disulfide bond was formed in solution using 20 mM potassium ferricyanide in a Tris buffer (pH 7.5). The resulting monocyclic peptide **3.1.C** was purified by RP-HPLC before the next folding step. Then, a simultaneous Acm group removal and oxidation was done using iodine oxidation to close the second disulfide linkage. To perform iodine oxidation, they dripped the monocyclic peptide and HPLC eluent into a solution of iodine:H_2O:trifluroacetic acid (78:2:20 by volume) and used ascorbic acid diluted 20-fold with 0.1% TFA to quench the reaction. Finally, a third purification step had to be performed using RP-HPLC to get a pure bicyclic product **3.1.D**.

Scheme 3.1 Published in-solution synthesis of α4/7-conotoxin LvIA

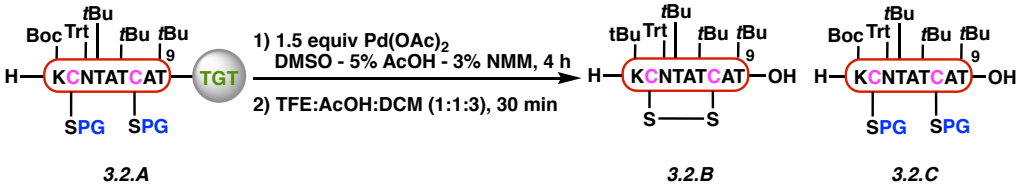


The above synthetic process contains total of three purifications steps, which consume more time and diminish the yield. In addition, there are two in-solution manipulations of the peptide, which introduces possibility of solubility and/or aggregation issues.

3.3 Orthogonality of Other Cysteine Protecting Groups Towards Pd-based Allocam removal

In order to access two disulfide systems like α4/7-conotoxin LvIA, in addition to Allocam group, another cysteine protecting group is needed for the regioselective synthesis. The suitable candidate must have stability towards $Pd^{0/II}$ - DMSO based cleavage-oxidation condition. Therefore, the orthogonality of common cysteine protecting groups such as Trt, Acm, Mmt and *t*Bu towards Pd-based Allocam removal condition was evaluated using the model peptide $IAPP_{1-9}$ (**Table 3.1**)

Table 3.1 Stability of cysteine protecting groups towards Pd-based Allocam removal condition



3.2.A *3.2.B* *3.2.C*

Entry	PG	*3.2.B*	:	*3.2.C*
1.	Trt	00		100
2.	Mmt	20		80
3.	Acm	90		10
4.	*t*Bu	00		100

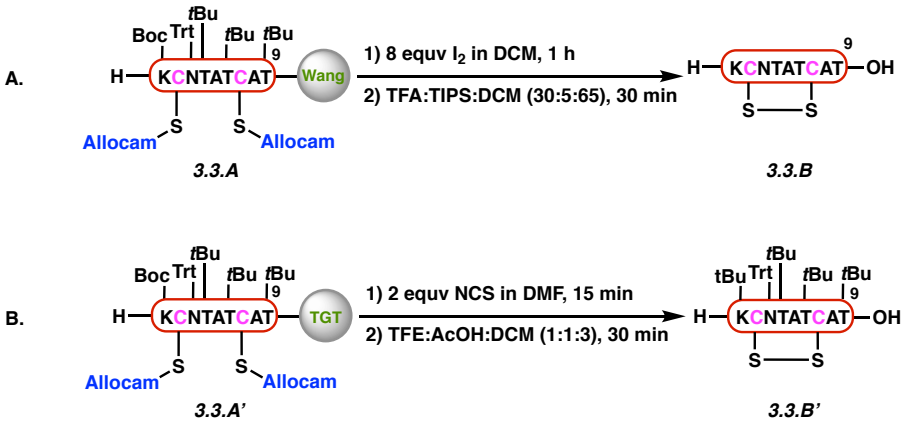
Four batches of the model peptide $IAPP_{1-9}$ were made using Cys(Trt), Cys(Mmt), Cys(Acm) and Cys(*t*Bu). Each peptide was subjected to the optimized $Pd^{0/II}$ - DMSO based Allocam removal condition discussed in Chapter 2. Peptides with Trt and *t*Bu protected cysteines remained unreacted (entry 1 and entry 4) while peptide with Mmt and Acm protected cysteines gave 20% (entry 2) and 90% (entry 3) of the expected oxidized product **3.2.B** respectively, compared to the starting peptide . This experiment concluded

that both Cys(Trt) and Cys(*t*Bu) are stable to the $Pd^{0/II}$ - DMSO based cleavage-oxidation condition and both Cys(Mmt) and Cys(Acm) groups can be cleaved during the Allocam removal process.

3.4 Stability of Allocam Group Towards Other On-Resin oxidants

After evaluating the orthogonality of other cysteine protecting groups towards Pd-based Allocam removal condition, next, the stability of Allocam group towards other on-resin oxidants was examined. Iodine[149] and NCS[150] are commonly used oxidants for on-resin disulfide formation. Therefore, the model peptide $IAPP_{1-9}$ with Allocam protected cysteines was subjected to iodine and NCS oxidation condition separately (**Scheme 3.2**).

Scheme 3.2 $IAPP_{1-9}$ under **A.** I_2 oxidation and **B.** NCS oxidation condition



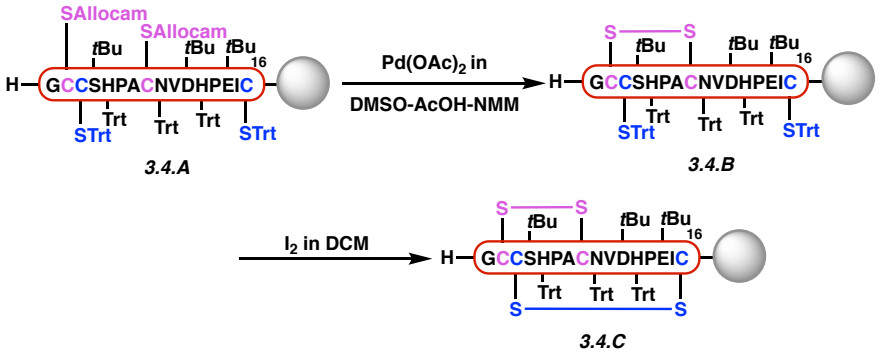
It was observed that Allocam is not stable under both iodine and NCS oxidation conditions. The model peptide **3.3.A** on Wang resin was subjected to general iodine-mediated oxidation conditions (**Scheme 3.2.A**). Interestingly, simultaneous Allocam cleavage and oxidation was observed. With Albericio conditions[79], 2 equivalents of NCS in DMF for 15 min (**Scheme 3.2.B**), 50% conversion of the oxidized product **3.3.B'** was observed.

3.5 On-Resin Synthesis of α4/7-Conotoxin LvIA

3.5.1 Synthesis of α4/7-Conotoxin LvIA using Cys(Allocam) and Cys(Trt) on CTC Resin

Considering results from both the experiments discussed in sections 3.1 and 3.2, our first attempt for on-resin synthesis of α4/7-conotoxin LvIA was use of Trt group as the second cysteine protecting group along with Cys(Allocam). In the synthesis procedure, simultaneous removal of Allocam and oxidation followed by Trt group removal and oxidation using I_2 in DCM can be done to obtain the two disulfide linkages selectively (**Scheme 3.3).** Stability of Trt group towards $Pd^{0/II}$ - DMSO based cleavage-oxidation condition allowed us to use it for the second disulfide bond formation. But, it cannot be used to form the first disulfide linkage since Allocam group is unstable to I_2 oxidation.

Scheme 3.3 On-Resin Synthesis of α4/7—Conotoxin LvIA using Cys(Allocam) and Cys(Trt)

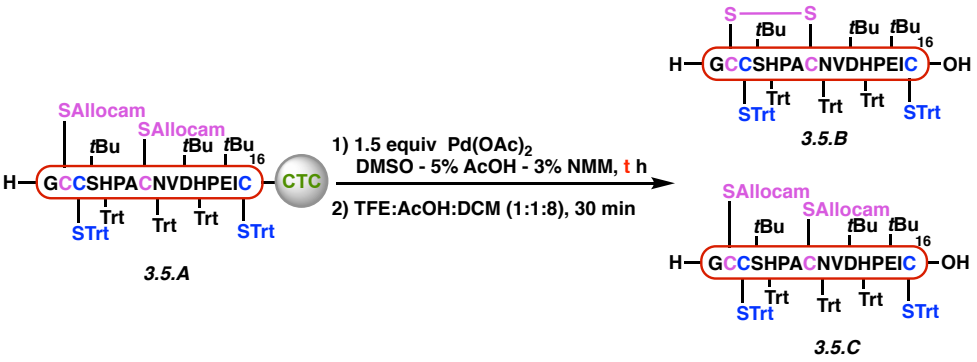


To evaluate feasibility of the proposed synthesis, linear synthesis of the peptide with Cys(Allocam) and Cys(Trt) was done on CTC resin. Although C-terminal end of α4/7-conotoxin LvIA is an amide, CTC resin, which gives C-terminal carboxylic acid after peptide cleavage, was used to facilitate analysis of the reaction. CTC resin cleavage requires mild acid condition such as TFE:AcOH:DCM (1:1:8), which results peptides with side chain protecting groups including Cys(Allocam) and Cys(Trt). This helps in reaction

optimization for each step of the synthesis. Reactions were monitored via analytical RP-HPLC-MS. The ratio of starting material to product was monitored and optimization was done until complete disappearance of the starting material in the system.

First, optimization of step one was done, which is Allocam removal and oxidation using $Pd^{0/II}$ - DMSO based cleavage-oxidation condition (**Table 3.2**).

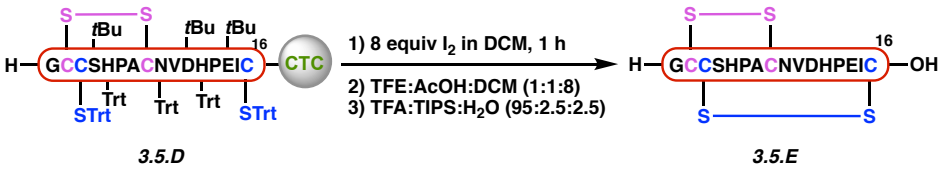
Table 3.2 Optimization for Pd-mediated Allocam cleavage and oxidation



Entry	Rxn time (h)	*3.X.B*	:	*3.X.C*
1.	4	80		20
2.	6	100		00

The linear peptide **3.5.A** was subjected to the optimized $Pd^{0/II}$ - DMSO based Allocam cleavage condition for IAPP $_{1-9}$ described on chapter 2. A ratio of 80:20, the desired folded product **3.5.B** and the starting peptide **3.5.C** was observed (entry 1). When the reaction time was increased from 4 hours to 6 hours, keeping equivalents of Pd the same, the reaction can be pushed towards 100% conversion of the product with desired disulfide bond **3.5.B** (entry 2). After optimizing the first step of the synthesis, the reaction was scaled up and the resulting solid supported peptide **3.5.D** was forward to the second step. Traditional I_2 oxidation was done using 8 equivalents of iodine in DCM to form the second disulfide bond. Here, Trt group removal and oxidation occur in a single step (**Scheme 3.4**).

Scheme 3.4 I_2 oxidation for second disulfide formation



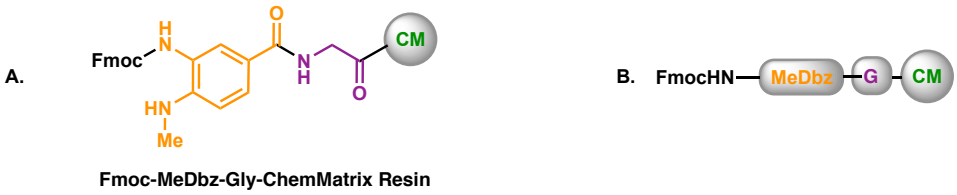
3.5.D *3.5.E*

After each step, the resin was washed thoroughly to remove all the reagents used. The final peptide was cleaved from the resin using TFA:AcOH:DCM (1:1:8) mixture and all the remaining side chain protecting groups were cleaved with 95% TFA to obtain fully deprotected carboxy–α4/7-conotoxin LvIA **3.5.E** with desired folding pattern. This illustrated the potential of orthogonal combination of Allocam and Trt groups for cysteine protection to form on-resin selective disulfide bonds to access carboxy–α4/7-conotoxin LvIA.

3.5.2 Synthesis of α4/7-Conotoxin LvIA using Cys(Allocam) and Cys(Trt) on MeDbz-G-ChemMatrix Resin

With the optimized conditions on hand, we wanted to synthesize α4/7-conotoxin LvIA on a resin which gives C-terminal carboxamide after cleavage. Our first choice was rink amide resin. Due to the failure in linear synthesis of the peptide on rink amide resin, we had to select another suitable resin for the synthesis. This problem let to development of a novel method for C-terminal modification of peptides,[151,152,153] while we employed as shown in **Figure 3.2**. ChemMatrix resin with MeDbz linker[154,155,156] (**Figure 3.2.A**) was employed to synthesize α4/7-conotoxin LvIA. Peptides on MeDbz linker found to be cleaved with a balloon of NH_3, after activating the linker, to achieve peptide carboxamide.[152]

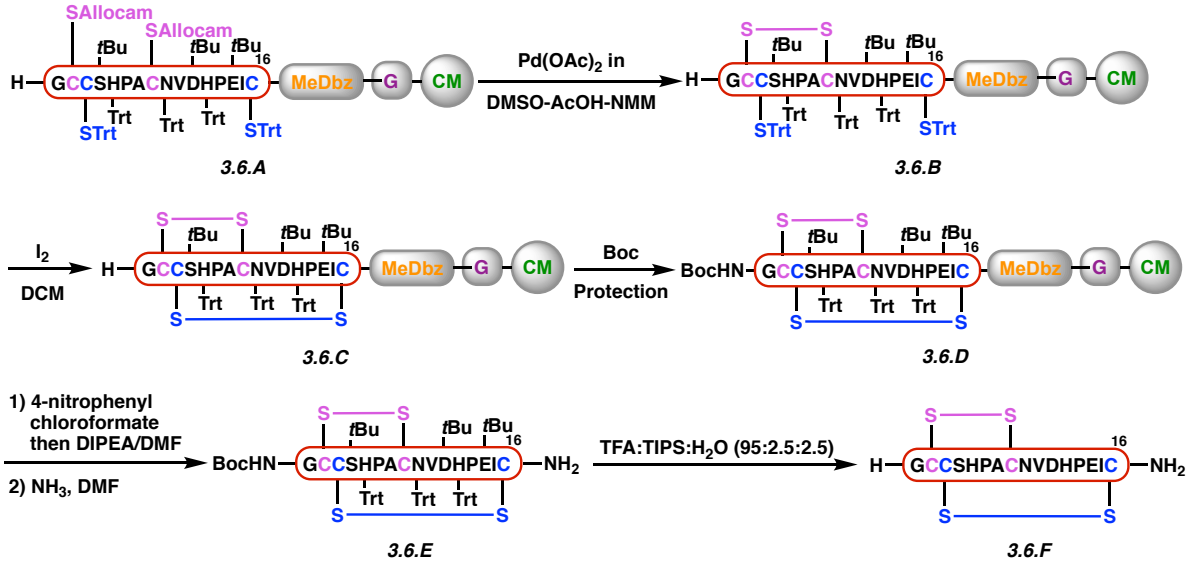
Figure 3.2 A. Structure of MeDbz linker-Gly-ChemMatrix Resin **B.** Graphical representation of MeDbz linker-Gly-ChemMatrix Resin



Fmoc-MeDbz-Gly-ChemMatrix Resin

When peptides are cleaved from MeDbz linker, the N-terminal needs to be protected to prevent acylation during linker activation.[153] On the other hand, for Pd-mediated Allocam removal process, the N-terminal of the peptide should be free in order to use less Pd loading.[157] Therefore, in the synthesis of α4/7-conotoxin LvIA on MeDbz linker-G-ChemMatrix resin, an additional step needed to be done to protect N-terminal of the peptide before cleavage from the resin (**Scheme 3.5**).

Scheme 3.5 On-Resin Synthesis of α4/7-Conotoxin LvIA on MeDbz-G-ChemMatrix Resin



After success in the linear synthesis of the peptide **3.6.A** on MeDbz-G-ChemMatrix resin, it was first subjected to optimized $Pd^{0/II}$ - DMSO based Allocam cleavage condition to form on-resin peptide **3.6.B** with first S-S bond**.** With 1.5 equivalences of Pd in DMSO-AcOH-NMM solvent system for 6 hours, 100% of the desired product **3.6.B** was obtained as

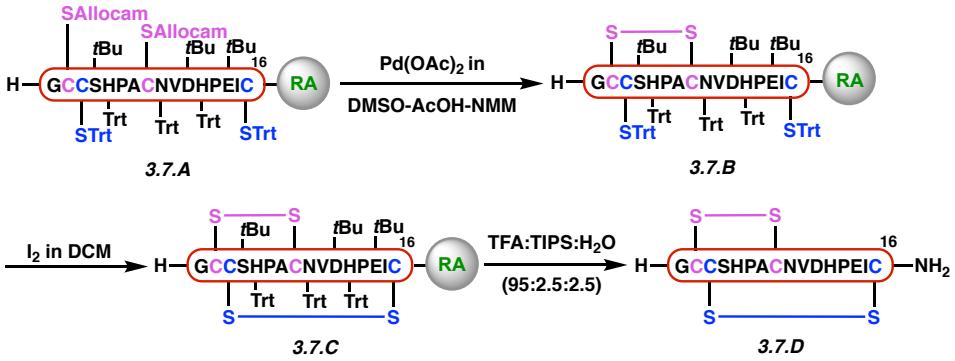
expected. The mono-cyclic peptide **3.6.B** was then exposed to I_2 oxidation for 1 hour to form the second S-S bond **3.6.C**. Before cleaving the peptide from resin, Boc protection was done to the N-teminal using $(Boc)_2O$ in DCM along with DIPEA as the base. Next, MeDbz linker was activated with 4-nitrophenyl chloroformate followed by bubbling NH_3 in to a solution of peptide in DMF to result Boc-protected and side chain protected peptide **3.6.E.** Finally, 95% TFA cleavage was done to obtain fully deprotected peptide **3.6.F** with desired disulfide bonds. All the steps in the synthesis proceeded as expected except the last step, the Boc group proved resistant to acidic cleavage. Even though the final product was subjected to 95% TFA for longer exposer time, Boc group deprotection was unsuccessful.

3.5.3 Synthesis of α4/7-Conotoxin LvIA using Cys(Allocam) and Cys(Trt) on Rink Amide Resin

After several attempts, we were able to optimize Fmoc-SPPS condition for the linear synthesis of α4/7-conotoxin LvIA with Cys(Allocam) and Cys(Trt) on rink amide resin. This led us to evaluate all the optimized on-resin folding steps on rink amide resin and synthesize α4/7-conotoxin LvIA with carboxyamide functionality (**Scheme 3.6**). First, the linear peptide **3.7.A** was subjected to the optimized $Pd^{0/II}$ - DMSO mediated Allocam cleavage condition for CTC resin. The reaction didn't go to completion with 1.5 equivalent of $Pd(OAc)_2$ loading. The reaction was repeated with 3.0 equivalent of $Pd(OAc)_2$ loading for 6 hours and complete disappearance of starting peptide was observed along with the product **3.7.B** with desired disulfide bond. Next, the resulting solid supported mono cyclic

peptide **3.7.C** was subjected to traditional I_2 oxidation to obtain the second disulfide bond **3.7.D**.

Scheme 3.6 On-Resin Synthesis of α4/7—Conotoxin LvIA using Cys(Allocam) and Cys(Trt) on Rink Amide Resin



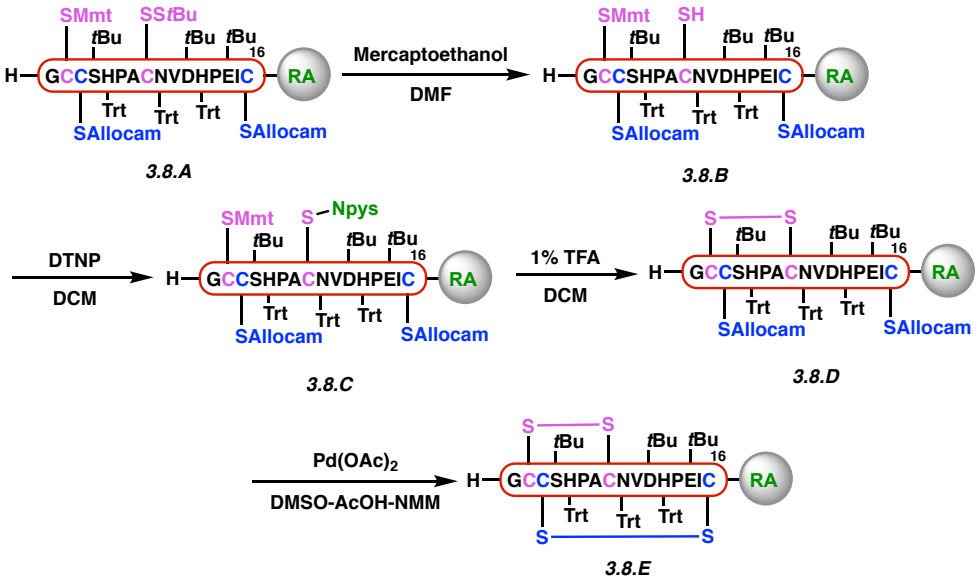
Treatment of 95% TFA followed by RP-HPLC purification gave the fully deprotected α4/7-conotoxin LvIA **3.7.E** in ~1% isolated yield.

3.5.4 Synthesis of α4/7-Conotoxin LvIA using Cys(StBu), Cys(Mmt) and Cys(Allocam) on Rink Amide Resin

After accessing α4/7-conotoxin LvIA with Cys(Allocam) and Cys(Trt), where simultaneous Allocam removal and oxidation followed by Trt group removal and oxidation was done on solid support, we wanted to evaluate the utility of Pd-based Allocam removal and oxidation method to generate a second S-S bond selectively in mono cyclic peptides. To form the first disulfide linkage, the method reported by Tung and co-workers was selected.[81] According to their method, S*t*Bu and Mmt was used as cysteine protecting groups, which facilitate on-resin disulfide bond formation without an oxidant. As the first step, the Cys(S*t*Bu) deprotection can be done using 20% mrecaptoethanol in DMF and the resulting free SH **3.8.B** can be activated with 10-fold excess of DTNP in DCM to form 5-Npys-activated disulfide **3.8.C**. Removal of Mmt group with mild TFA condition in

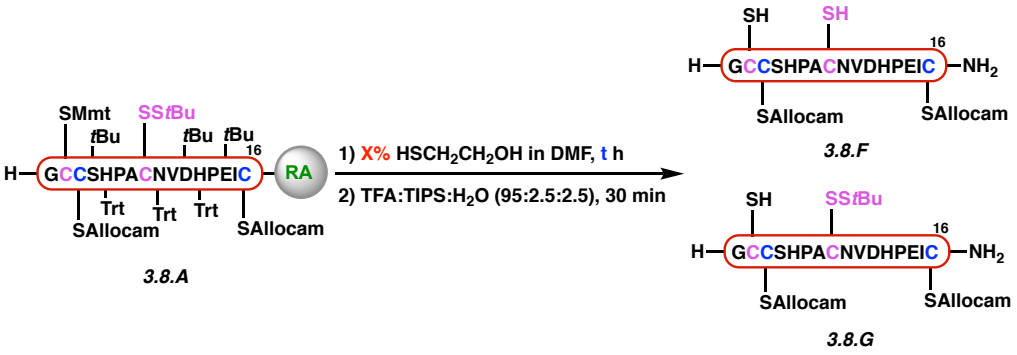
presence of TIPS facilitates the disulfide bond formation in the peptide **3.8.D**. After formation of first disulfide bond, our Pd-based simultaneous removal of Allocam and oxidation method can be done to form the second disulfide bond (**3.8.E)** (**Scheme 3.7**).

Scheme 3.7 On-Resin Synthesis of α4/7-Conotoxin LvIA with Cys(S*t*Bu), Cys(Mmt) and Cys(Allocam)



Linear synthesis of α4/7-conotoxin LvIA with Cys(S*t*Bu), Cys(Mmt) and Cys(Allocam) was successfully done using optimized Fmoc-SPPS conditions. Optimization of each step was then begun starting with thiol exchange of the S*t*Bu group (**Table 3.3**). Linear α4/7-conotoxin LvIA on rink amide resin **3.8.A** was first subjected to 20% mercaptoethanol at room temperature for different time intervals. In 3 hours, it gave 40% conversion of the desired product **3.8.F** (entry 1)**.** When increase the time to 6 hours, 80% product conversion was observed (entry 2). More than 95% of product was observed when the reaction run for 12 hours (entry 3). Grøtli and co-workers have reported microwave conditions for S*t*Bu – Mmt mediated on-resin non-oxidative disulfide bond formation strategy.[82]

Table 3.3 Reaction optimization for Cys(StBu) deprotection



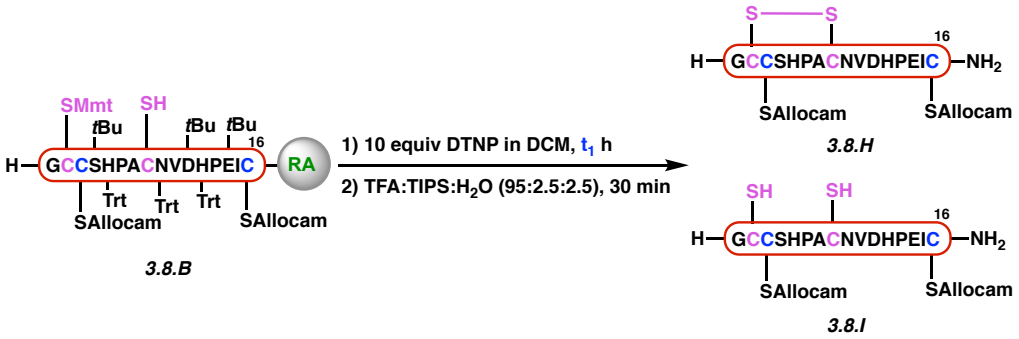
Entry	X%	Temperature	time (h)	*3.8.F* :	*3.8.G*
1.	20	R.T.	3	40	60
2.	20	R.T.	6	80	20
3.	20	R.T.	12	>95	<5
4.	30	60 °C, MW	2	>95	<5
5.	30	60 °C, MW	3	>98	<2

For Cys(S*t*Bu) deprotection, they used microwave heating at 60 ºC with 30% mercaptoethanol. In order to lower the reaction time for Cys(S*t*BU) deprotection, a reaction with 30% mercaptoethanol at 60 ºC under microwave heating was set up for 2 hours. This reaction gave 95% conversion of product (entry 4), while heating for 3 hours gave more than 98% of product conversion (entry 5). Therefore, it was concluded that 30% mercaptoethanol at 60 ºC under microwave heating for 3 hours is the optimal Cys(S*t*Bu) deprotection condition for the substrate **3.8.A**.

According to Tung and co-workers' displacement method for on-resin disulfide bond formation, the resulting free SH, after Cys(S*t*Bu) deprotection, needs to be activated using 10-fold excess of DTNP in DCM. Optimization for DTNP activation was done for Cys(S*t*Bu) deprotected peptide **3.8.B**. In terms of monitoring the reaction, we analyzed ratio of the desired folded product **3.8.H** and the fully reduced product **3.8.I**. If the reaction goes to 100% conversion, only desired folded product **3.8.H** should be observed in MS

after 95% TFA cleavage. Partially activated resins give mixture of folded and reduced products since, only peptides with 5-Npys-activated thiol group facilitates disulfide bond formation. First, reactions were done at room temperature for different time intervals (**Table 3.4**). Reactions which run for 1 hour (entry 1) and 3 hour (entry 2) time intervals gave about 50% and 65% of folded product respectively while 6 hour reaction (entry 3) gave 100% folded product, indicating all the thiol groups on the resin have been activated with 5-Npys group.

Table 3.4 Reaction optimization for activation of free thiol using DTNP



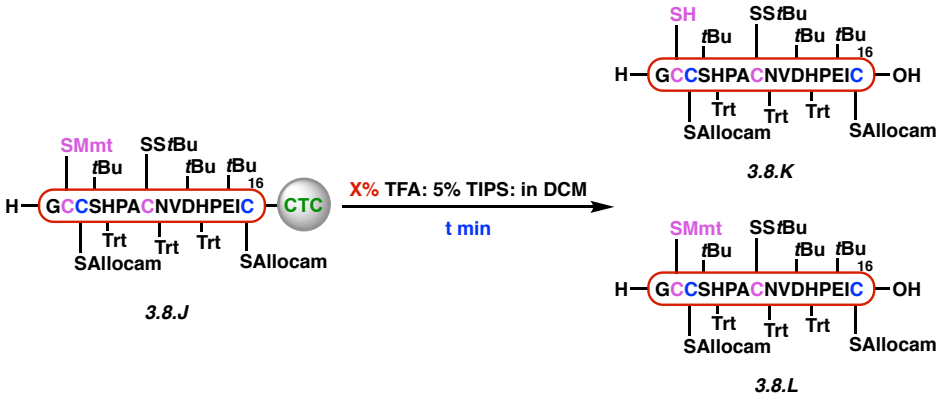
Entry	Temperature	time (h)	*3.8.H*	:	*3.8.I*
1.	R.T.	1	50		50
2.	R.T.	3	65		35
3.	R.T.	6	100		00
4.	60 °C, MW	1	100		00

In addition, microwave condition was tried similar to Grøtli and co-workers have reported in their studies. Under microwave heating at 60 °C, 100% activation of free SH from DTNP can be achieved within an hour (entry 4). When scaling up the reaction, microwave conditions didn't give consistent results. Hence, we concluded 6 hour room temperature conditions as the optimal conditions for this reaction.

According to the synthetic procedure, next step is to remove Mmt group using mild TFA condition to facilitate S-S bond formation. Although commonly used Cys(Mmt)

cleavage condition is 1% TFA in DCM, in our system, Mmt group is found to be not labile to such mild TFA condition. Therefore, reaction optimization had to be done to Mmt group removal and α4/7-conotoxin LvIA was synthesized on CTC resin as the substrate for the optimization. After the success in linear synthesis of α4/7-conotoxin LvIA on CTC, the peptide **3.8.J** was subjected to various percentages of TFA solutions in DCM with 5% TIPS for different time intervals (**Table 3.5**).

Table 3.5 Reaction optimization for Cys(Mmt) deprotection



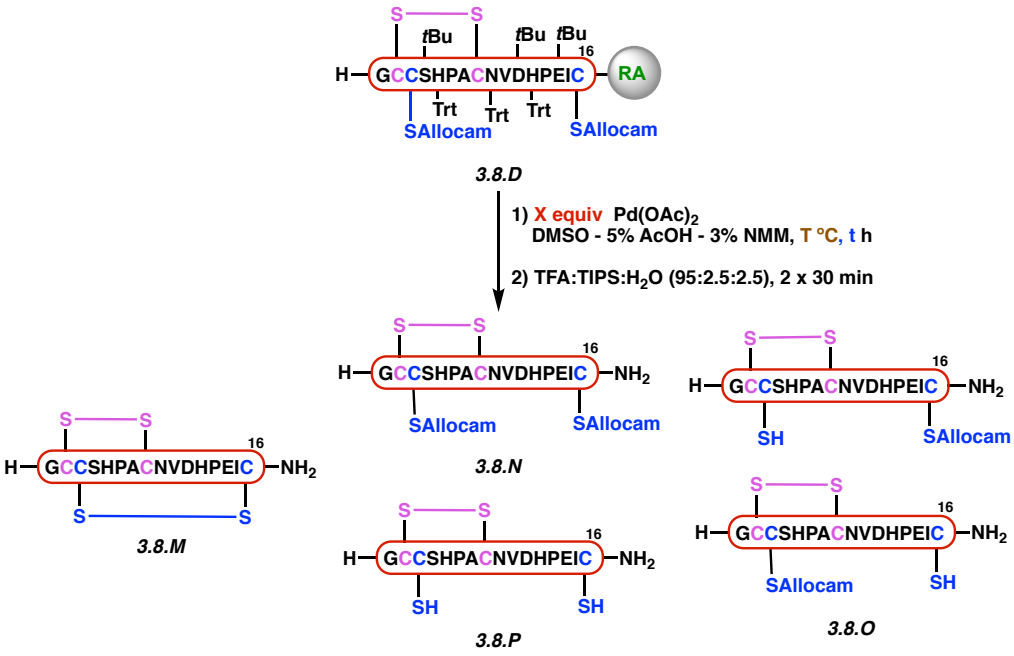
Entry	X%	time (min)	*3.8.K*	:	*3.8.L*
1.	2	30	✓		✗
2.	2	60	✗		✓
3.	3	15	✗		✓
4.	4	10	✗		✓
5.	4	5	✗		✓
6.	5	5	✗		✓

As CTC resin is sensitive to mild acidic conditions, the peptide cleavage can also be achieved in the same TFA condition as the reaction condition. The resulting peptides are immediately precipitated using cold diethyl ether, centrifuged, the ether layer was decanted, resulting precipitate was dissolved in 1:1 MeCN:H_2O and analyzed by MS directly for 100% product **3.8.K** peaks. With increased TFA concentration, Cys(Mmt) removal can be seen in shorter period of time. In order to have a minimal percentage of

TFA to remove Mmt group, 3% TFA condition was selected as the optimal reaction condition for this substrate (entry 3). For peptides with 5-Npys-activated disulfide group, 3% TFA treatment was done for 2 x 15 min to ensure total Mmt group cleavage and it was then subjected to DCM wash for 3 x 30 min until no yellow color in the washings. As the byproduct 5-nitropyridine-2-thione has an absorbance at 386 nm,[14] disappearance of yellow color in the washings indicates completion of disulfide bond formation. In addition, Ellman's reagent was used to analyze unreacted free thiol groups in the system. These steps led to the first folding of α4/7-conotoxin LvIA. The resulting peptide was then subjected to Pd-DMSO Allocam removal condition to obtain the second folding of the peptide (**Table 3.6**). For an incomplete reaction, in addition to the desired peptide **3.8.M**, starting peptide with both Allocam group on, **3.8.N**, and starting peptides with partial Allocam cleavage, **3.8.O**, were observed by MS. Therefore, when monitoring the reaction for completion, all the above structures were evaluated. Optimization was started with previously optimized conditions for caboxy-α4/7-conotoxin LvIA. With 1.5 equivalences of $Pd(OAc)_2$ at room temperature for 6 hours, reaction didn't go to completion as expected (enrty 1). Neither increased time nor increased Pd loading at room temperature pushed the reaction for 100% product conversion (entry 2 – entry 4). Assuming additional energy might improve reactivity of the reaction, microwave heating was applied to the system (entry 5 – entry 9). Various Pd loadings and reaction times were tried, but none of them led to complete disappearance of starting material in MS. In addition to the Pd-based Allocam removal condition, NCS oxidation condition was also used to achieve a complete conversion of the desired product. Different equivalencies of NCS and reaction times

were tried (**Table 3.7**), and no complete conversion of the reaction was observed. Interestingly, in most of these reaction conditions tried, major substrate observed was the peptide with one Allocam cleavage **3.8.O**.

Table 3.6 Reaction optimization for Pd-based Allocam removal and oxidation



Entry	X equiv.	Temperature	time (h)	*3.8.M*	: *3.8.N*	: *3.8.O*	: *3.8.P*
1.	1.5	R.T.	6	05	00	95	00
2.	1.5	R.T.	12	n.d.	n.d.	n.d.	n.d.
3.	3.0	R.T.	6	05	00	95	00
4.	7.0	R.T.	6	15	00	85	00
5.	3.0	60 °C MW	6	55	00	45	00
6.	3.0, 1.5	60 °C MW	6, 3	n.d.	n.d.	n.d.	n.d.
7.	3.0	60 °C MW	7	50	00	50	00
8.	3.0	60 °C MW	6	45	00	55	00
9.	5.0	60 °C MW	6	20	00	80	00

Table 3.7 Reaction optimization for NCS-mediated Allocam removal and oxidation

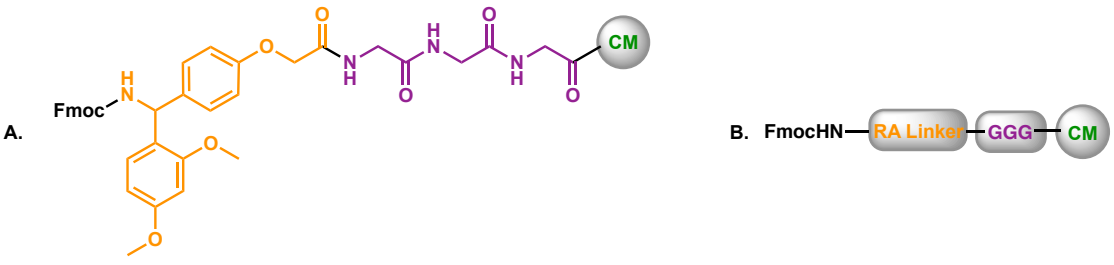
Entry	X equiv.	time (h)	*3.8.M*	: *3.8.N*	: *3.8.O*	: *3.8.P*
1.	5.0	6	02	00	95	00
2.	5.0	10	60	00	40	00
3.	7.0	6	85	00	15	00

The results of these reactions indicate that removal of one of the Allocam groups' is difficult. If we analyze the peptide structure, one of the Allocam protected cysteines is closer to the resin and sterically more hindered. Due to the fact Cys^{16}(Allocam) is closer to the resin, deprotection might be challenging.

3.5.5 Synthesis of α4/7-Conotoxin LvIA using Cys(StBu), Cys(Mmt) and Cys(Allocam) on Rink Amide Linker-GGG-ChemMatrix Resin

In order to overcome the challenge of terminal Cys^{16}(Allocam) removal we hypothesized that the steric hindrance around the peptide needed to be minimized. Therefore, we endeavor to increase the distance between the resin and the peptide by introducing a long linker in between them (**Figure 3.3**).

Figure 3.3 A. Structure of RA linker-Gly—Gly-Gly-ChemMatrix Resin
B. Graphical representation of RA linker-Gly-Gly-Gly-ChemMatrix Resin

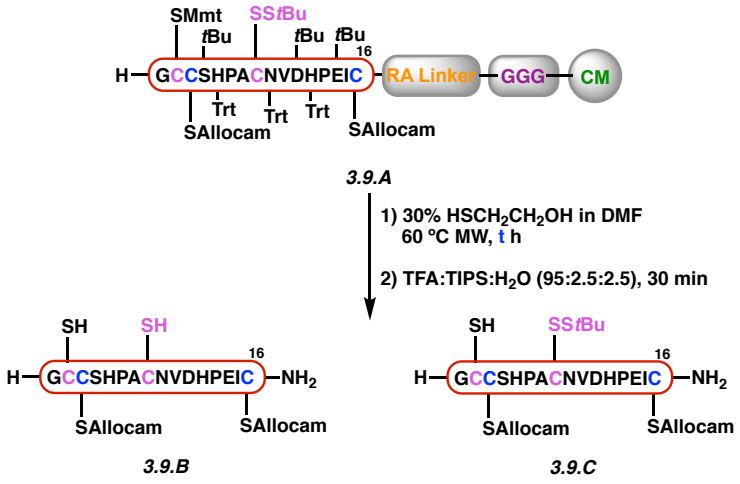


Fmoc—Rink amide linker-Gly-Gly-Gly-ChemMatric Resin

The linker (**A. Figure 3.3**) was synthesized successfully on ChemMatrix resin and then, linear synthesis of the peptide **3.9.A** was done, using optimized Fmoc-SPPS conditions, on the linker. Thereafter, reaction conditions for each step was evaluated. First, the

peptide **3.9.A** was subjected to microwave assisted optimized Cys(S*t*Bu) deprotection conditions described in section 3.6 (**Table 3.8**). With 3 hours, a total deprotection was observed as expected (entry 1). In chapter 02, we evaluated that PEG based ChemMatrix resin can proceed reactions faster than polystyrene based resins due to their superior swelling power. Therefore, we set up reactions with lower reaction times leaving microwave heating conditions the same. Interestingly, complete S*t*Bu removal was observed in 1.5 hours for our new system (entry 2).

Table 3.8 Reaction optimization for Cys(StBu) deprotection

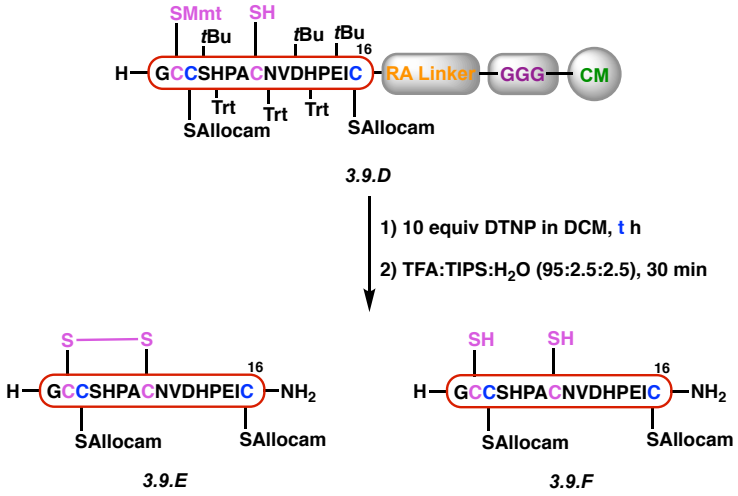


Entry	time (h)	*3.9.B*	:	*3.9.C*
1.	3	100		00
2.	1.5	100		00
3.	1	95		05

After S*t*Bu removal, the resulting free SH needed to be activated by DTNP. The reaction optimization was done for the peptide **3.9.D** with 10 equivalences of DTNP in DCM for different time intervals (**Table 3.9**). Even though it took 6 hours for the peptide on rink amide resin to get fully activated, for the same peptide on rink amide linker-Gly-Gly-Gly

ChemMatrix resin, it only took 1 hour (entry 3). Remarkably, the reaction time dropped drastically for this reaction.

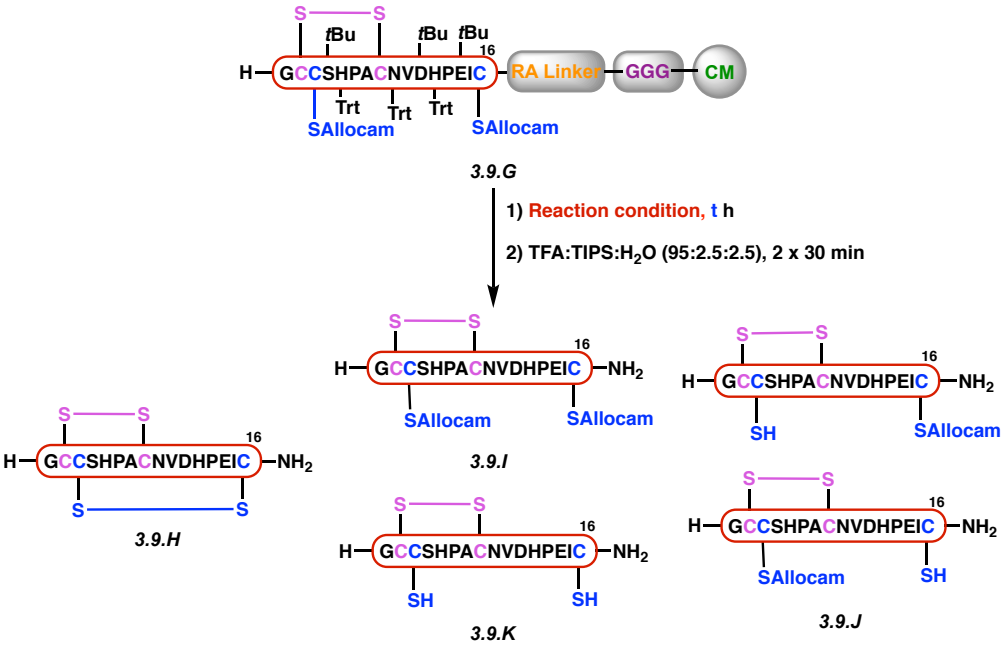
Table 3.9 Reaction optimization for Activation of free thiol using DTNP



Entry	time (h)	*3.9.E*	:	*3.9.F*
1.	6	100		00
2.	3	100		00
3.	1	100		00

After activating the free thiol group on the peptide, Mmt removal needed to be done to form the first disulfide bond after release of Npys group. In section 3.6, the selected condition for Mmt removal was 3% TFA in DCM for 15 min. Due to a significant peptide cleavage observed with longer exposer time, we switched the condition for shorter time by using 5% TFA in DCM for 5 x 1 min. After TFA treatment, the resin was allowed to agitate with DCM wash an hour to ensure complete displacement of 5-Npys group. Additionally, DCM wash for 3 x 30 min was done until no yellow color was observed in the washings. Furthermore, absence of free thiol groups was tested with Ellman's reagent to confirm complete folding of first S-S bond.

Table 3.10 Reaction optimization for Allocam removal and oxidation



Entry	Rxn condition	time (h)	*3.9.H (Yield%)*	:	*3.9.I*	:	*3.9.J*	:	*3.9.K*
1.	3.0 equiv $Pd(OAc)_2$ DMSO-5%AcOH-3%NMM	6	>95	(7)	00		<5		00
2.	5.0 equiv NCS in DMF	6	>99	(n.d.)	00		<1		00
3.	5.0 equiv I_2 in DCM	6	50		00		00		50
4.	8.0 equiv I_2 in DCM	6	>99	(18)	00		<1		00

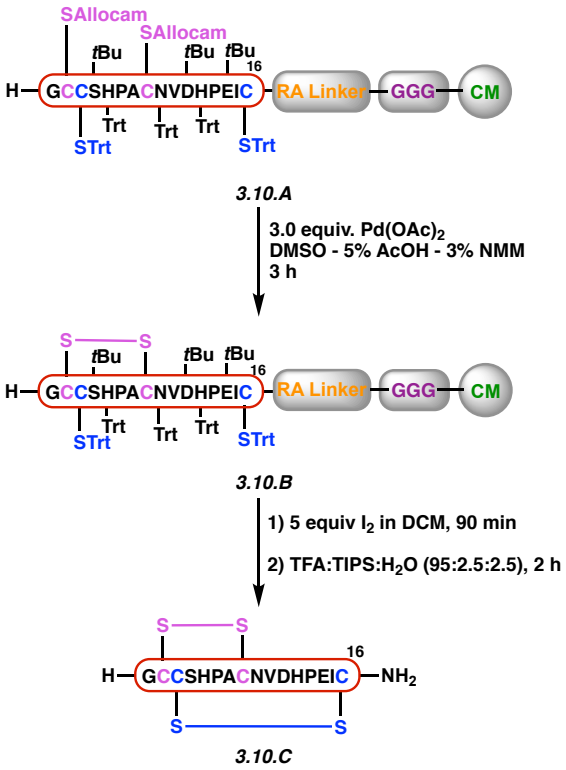
Three different procedures were tried simultaneously to form the second on-resin disulfide bond (**Table 3.10**). Previously optimized Pd-DMSO Allocam removal and oxidation condition gave about 95% conversion of desired product with 7% overall yield and significantly less partial Allocam cleavage substrate **3.9.J** (entry 1). The NCS condition also worked well for the new design where about 98% conversion was achieved with 5.0 equivalences of NCS in 6 hours reaction time (entry 2). This is a great improvement from what we observed in section 3.6. For the first time, we tried iodine condition for Allocam removal and oxidation. With 5.0 equivalences of iodine in DCM for 6 hours, about 50% oxidized product formation was observed (entry 3). When increase the iodine loading from

5.0 to 8.0 equivalences the reaction went to 100% conversion of the product with desired S-S pattern in 18% overall yield (entry 4).

3.5.6 Synthesis of α4/7-Conotoxin LvIA using Cys(Allocam) and Cys(Trt) on Rink Amide Linker-GGG-ChemMatrix Resin

After the success in the synthesis of α-Conotoxin LvIA on Rink amide linker-GGG-ChemMatrix resin (see section 3.5.5), we wanted to revisit the synthesis protocol discussed in section 3.5.3 on Rink amide linker-GGG-ChemMatrix resin.

Scheme 3.8 Synthesis of of α4/7—Conotoxin LvIA using Cys(Allocam) and Cys(Trt) on Rink Amide Linker-GGG-ChemMatrix Resin



The linear synthesis of the peptide was done using Cys(Allocam) and Cys(Trt) on Rink amide linker-GGG-ChemMatrix resin and the optimized conditions for each step were rerun for the new system (**Scheme 3.8**). Similar to what we observed in section 3.5.5, an increment in the reaction rates was observed. First disulfide bond formation can be

obtained in 3 hours with 3.0 equivalent of $Pd(OAc)_2$ in DMSO-AcOH-NMM solvent system, which required 6 hours for rink amide resin without the linker. An iodine oxidation was done to achieve the formation of second disulfide bond. Final TFA cleavage was done to access the fully deprotected α-Conotoxin LvIA, **3.10.C** with desired disulfide bonds.

3.6 Summary

In this chapter, we demonstrated the utility of Allocam group to access α4/7-conotoxin LvIA in combination with other cysteine protecting groups such as Trt, S*t*Bu and Mmt. Two routes were introduced based on position of the Allocam protected cysteines and selection of compatible other cysteine protecting groups for each of the synthetic routes. The linear synthesis of the α4/7-conotoxin LvIA and its reactivity towards folding were improved by using a three glycine linker in between the resin and the peptide. In addition to Pd-DMSO based simultaneous on-resin Allocam removal and oxidation condition, both I_2 oxidation and NCS oxidation can be used in on-resin Allocam removal and oxidation. Among the three deprotection-oxidation conditions, I_2 mediated simultaneous Allocam removal and oxidation gave the highest yield for selective on-resin synthesis of α4/7-conotoxin LvIA. The utility of Allocam group can be extended to synthesize peptides with more than two disulfide bonds by careful selection of orthogonally compatible other cysteine protecting groups.

3.6 Materials and methods

3.6.1 General Information

Unless otherwise specified, all commercially available reagents were purchased from Sigma-Aldrich and used without further purification. Anhydrous Et_2O, MeCN, DMF, DMSO, CH_2Cl_2 were purchased from Fisher. These were passed through a commercial solvent purification system (2 columns of alumina) and used without further drying. Hünig's base was distilled over CaH_2 immediately prior to use. All amino acids were purchased from Chem-Impex Int'l. Inc. unless otherwise noted. HATU were purchased from Chem-Impex Int'l. Inc. Unless otherwise noted, all reactions were performed in 2 mL, 5 mL and 10 mL Biotage reactor vials with PTFE frit (depend on reaction volume) at room temperature. All yields refer to chromatographically and spectroscopically pure products. All HPLC analyses and purifications were performed on a Custom Reverse Phase Shimadzu Liquid Chromatograph Mass Spectrometer (LCMS-2020), which can toggle between analytical and semi-preparative columns. This instrument has a photodiode array (PDA) detector (D2 & W lamp), which collects a range of wavelengths, in place of a traditional single channel UV detector. RP-HPLC-MS mobile phases (MeCN and H_2O) contained 0.1% Formic Acid. Analytical HPLC was performed on a Phenomenex Kinetex C18 column (5 μm, 250 x 4.6 mm) and a Thermo Scientific Hypersil Gold C8 column (5 μm, 250 x 4.6 mm). Semi-Preparative HPLC was conducted using a Thermo Scientific Hypersil Gold C8 column (5 μm, 150 x 10 mm). All peptide yields are calculated based on the final loading.

SPPS General Information. Solid-phase peptide synthesis was executed on a Biotage Isolera+ semi-automated synthesizer with microwave heating.

- *Reactor Vials [Vial size (Volume range allowed)]:* 2 mL reactor vial (0.8-1.1 mL), 5 mL reactor vial (1.6-3.2 mL), and 10 mL reactor vial (3.2-6.4 mL)
- *Swelling + Heat:* DMF was added and vortexed at 1200 RPM for 20 m at 70 ºC. The solvent was then removed over 1 m followed by two DMF washes (DMF was added and the suspension was vortexed at 600 RPM for 45 s, followed by the removal of solvent (over 2 m)).
- *Coupling:* A solution of Fmoc-aa-OH (3 equiv), HATU (3 equiv), and DIPEA (6 equiv) in DMF was made immediately prior to addition to the reaction vial containing the resin. Once the solution was added, the suspension was heated to 75 ºC (except for Fmoc-Cys-OH which was heated to 50 ºC) for 5 minutes with a vortex rate of 1200 RPM. After the reaction, the solution was removed (over 2 m) and the resin was rinsed with DMF 4 times (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).
- *Fmoc Removal (Deprotection):* The reactor vial was filled with 20% piperidine in DMF. The suspension was vortexed at 1200 RPM for 3 m at RT. The solvent is removed followed by addition of 20% piperidine in DMF. The suspension is vortexed again at 1200 RPM for 10 m at RT. The solvent was removed over 2 m, followed by 4 DMF washes (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).

- *Wash*: DMF was added to the reaction vial and agitated at a vortex rate of 1200 RPM for 1 m. The solvent was removed over 1 m and repeated for a total of 4 times.
- *Final Wash:* Resin was rinsed with CH_2Cl_2 (3 x 1 mL) and MeOH (3 x 1 mL).
- *Drying for Storage/Weighing:* After the final wash, the resin was placed on the lyophilizer overnight for drying.

General Procedure for Capping - First, 25% acetic anhydride in DMF (3 mL) was added and agitated for 5 min. Then,1.5 eqiuv. of DIPEA was added to the reaction and agitated for 30 min. Finally, the reaction solution was filtered and the resin was washed with DMF (5 x 3 mL).

Procedure for Resin Loading Analysis. Initial and final loading of resin was analyzed based on amount of Fmoc group.

Fmoc Deprotection – 10 mg of the resin was weighed out and swelled in DMF in the peptide synthesizer. Then, 1.00 ml of 20% piperidine in DMF was added and it was allowed to vortex in the peptide synthesizer at room temperature for 3 min. After 3 min, the filtrate was collected in to a separate tube, another 1.00 ml of 20% piperidine in DMF was added to it and allowed to vortex in the peptide synthesizer at room temperature for 10 min. The resulting filtrate was collected to the same tube and the deprotected resin was washed with DMF (4 x 1.75 ml).

Sample preparation for UV analysis – From the collected ”deprotection solution” above, 100 μL was transferred in to an eppendorf tube and it was diluted with 900 μl of DMF. The resulting diluted sample was used to get the absorbance at 301 nm versus a DMF blank using a UV spectrometer with 1 cm cuvette.

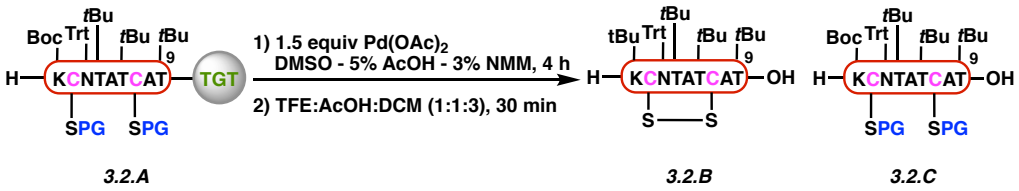
General Procedure for Pd-DMSO Cleavage/Oxidation. The peptide on the stated resin (10 mg) was swelled in DMF at room temperature for 30 minutes. After removing excess DMF, the cartridge was capped on the bottom. A solution of $Pd(OAc)_2$ in 5% AcOH/DMSO solution was added, and then the stated amount of NMM was added. The cartridge was capped on the top, attached to a magnetic stirbar retriever using a rubber band, and agitated for the stated amount of time. The reaction was filtered, and the resin was washed successively with with DMF (3 x 2 min), CH_2Cl_2 (3 x 2 min), 0.2 M sodium diethyldithiocarbamate in DMF (3 x 30 min), MeOH (3 x 1 min), DMF (5 x 1 min) and CH_2Cl_2 (3 x 1 min). *Note: resin changes from dark orange/brownish to yellowish/tan. The resin was then subjected to a stated cleavage mixture for 30 minutes.

Product analysis was done directly (with no lyophilization) by analytical RP-HPLC-MS using a Hypersil GOLD column (5 L, 150 x 4.6 mm) at a flow rate of 1 mL/min with stated gradient and time.

Ammonia Cleavage of Peptides on Resin: The stated amount of the peptide on resin (dry) was weighed out into a fritted reaction vial with an attached needle and rubber stopper (suggested selection of SPPS vial: 10-40 mg of peptide on resin goes into the 2 mL reaction vial, 40-200 mg in the 5 mL reaction vial and ≥200 mg goes into the 10 mL reaction vial). The resin was swelled in DCM (500 μL – 4 mL) for 0.5-1 h. The MeDbz linker was activated by a previously established protocol.[1] Next, 500 μL of DMF was added and the reaction vial was closed off with a septum (needle and stopper still attached at the bottom). A double balloon was made and filled with ammonia. A hypodermic needle was attached to the balloon and inserted into the septum and submerged into the DMF

solution. The smallest (blue) gauge needle was placed into the septum to allow for the slow exiting of gas. The ammonia gas was bubbled into DMF for 1.5 h. Then the solvent was removed and collected. Additional rinsing was required to completely remove the desired peptide from resin. DCM (3 x 1 mL) was used to rinse the resin, followed by MeCN (3 x 1 mL), and lastly DCM (3 x 1 mL). The washes were combined with the initial DMF solution, blown down with air, subjected to 1:1 MeCN:H_2O, frozen (LN_2), and lyophilized to yield the crude product.

3.6.2 Experimental Procedures and Spectroscopic Data



3.2.A *3.2.B* *3.2.C*

General Procedure for Table 3.1. The general procedure for Pd-DMSO cleavage-oxidation was followed. The reaction mixture was agitated in a closed vial at room temperature for 4 hours. After the wash, the dried resin was subjected to 300 μL of the cleavage mixture (1:1:3 TFE:AcOH:DCM) for 30 min. The filtrates were collected, concentrated and analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min.

Entry 1. The $IAPP_{1-9}$ with Cys(Trt) was used. The relevant quantities are: Resin **3.2.A** (10 mg, 2 μmol), $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (7.5 μL, 68.4 μmol) and DMSO-5% AcOH (250 μL). The reaction was analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-90% MeCN/H_2O

gradient for 30 minutes. m/z ES calc'd for peptide 3.2.C $[C_{108}H_{135}N_{11}O_{16}S_2 + 1]^+$: 1907.45; observed: 1908.40. m/z ES calc'd for peptide 3.2.C-Trt $[C_{89}H_{121}N_{11}O_{16}S_2 + 1]^+$: 1665.13; observed: 1665.70.

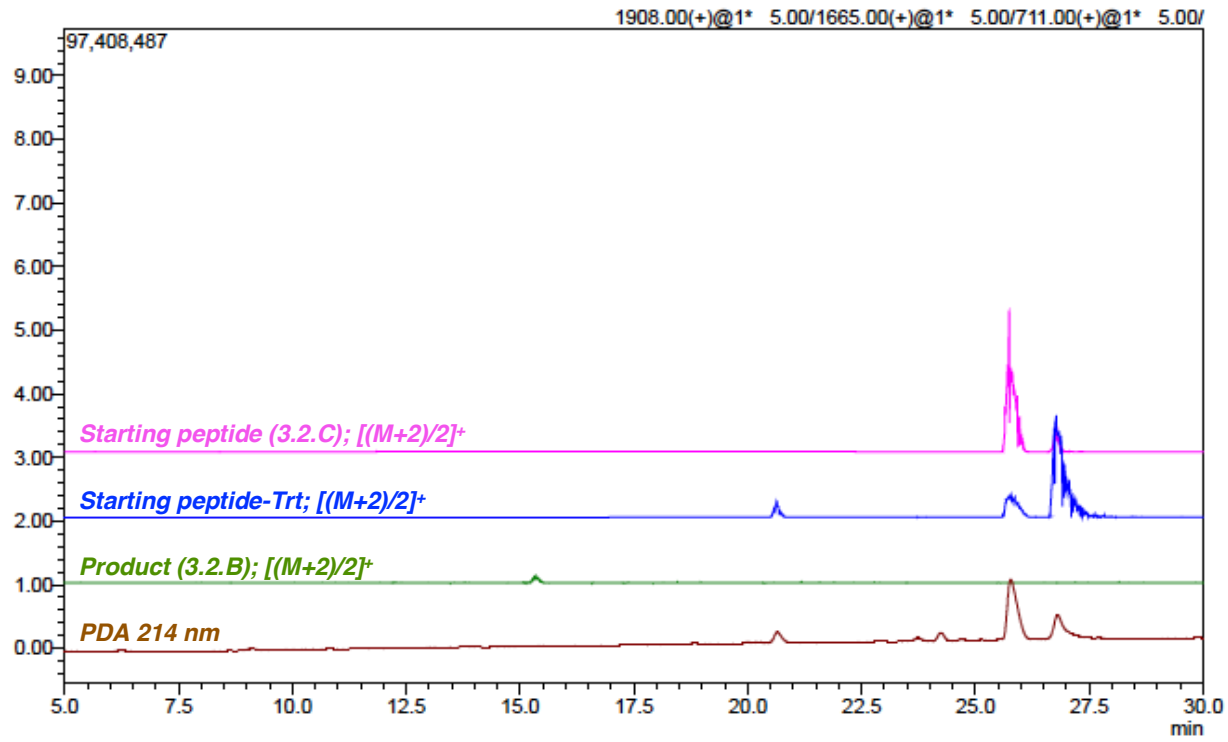


Figure 3.4 Analytical Column PDA for Table 3.1 – Entry 1

Entry 2. The $IAPP_{1-9}$ with Cys(Mmt) was used. The relevant quantities are: Resin **3.2.A** (10 mg, 2 μmol), $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (7.5 μL, 68.4 μmol) and DMSO-5% AcOH (250 μL). The reaction was analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-80% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.2.C $[C_{110}H_{139}N_{11}O_{18}S_2 + 1]^+$: 1967.50; observed: 1968.40. m/z ES calc'd for peptide 3.2.C-Mmt $[C_{90}H_{123}N_{11}O_{17}S_2 +$

1]$^+$: 1695.15; observed: 1695.75. m/z ES calc'd for peptide 3.2.B [($C_{70}H_{105}N_{11}O_{16}S_2$ + 2)/2]$^+$: 711.40; observed: 711.25.

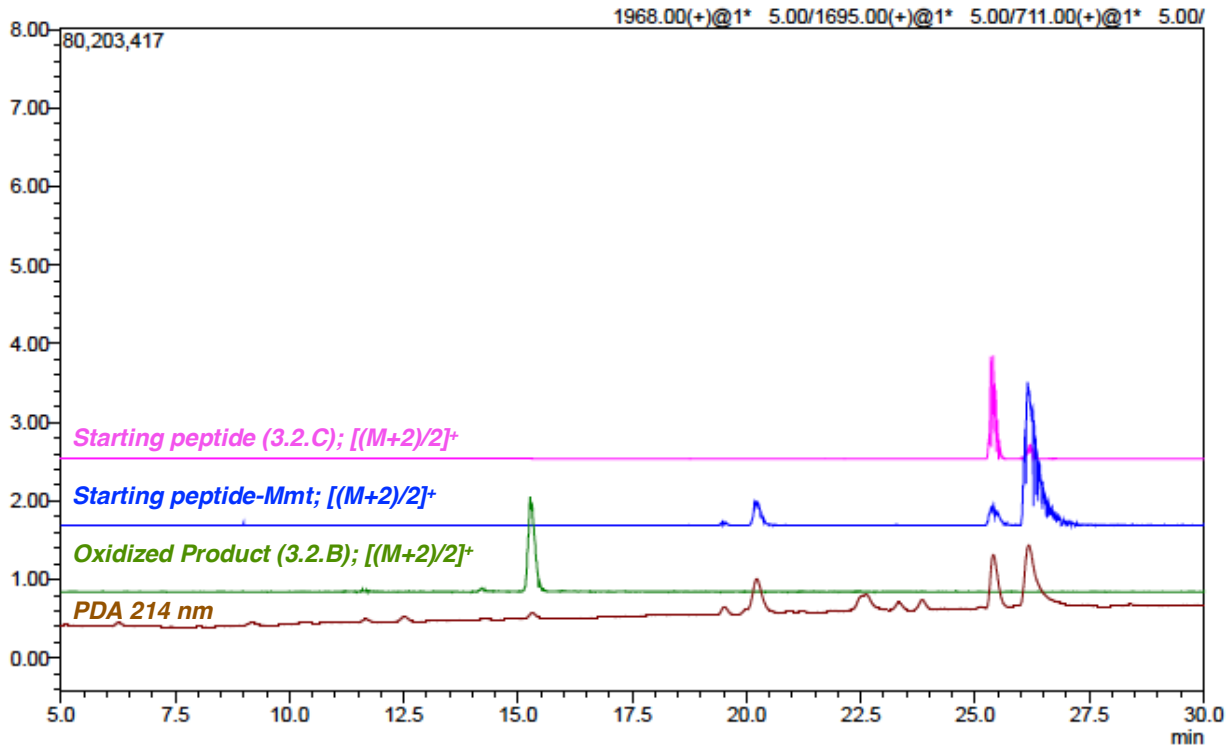


Figure 3.5 Analytical Column PDA for Table 3.1 – Entry 2

Entry 3. The $IAPP_{1-9}$ with Cys(Acm) was used. The relevant quantities are: Resin **3.2.A** (10 mg, 2 μmol), $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (7.5 μL, 68.4 μmol) and DMSO-5% AcOH (250 μL). The reaction was analyzed by analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-80% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.2.C [($C_{76}H_{117}N_{13}O_{18}S_2$ + 2)/2]$^+$: 783.48; observed: 783.20. m/z ES calc'd for peptide 3.2.8 [($C_{70}H_{105}N_{11}O_{16}S_2$ + 2)/2]$^+$: 711.40; observed: 711.20.

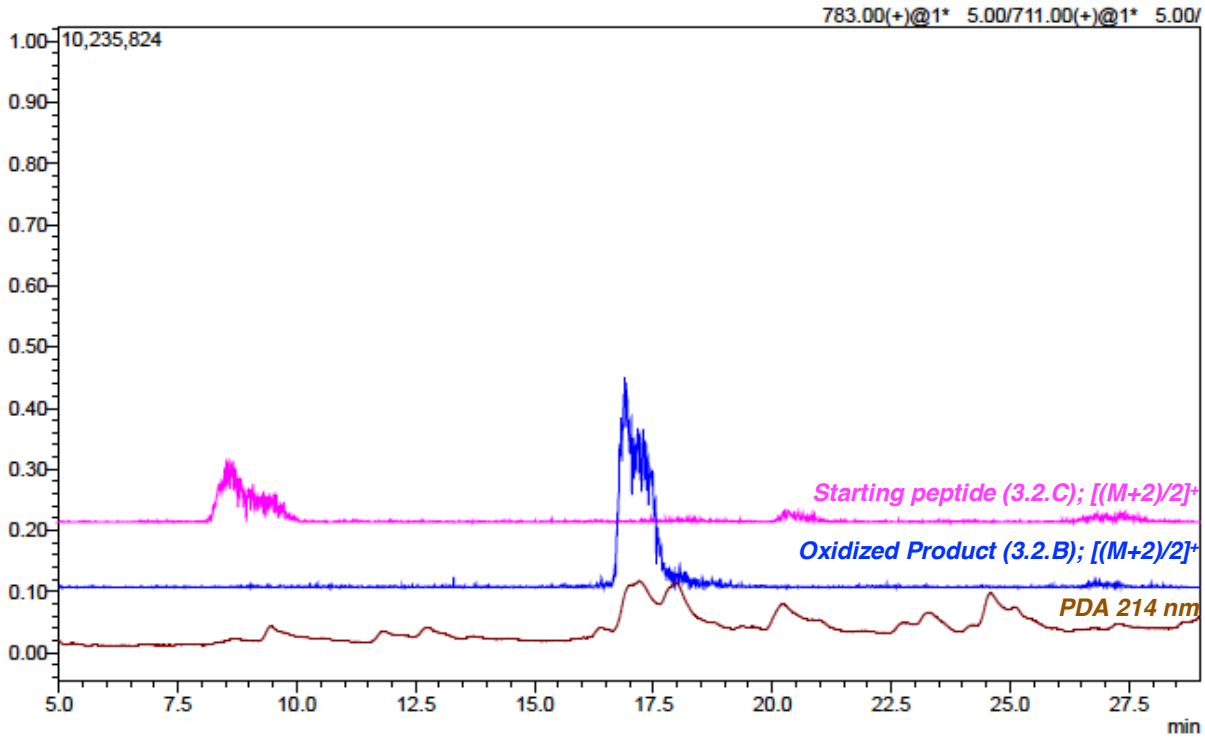


Figure 3.6 Analytical Column PDA for Table 3.1 – Entry 2

Entry 4. The $IAPP_{1-9}$ with Cys(*t*Bu) was used. The relevant quantities are: Resin **3.2.A** (10 mg, 2 μmol), $Pd(OAc)_2$ (0.67 mg, 3 μmol), NMM (7.5 μL, 68.4 μmol) and DMSO-5% AcOH (250 μL). The reaction was analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-90% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 4.X.B $[C_{78}H_{123}N_{11}O_{16}S_2 + 1]^+$: 1535.02; observed: 1535.65. m/z ES calc'd for $[(C_{78}H_{123}N_{11}O_{16}S_2 + 2)/2]^+$: 768.51; observed: 768.25.

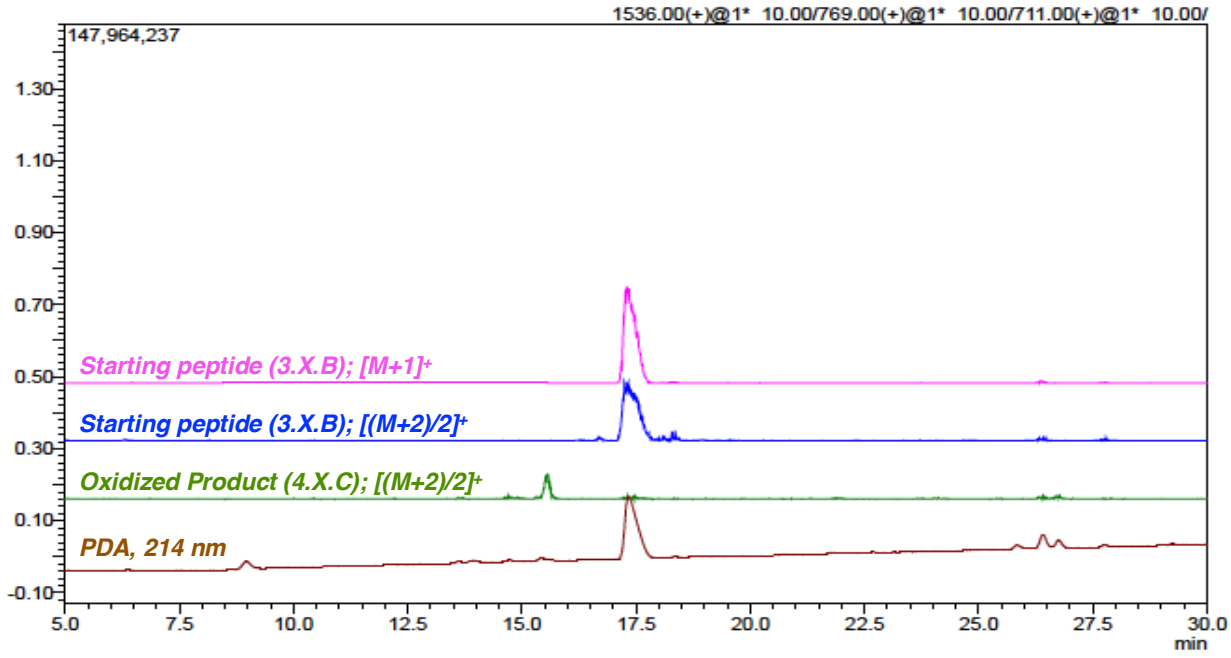
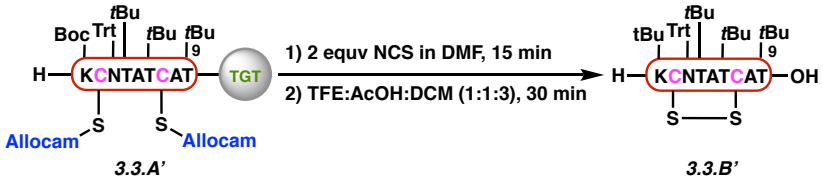


Figure 3.7 Analytical Column PDA for Table 3.1 – Entry 4



3.3.A' *3.3.B'*

Test Reaction (Scheme 3.2.B). The resin 3.3.A' (10 mg, 2 μmol) was swelled in DMF (300 μL). Then, a solution of NCS (0.5 mg, 4 μmol) in DMF (200 μL) was added and it was agitated at room temperature for 15 min. The reaction solution was filtered and the resin was rinsed with DMF (3 x 1 mL) and DCM (3 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture (1:1:3 TFE:AcOH:DCM) for 30 min. The resulting solution was collected, concentrated and analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.3.A'

$[(C_{80}H_{121}N_{13}O_{20}S_2 + 2)/2]^+$: 825.52; observed: 824.90. m/z ES calc'd for peptide 3.3.B' $[(C_{70}H_{105}N_{11}O_{16}S_2 + 2)/2]^+$: 711.40; observed: 711.15.

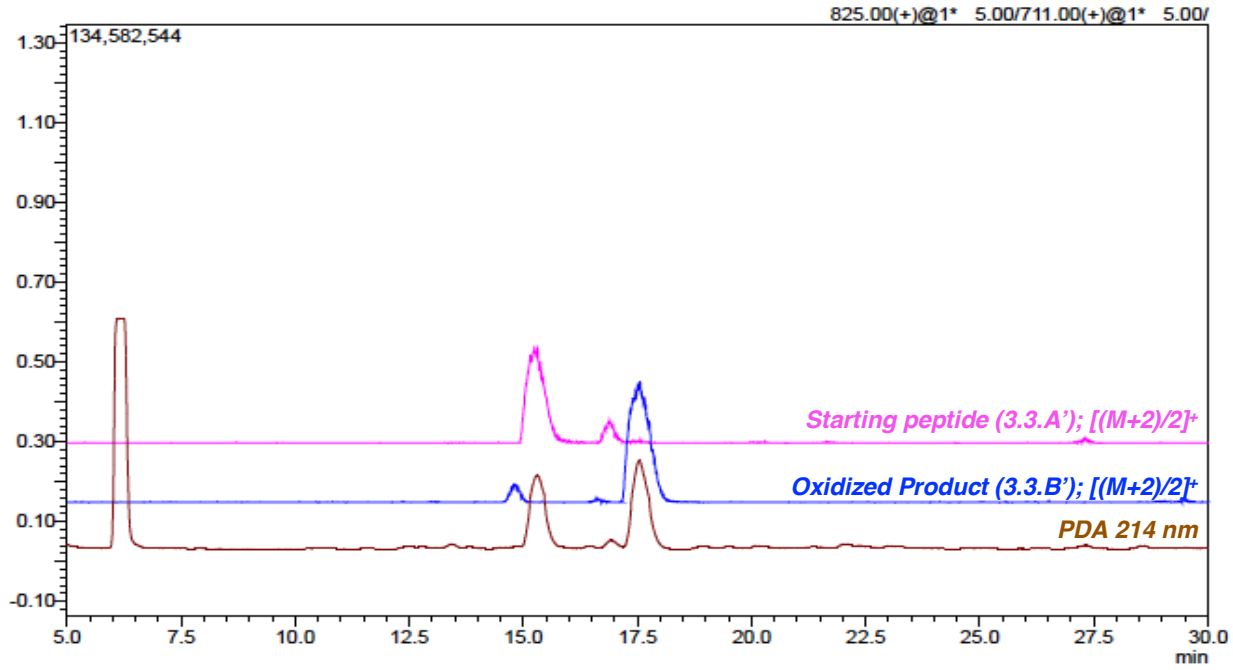
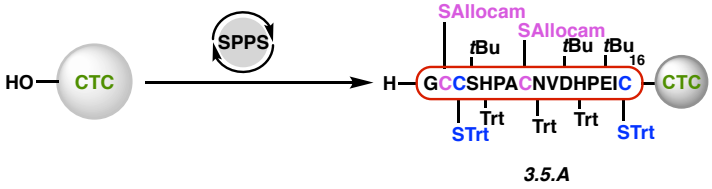


Figure 3.8 Analytical Column PDA for Scheme 3.2 Reaction B



SPPS of Peptide 3.5.A. The standard Fmoc-SPPS protocol was used to synthesize peptide **3.5.A** on CTC resin, except no HATU was used in the first amino acid coupling. To analyze the peptide, 10 mg of the resin was subjected to 300 μL of the cleavage mixture (1:1:8 TFE:AcOH:DCM) for 30 min. The filtrates were collected, concentrated and analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8

column at a flow rate of 1 mL/min with 50-75-95% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 4.X.B $[(C_{183}H_{209}N_{23}O_{27}S_4 + 2)/2]^+$: 1645.23; observed: 1645.35.

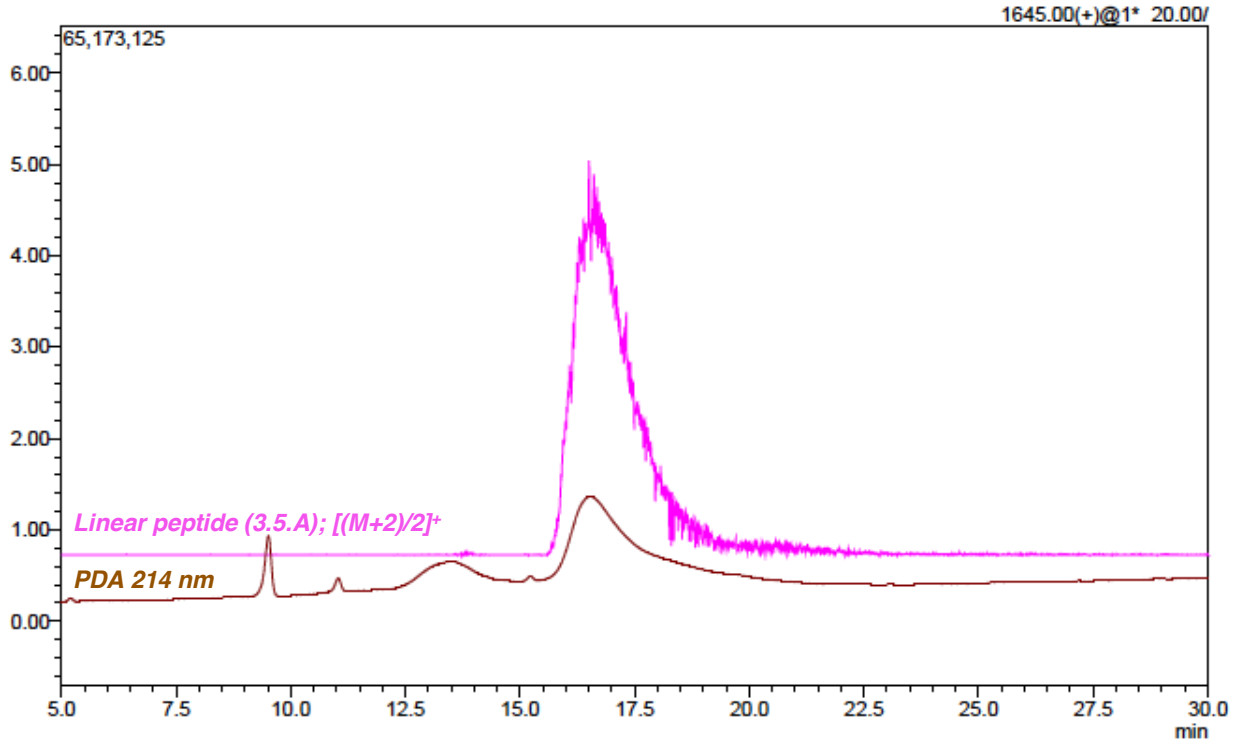


Figure 3.9 Analytical Column PDA for linear peptide **3.5.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 16.81 min

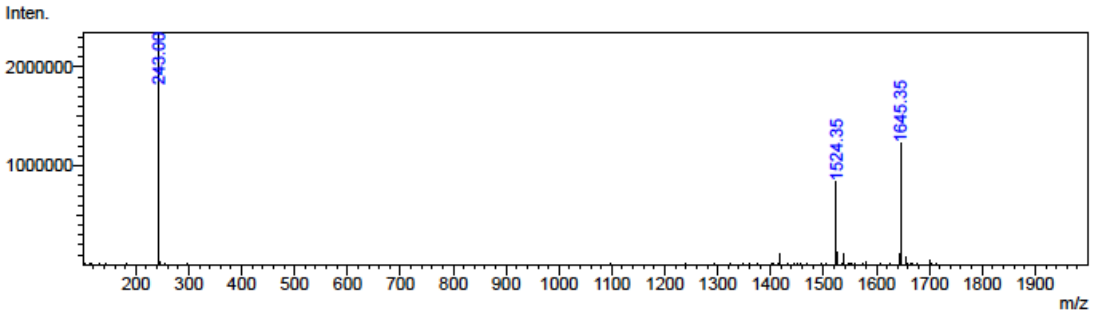
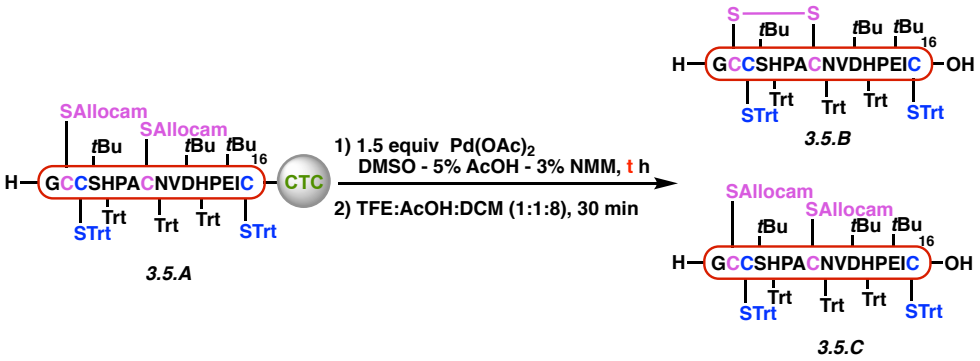


Figure 3.10 ES-MS spectrum for linear peptide **3.5.A** at retention time 16.81 min



General Procedure for Table 3.2. For each of the reaction, the general procedure for Pd-DMSO cleavage-oxidation was followed using the peptide 3.5.A (10 mg, 4 μmol), $Pd(OAc)_2$ (1.4 mg, 6 μmol), NMM (15.0 μL, 68.4 μmol) and DMSO-5% AcOH (500 μL). The reaction mixture was then agitated in a closed vial at room temperature for stated amount of time. After the wash, the dried resin was subjected to 300 μL of the cleavage mixture (1:1:8 TFE:AcOH:DCM) for 30 min. The filtrates were collected, concentrated and analyzed by RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-75-95% MeCN/H_2O gradient for 30 minutes.

Entry 1. The reaction was agitated for 4 h. m/z ES calc'd for peptide 3.5.C $[(C_{183}H_{209}N_{23}O_{27}S_4 + 2)/2]^+$: 1645.23; observed: 1646.00. m/z ES calc'd for peptide 3.5.B $[(C_{173}H_{193}N_{21}O_{23}S_4 + 23 + 2)/2]^+$: 1543.41; observed: 1544.41

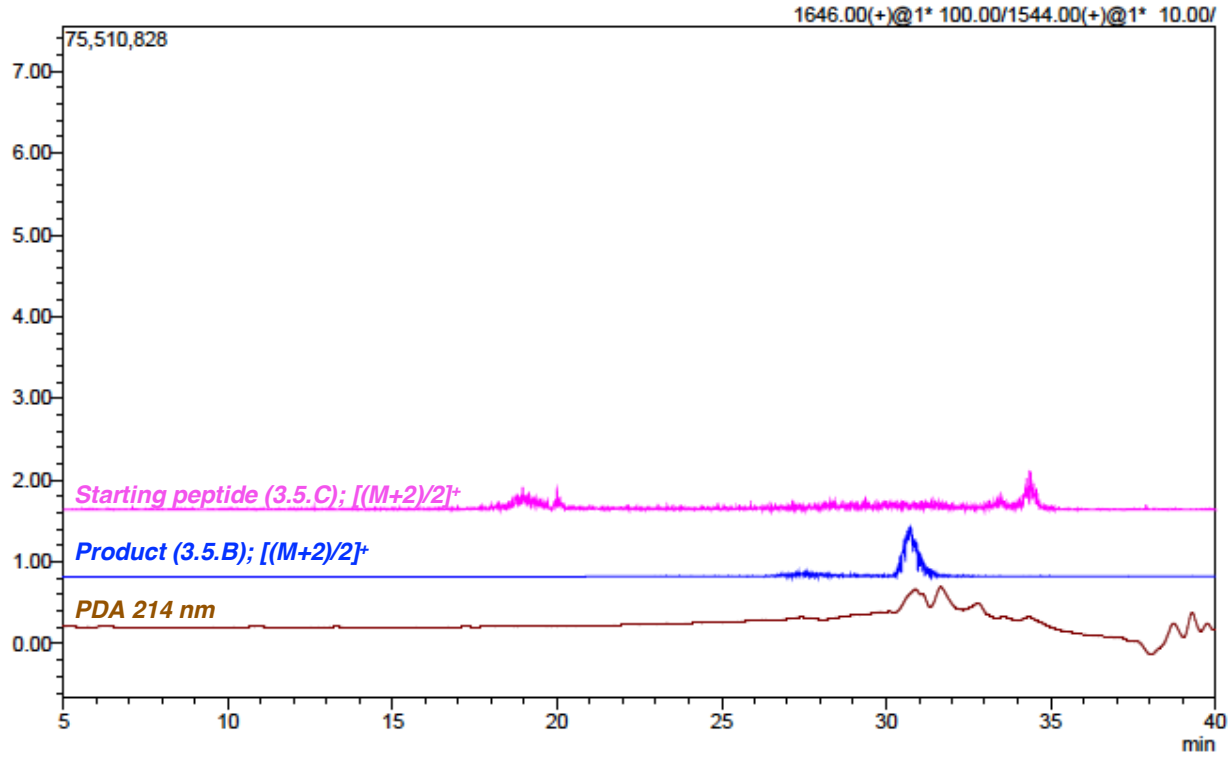


Figure 3.11 Analytical Column PDA for determination of conversion; Table 3.2. Entry 1. Gradient: 50-95% MeCN/H_2O + 1% HCOOH over 40 min.

Entry 2. The reaction was agitated for 6 h. m/z ES calc'd for peptide 3.5.B $[(C_{173}H_{193}N_{21}O_{23}S_4 + 23 + 2)/2]^+$: 1532.41; observed: 1531.45

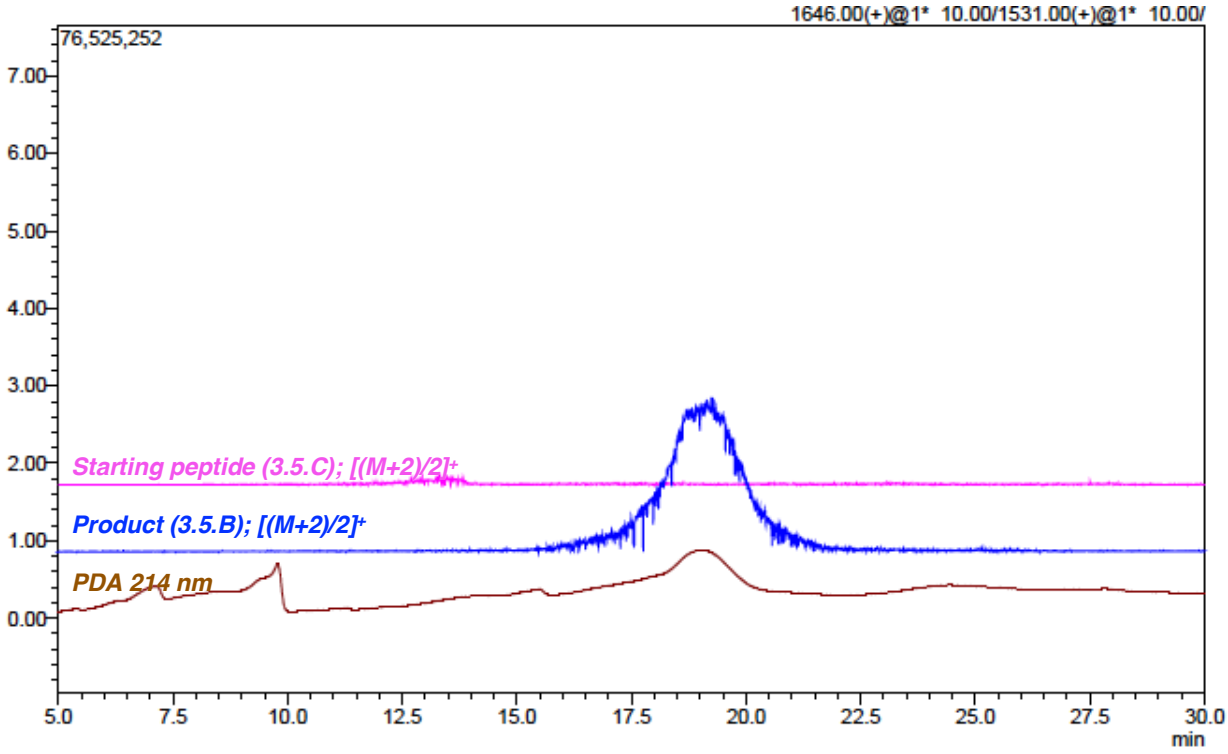
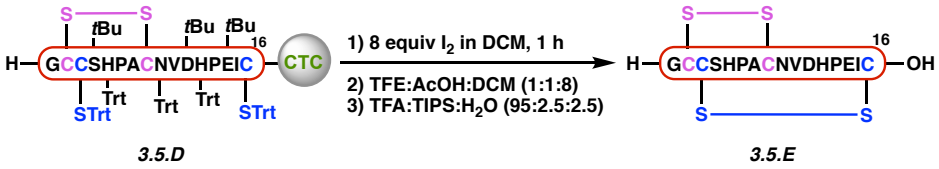


Figure 3.12 Analytical Column PDA for determination of conversion; Table 3.2. Entry 2. Gradient: 50-75-95% MeCN/H_2O + 1% HCOOH over 30 min.



3.5.D *3.5.E*

Carboxy α4/7-Conotoxin LvIA (3.5.E). The resin 3.5.D (30 mg, 12 μmol) was swelled in DMF (500 μL). Then, Iodine (24.4 mg, 96 μmol) in DCM (360 μL) was added and the reaction mixture was agitated at room temperature for 1 hour. Next, the reaction mixture was filtered out and few drops of saturated ascorbic acid in water was added until the purple color of iodine disappeared. The resulting resin was washed with saturated ascorbic acid in water. After that, 300 μL of the cleavage mixture (1:1:8 TFE:AcOH:DCM) was introduced and kept for 2 x 15 min. The filtrates were collected, concentrated and subjected to TFA:TIPS:H_2O (95:2.5:2.5) cleavage mixture for 1 hour. Finally, the resulting solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 5% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 5-95% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.5.E $[(C_{66}H_{97}N_{21}O_{23}S_4 + 2)/2]^+$: 841.44; observed: 840.90

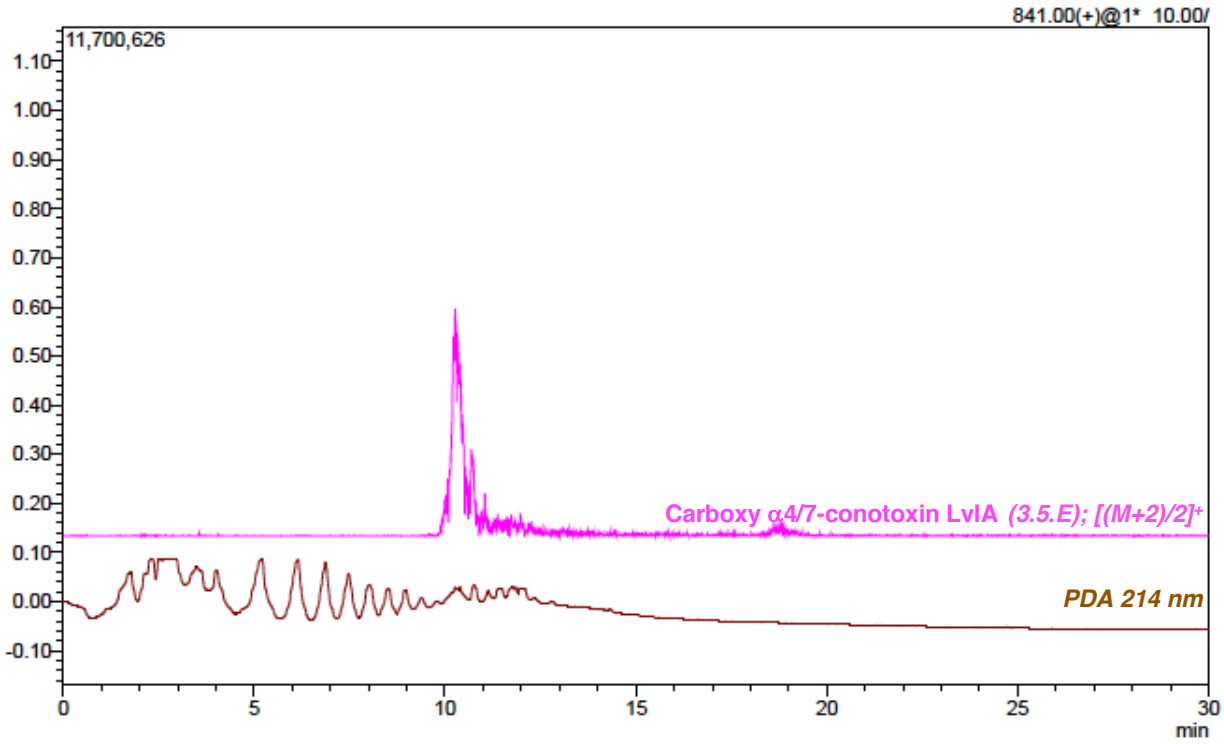


Figure 3.13 Analytical Column PDA for crude product **3.5.E**; Gradient: 5-95% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 10.34 min

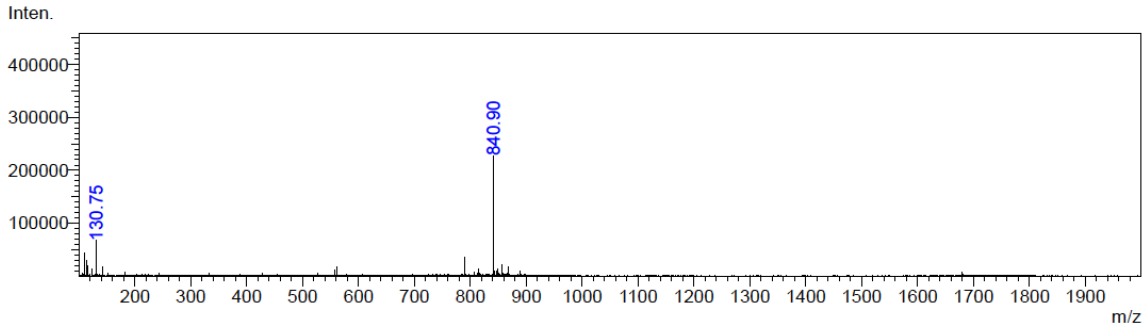
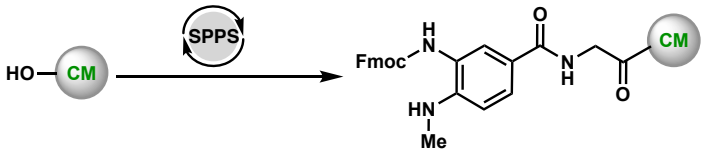
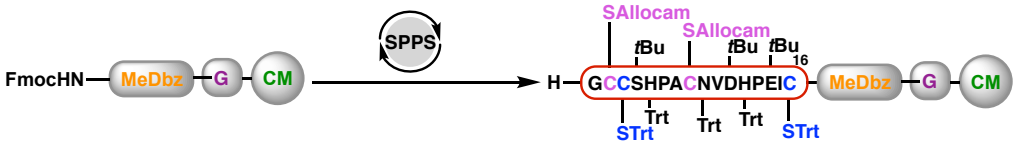


Figure 3.14 ES-MS spectrum for crude product **3.5.E** at retention time 10.34 min



Fmoc-MeDbz-Gly-ChemMatrix Resin

MeDbz Linker-Gly-ChemMatrix Resin. The standard Fmoc-SPPS protocol described was used to synthesize the linker on ChemMatrix resin. The loading of MeDbz linker: 0.3 mmol/g.



3.6.A

Linear Peptide 3.6.A. The standard Fmoc-SPPS protocol described was used to synthesize peptide **3.6.A** on MeDbz linker-Gly-ChemMatrix resin. The peptide was capped before the last coupling step. To analyze the peptide, 10 mg of the resin was subjected general ammonia cleavage protocol and the resulting crude product was dissolved in 50% MeCN:Water in presence of 0.05%TFA and analyzed via direct ES-MS. Final loading of the resin: 0.01 mmol/g. m/z ES calc'd for peptide 3.6.A $[(C_{183}H_{210}N_{24}O_{26}S_4 + 2)/2]^+$: 1646.04; observed: 1645.30.

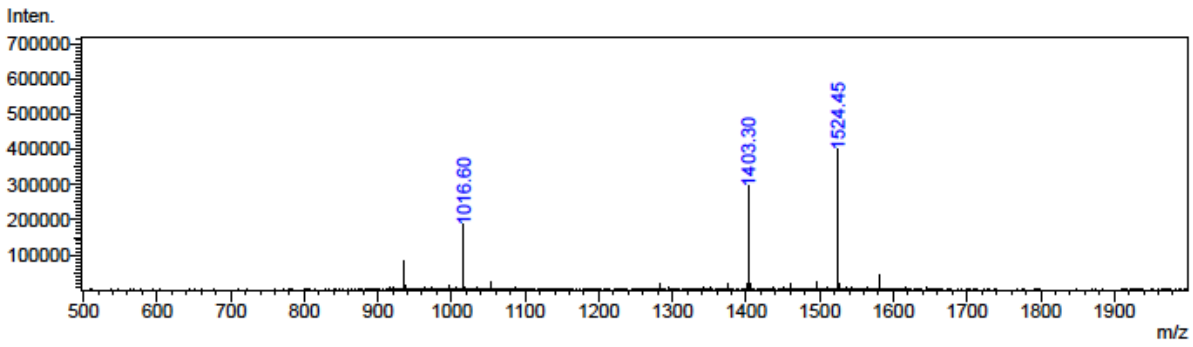
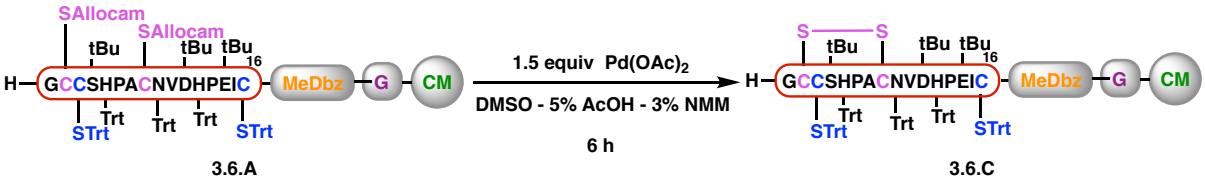


Figure 3.15 ES-MS spectrum for linear peptide **3.6.A**



Mono Cyclic Peptide 3.6.C. The general procedure for Pd-DMSO cleavage-oxidation was followed. The relevant quantities are: Resin **3.6.A** 50 mg, 15 μmol), $Pd(OAc)_2$ (5.1 mg, 22.5 μmol), NMM (56.5 μL, 513 μmol) and DMSO-5% AcOH (1.875 mL). The reaction mixture was agitated in a closed vial at room temperature for 6 hours. To analyze the peptide, first, 10 mg of the peptide (3 μmol) was treated with di-*tert*-butyl dicarbonate (32.7 mg, 150 μmol) in DCM (300 μL). Then DIPEA (1.5 μL, 9 μmol) was added to the reaction mixture and agitated for 12 hours. Thereafter, the resin was washed with DCM (3 x 1 mL) and DMF (3 x 1 mL). After Boc protection step, the resulting peptide was subjected to the general procedure for ammonia cleavage of peptide on resin. The resulting crude product was then treated with TFA:TIPS:H_2O (95:2.5.2.5) for 2 hours. Finally, the TFA solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 50% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-90% MeCN/H_2O gradient for 30 minutes. Final loading of the resin: 0.01 mmol/g. m/z ES calc'd for peptide 3.6.C+Boc-Trt $[(C_{159}H_{188}N_{22}O_{24}S_4 + 2)/2]^+$: 1460.81; observed: 1460.40. m/z ES calc'd for peptide 3.6.C+Boc-2Trt $[(C_{140}H_{174}N_{22}O_{24}S_4 + 2)/2]^+$: 1339.65; observed: 1339.15.

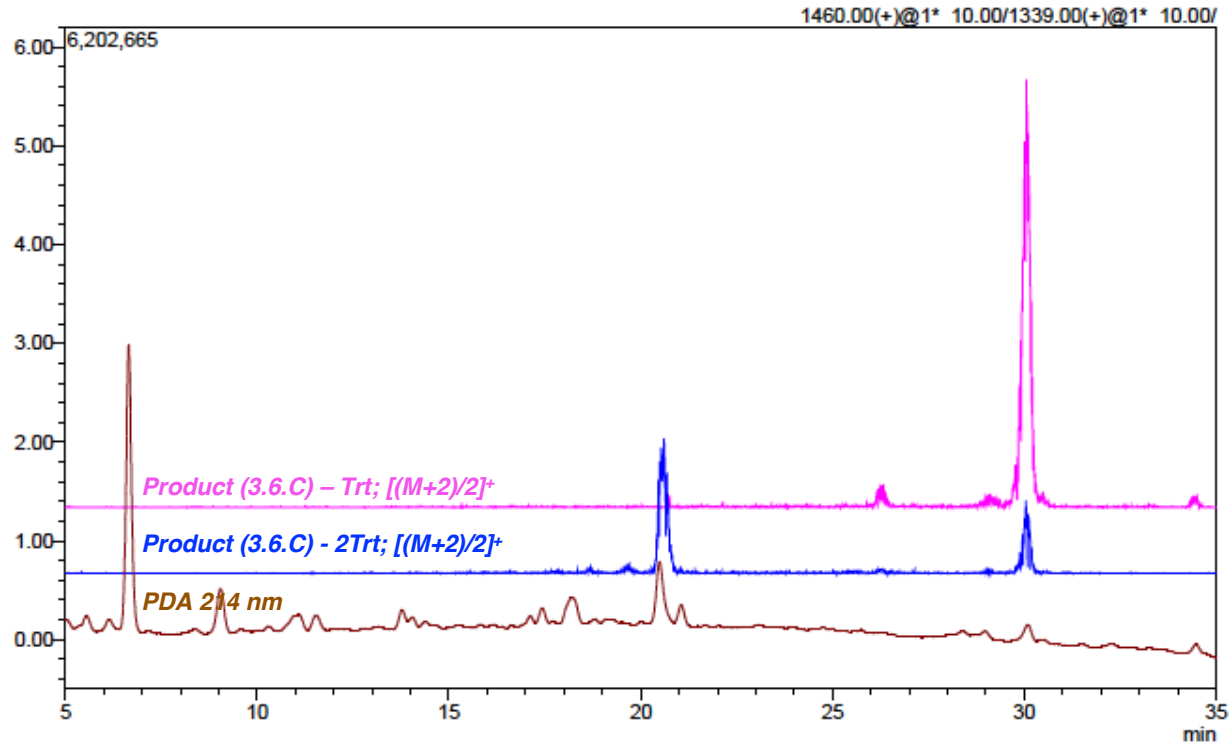
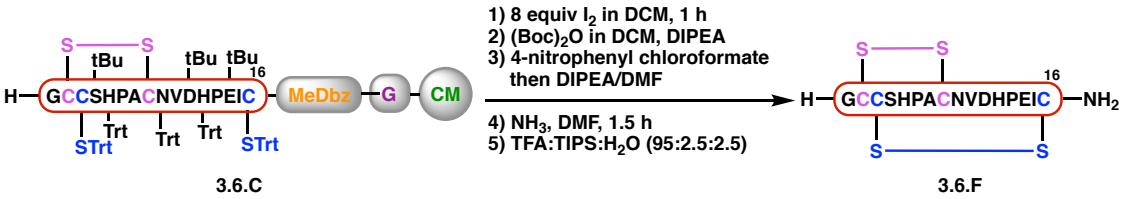


Figure 3.16 Analytical Column PDA for determination of conversion. Gradient: 50-90% MeCN/H_2O + 1% HCOOH over 30 min.



α4/7-conotoxin LvIA 3.6.F. First, the peptide **3.6.C** (50 mg, 15 μmol) was swelled in DMF (500 μL). Then, Iodine (30.5 mg, 120 μmol) in DCM (400 μL) was added and the reaction mixture was agitated at room temperature for 1 hour. Then, the reaction mixture was filtered out and few drops of saturated ascorbic acid in water was added until the purple color of iodine disappeared. The resulting resin was washed with saturated ascorbic acid in water. Next, the peptide was treated with di-*tert*-butyl dicarbonate (163.5 mg, 750 μmol) in DCM (1.5 mL). Then DIPEA (8.0 μL, 45 μmol) was added to the reaction mixture and

agitated for 12 hours. After Boc protection step, the resulting peptide was subjected the general procedure for ammonia cleavage of peptide on resin. The resulting crude product was then treated with TFA:TIPS:H_2O (95:2.5.2.5) for 2 hours. Finally, the TFA solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 50% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 20-90% MeCN/H_2O gradient for 30 minutes. Final loading of the resin: 0.01 mmol/g. m/z ES calc'd for peptide 3.6.F+Boc $[(C_{71}H_{106}N_{22}O_{24}S_4 + 2)/2]^+$: 891.00; observed: 890.20. m/z ES calc'd for $[(C_{140}H_{174}N_{22}O_{24}S_4 + 3)/3]^+$: 594.33; observed: 593.80.

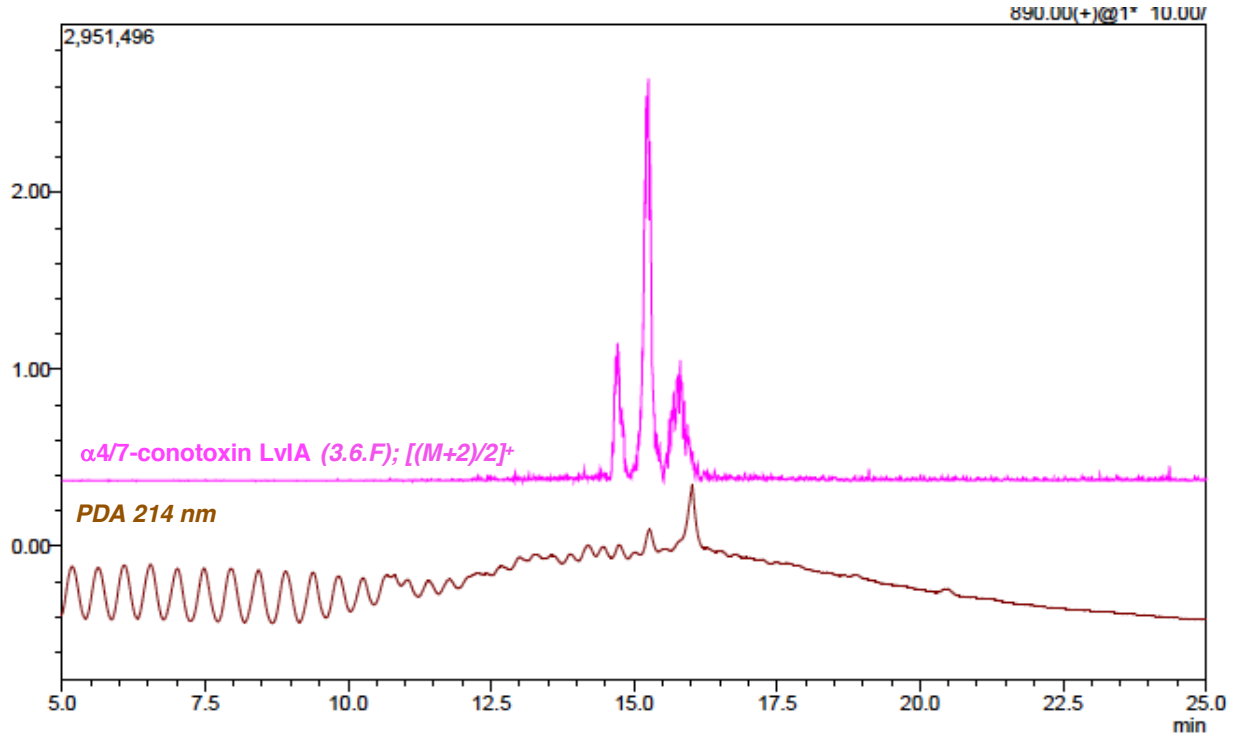


Figure 3.17 Analytical Column PDA for crude product **3.6.F**; Gradient: 20-90% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 15.26 min

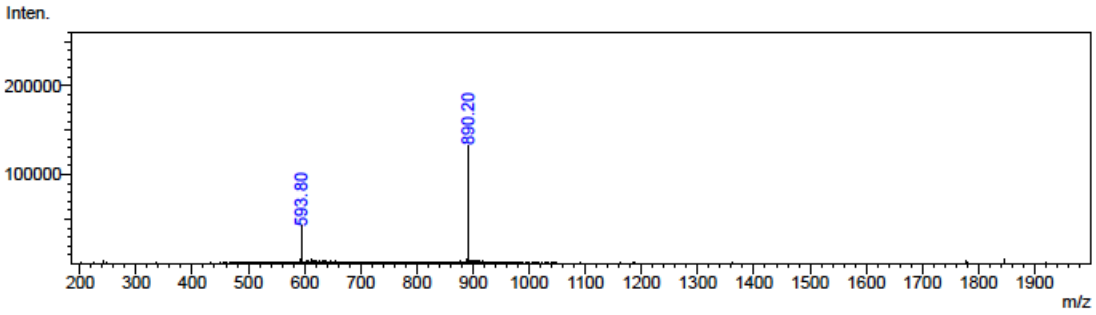
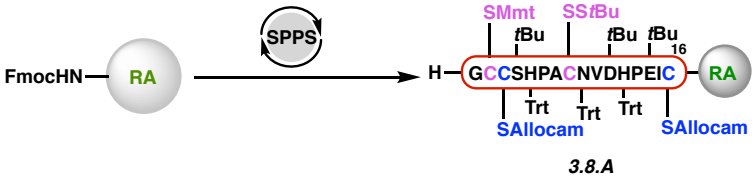


Figure 3.18 ES-MS spectrum for crude product **3.6.F** at retention time 15.26 min



3.8.A

SPPS of Linear Peptide 3.8.A. The standard Fmoc-SPPS protocol described was used to synthesize peptide **3.8.A** on rink amide resin. The peptide was capped before the last coupling step. To analyze the final peptide, 10 mg of the resin was subjected to cleavage solution of TFA:TIPS:H_2O (95:2.5.2.5) for 30 min. Finally, the TFA solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.A $[(C_{80}H_{124}N_{24}O_{26}S_5 + 2)/2]^+$: 1000.15; observed: 999.90. m/z ES calc'd for $[(C_{74}H_{116}N_{24}O_{26}S_5 + 3)/3]^+$: 667.10; observed: 667.00.

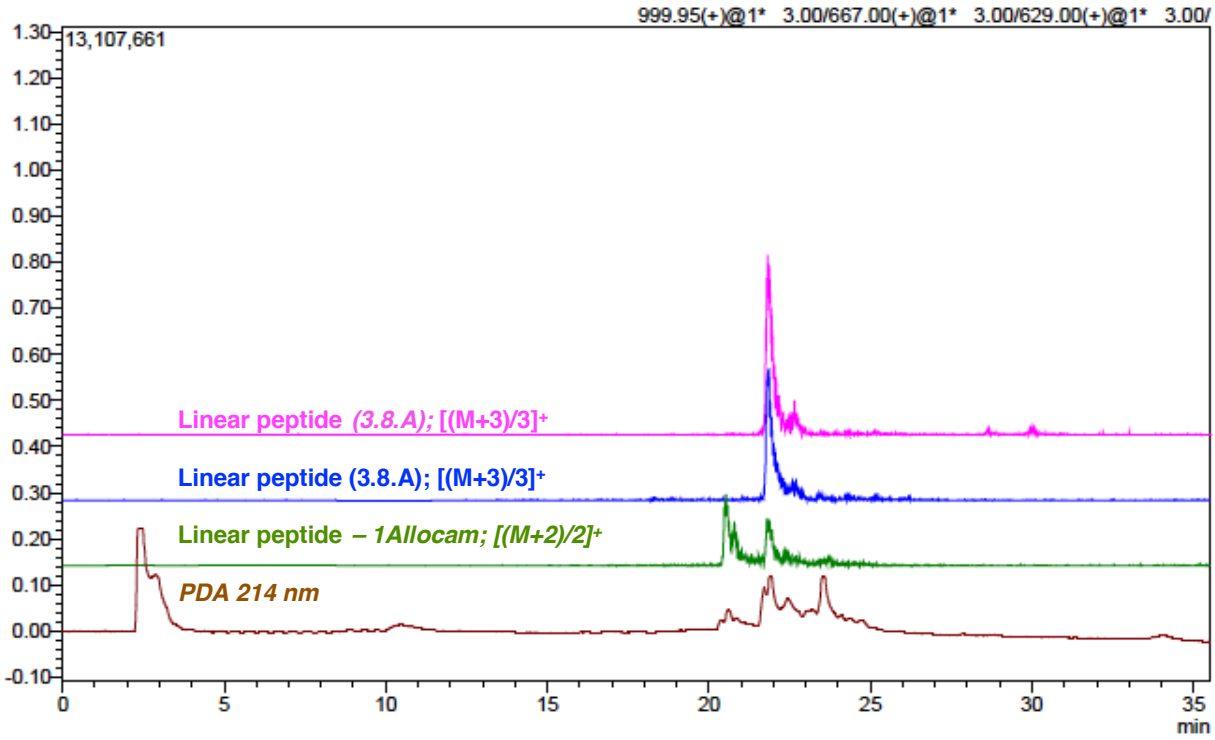


Figure 3.19 Analytical Column PDA for linear peptide **3.8.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 21.87 min

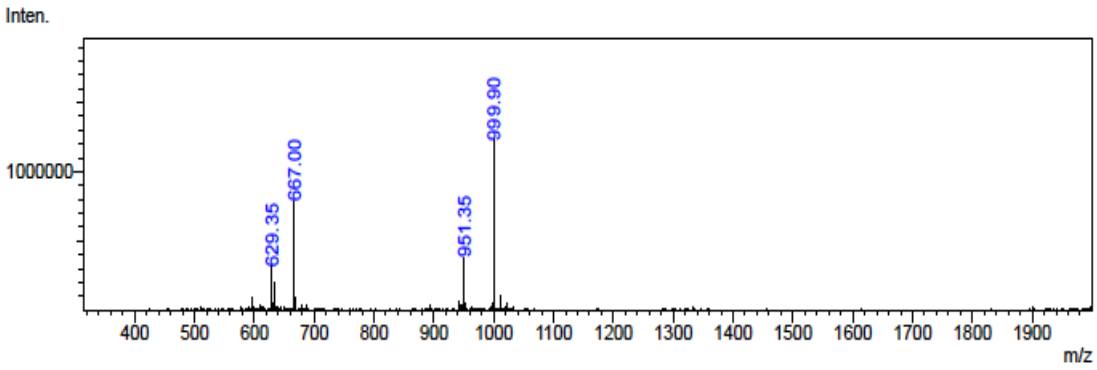
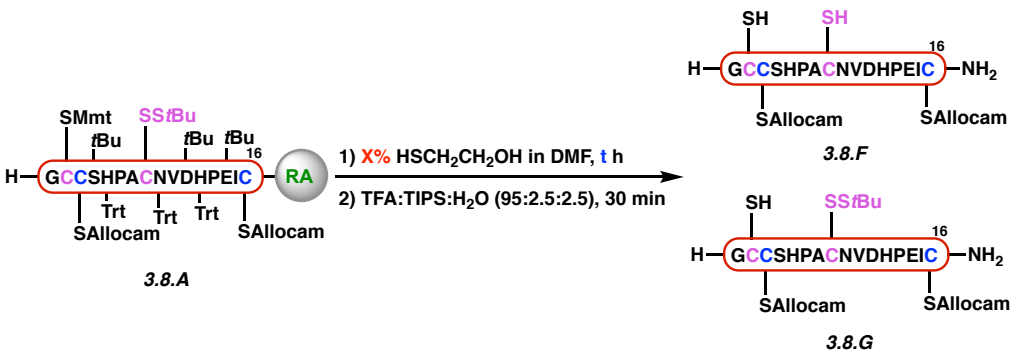


Figure 3.20 ES-MS spectrum for linear peptide **3.8.A** at retention time 21.87 min



General Procedure for Reaction Optimization Table 3.3. For each of the reactions, the peptide **3.8.A** (10 mg, 4 μmol) was first swelled in DMF (500 μL). Then, a solution of 20% mercaptoethanol in DMF (1.0 mL) was added and agitated for stated amount of time either at room temperature or at 60 ^{o}C under microwave heating. The reaction solution was then filtered and the resin was washed with DMF (5 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture (95:2.5:2.5 TFA:TIPS:H_2O) for 30 min. The resulting TFA solution was collected, concentrated, and the peptide was crashed out with cold diethyl ether. The precipitate was centrifuged, ether discarded, and the pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.G $[(C_{80}H_{124}N_{24}O_{26}S_5 + 2)/2]^+$: 1000.15; observed: 999.80. m/z ES calc'd for $[(C_{80}H_{124}N_{24}O_{26}S_5 + 3)/3]^+$: 667.10; observed: 666.95. m/z ES calc'd for peptide 3.8.F $[(C_{76}H_{116}N_{24}O_{26}S_4 + 2)/2]^+$: 956.08; observed: 955.70. m/z ES calc'd for $[(C_{76}H_{116}N_{24}O_{26}S_4 + 3)/3]^+$: 637.72; observed: 637.55.

Entry 1. The reaction was agitated at room temperature for 3 hours.

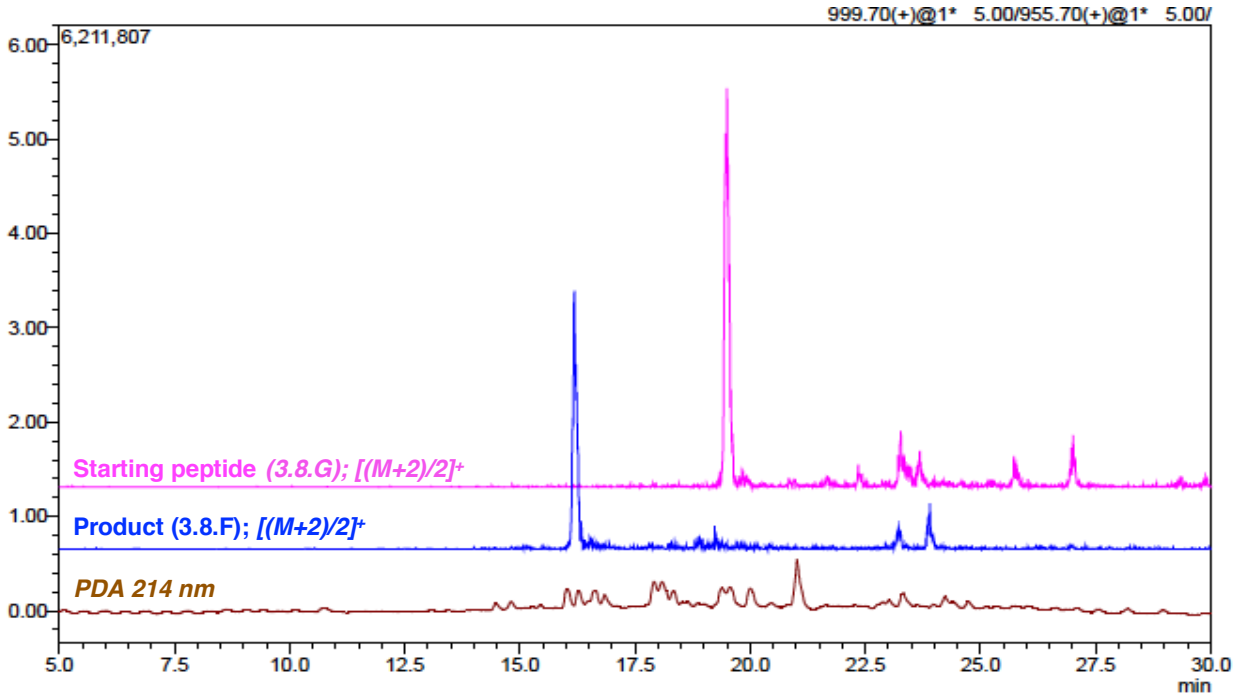


Figure 3.21 Analytical Column PDA for Table 3.3. Entry 1.

Entry 2. The reaction was agitated at room temperature for 6 hours.

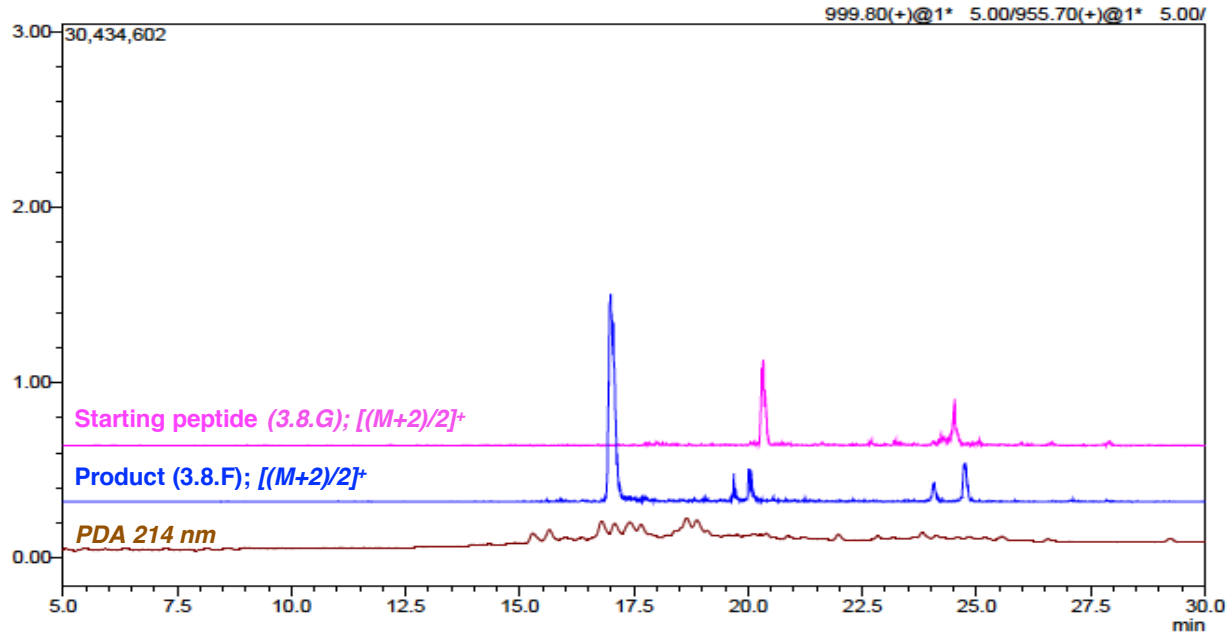


Figure 3.22 Analytical Column PDA for Table 3.3. Entry 2.

Entry 3. The reaction was agitated at room temperature for 12 hours.

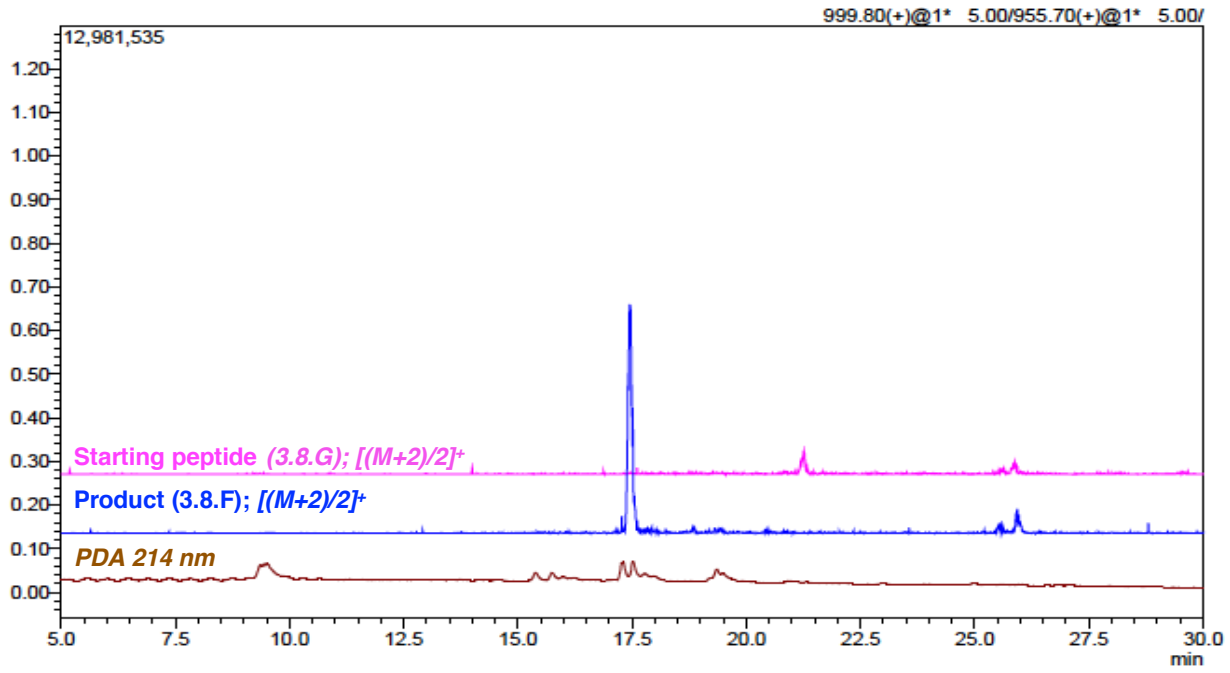


Figure 3.23 Analytical Column PDA for Table 3.3. Entry 3.

Entry 4. The reaction was agitated in a microwave at 60 ºC for 2 hours.

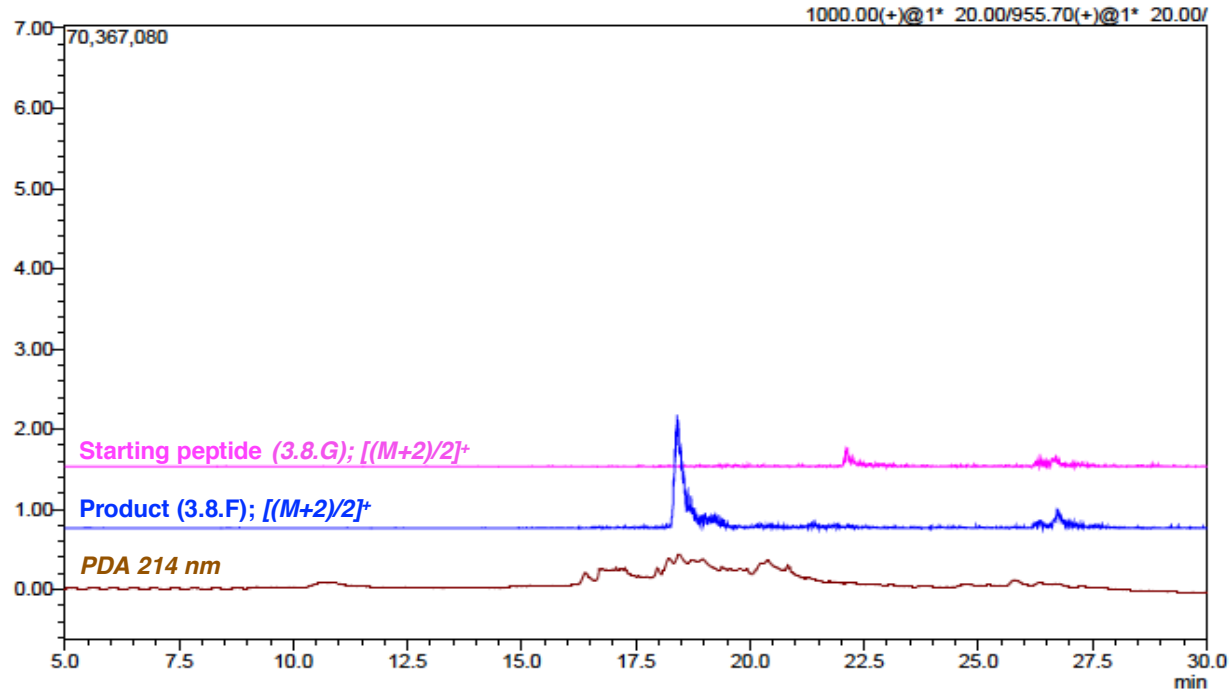


Figure 3.24 Analytical Column PDA for Table 3.3. Entry 4.

Entry 5. The reaction was agitated in a microwave at 60 ºC for 3 hours.

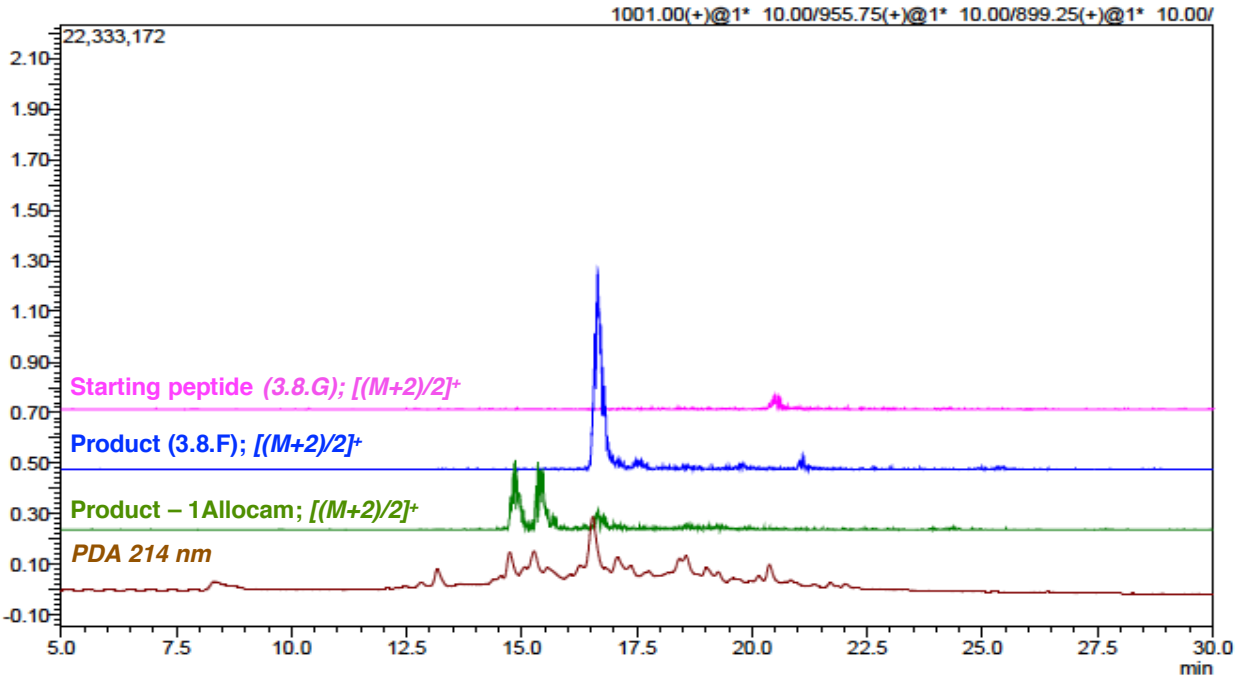
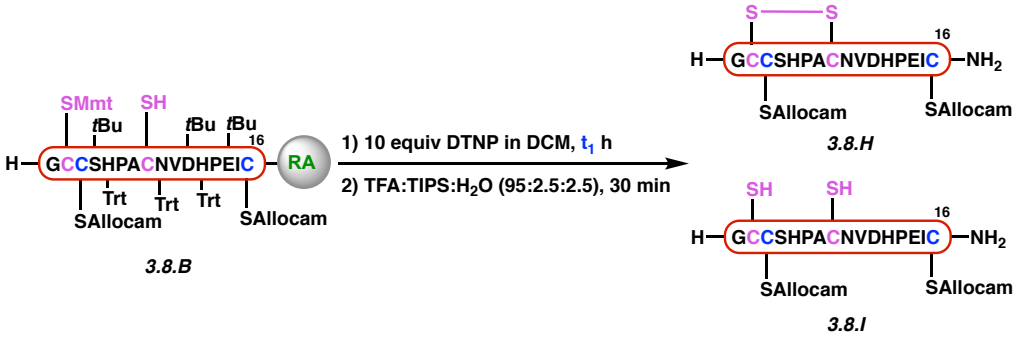


Figure 3.25 Analytical Column PDA for Table 3.3. Entry 5.



General Procedure for Reaction Optimization Table 3.4. For each of the reactions, the resin **3.8.B** (10 mg, 4 μmol) was first swelled in DMF (500 μL). Then, a solution of DTNP (12.4 mg, 40 μmol) in DCM (1.0 mL) was added and allowed to agitate the stated amount of time either at room temperature or at 60 ºC under microwave heating. The reaction solution was filtered and the resin was washed with DCM (5 x 1 mL). The resulting resin

was subjected to 300 μL of the cleavage mixture (95:2.5:2.5 TFA:TIPS:H_2O) for 30 min. The resulting TFA solution was collected, concentrated, and the peptide was crashed out with cold diethyl ether. The precipitate was centrifuged, ether discarded, and the pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.H $[(C_{76}H_{116}N_{24}O_{26}S_4 + 2)/2]^+$: 956.08; observed: 955.75. m/z ES calc'd for $[(C_{76}H_{116}N_{24}O_{26}S_4 + 3)/3]^+$: 637.72; observed: 637.45. m/z ES calc'd for peptide 3.8.I $[(C_{76}H_{114}N_{24}O_{26}S_4 + 2)/2]^+$: 955.06; observed: 954.85. m/z ES calc'd for $[(C_{76}H_{114}N_{24}O_{26}S_4 + 3)/3]^+$: 637.04; observed: 636.95.

Entry 1. The reaction was agitated at room temperature for 1 hour.

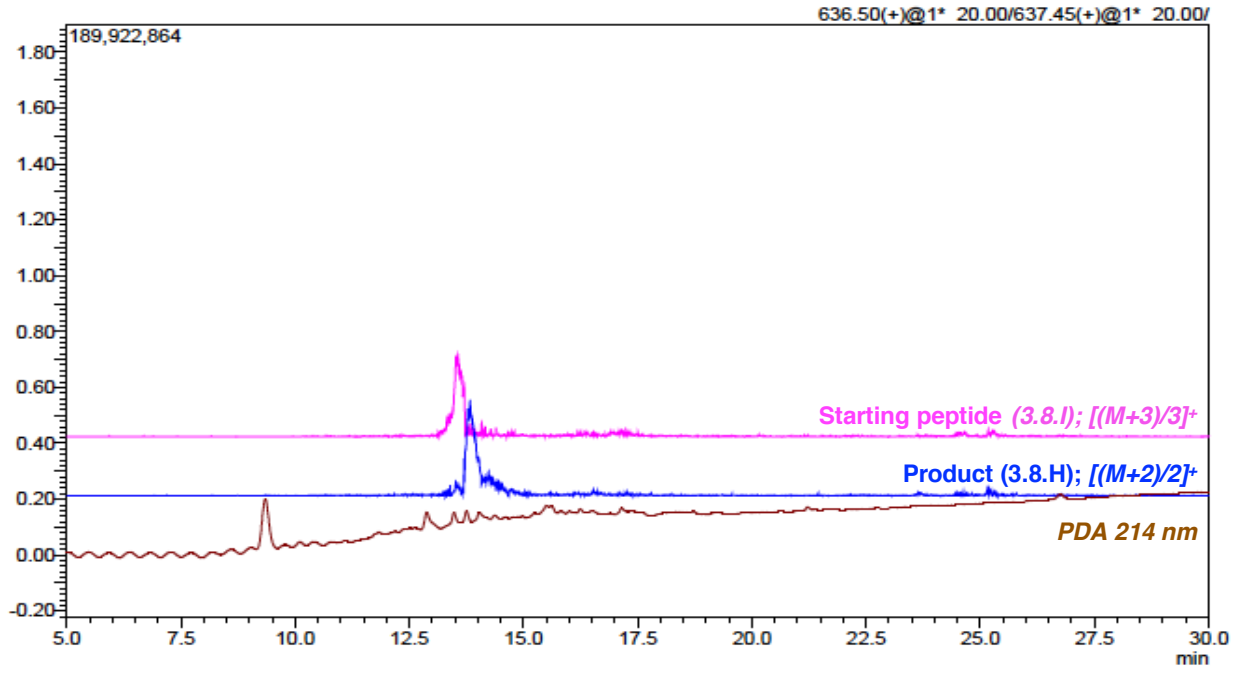


Figure 3.26 Analytical Column PDA for Table 3.4. Entry 1.

Entry 2. The reaction was agitated at room temperature for 3 hours.

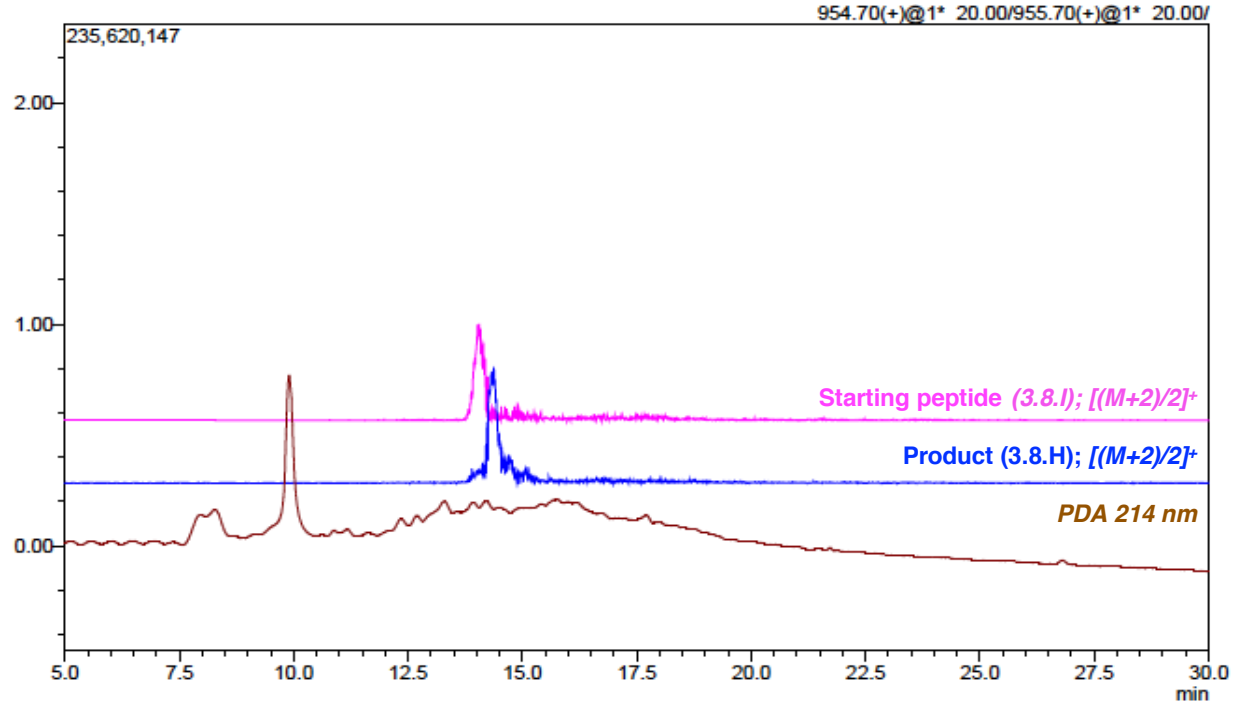


Figure 3.27 Analytical Column PDA for Table 3.4. Entry 2.

Entry 3. The reaction was agitated at room temperature for 6 hours.

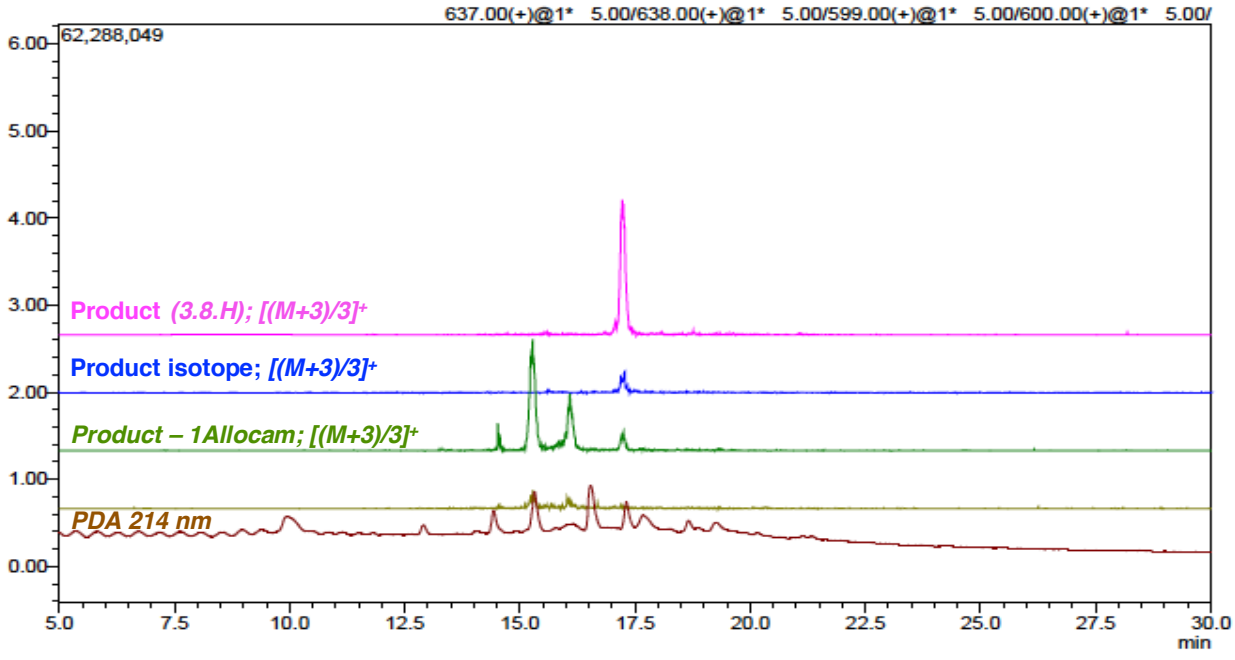


Figure 3.28. Analytical Column PDA for Table 3.4. Entry 3.

Entry 4. The reaction was agitated in a microwave at 60 ºC for 1 hour.

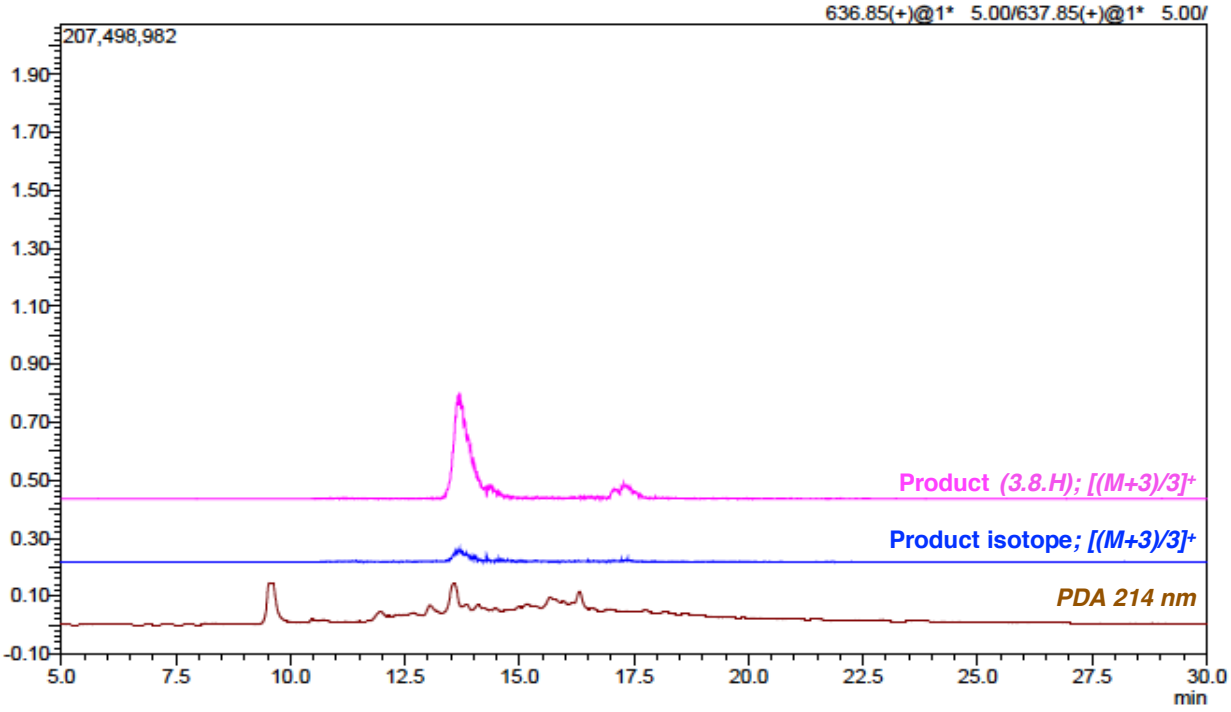
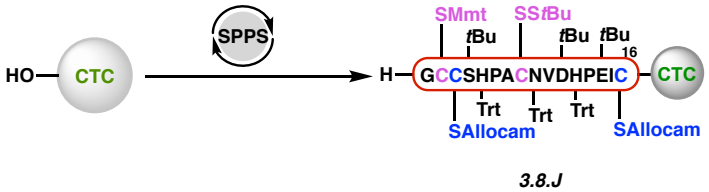


Figure 3.29 Analytical Column PDA for Table 3.4. Entry



3.8.J

SPPS of Peptide 3.8.J. The standard Fmoc-SPPS protocol described was used to synthesize peptide **3.8.J** on CTC resin, except no HATU was used in the first amino acid coupling. To analyze the peptide, 10 mg of the resin was subjected to 300 μL of the cleavage mixture (1:1:8 TFE:AcOH:DCM) for 30 min. The filtrates were collected, concentrated and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 50-75-95% MeCN/H_2O gradient

for 30 minutes. . m/z ES calc'd for peptide 3.X.B [($C_{169}H_{206}N_{24}O_{27}S_5$ + 2)/2]$^+$: 1583.97; observed: 1584.35. m/z ES calc'd for [($C_{169}H_{206}N_{24}O_{27}S_5$ + 3)/3]$^+$: 1056.32; observed: 1056.55.

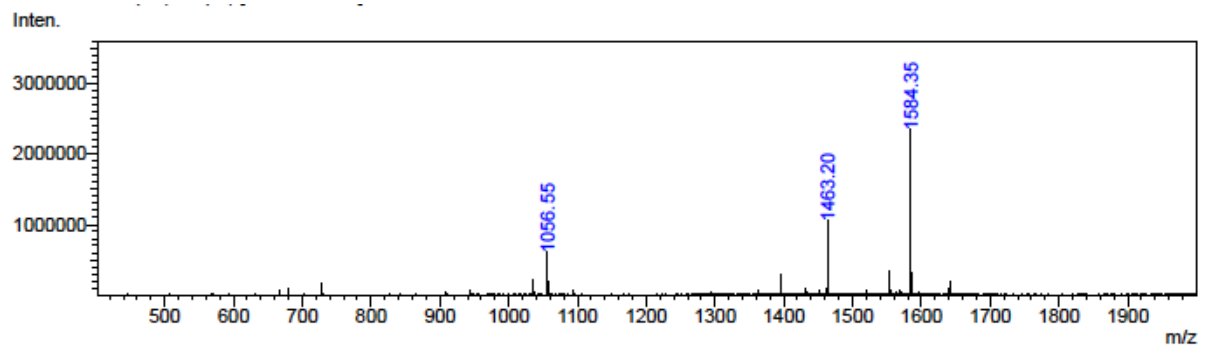
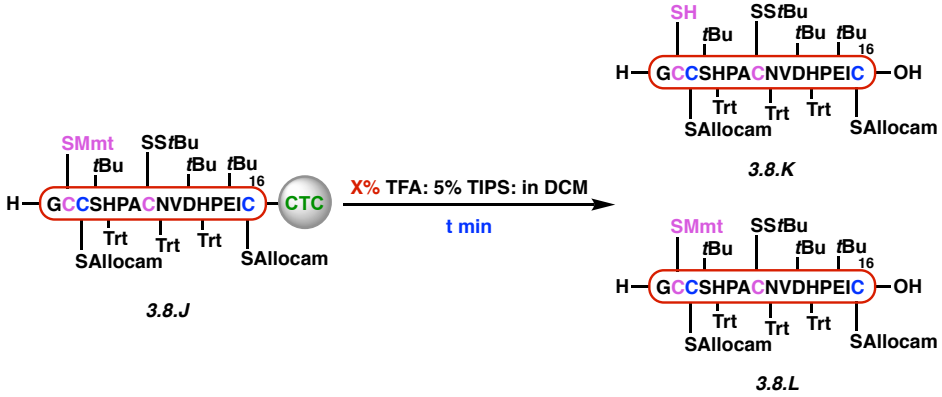


Figure 3.30 ES-MS spectrum for linear peptide **3.8.J**



General Procedure for Reaction Optimization Table 3.5. For each of the reactions, the peptide **3.8.J** (10 mg, 4 μmol) was first swelled in DCM (500 μL). A freshly prepared stated percentage of TFA solution in DCM was added and the reaction mixture was agitated at stated amount of time. About 2 – 3 drops of TIPS were added during the reaction until the orange-yellow color disappeared in the reaction mixture. The resulting reaction solution

was filtered in to a tube. The peptide was immediately crashed out with cold diethyl ether and centrifuged, ether discarded, and the pellet was dissolved in 50% MeCN:Water in presence of 0.05%TFA. This solution was analyzed directly by MS. m/z ES calc'd for peptide 3.8.L $[(C_{169}H_{206}N_{24}O_{27}S_5 + 2)/2]^+$: 1583.97; observed: 1584.30. m/z ES calc'd for peptide 3.8.K $[(C_{149}H_{190}N_{24}O_{26}S_5 + 2)/2]^+$: 1447.80; observed: 1448.05.

Entry 1. A freshly prepared solution of TFA:TIPS:DCM (2:5:93, 300 μL) was added and the reaction mixture was agitated at room temperature for 30 min.

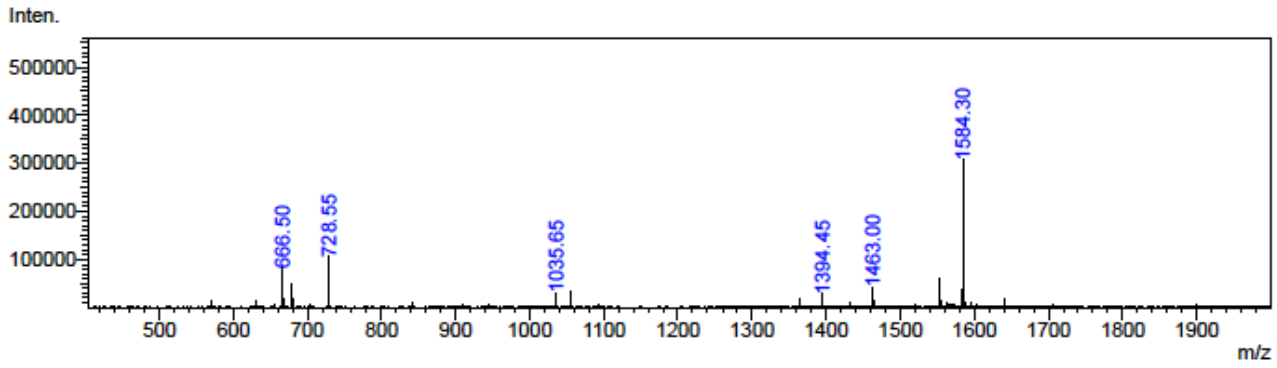


Figure 3.31 ES-MS spectrum for direct injection. Table 3.5. Entry 1

Entry 2. A freshly prepared solution of TFA:TIPS:DCM (2:5:93, 300 μL) was added and the reaction mixture was agitated at room temperature for 60 min.

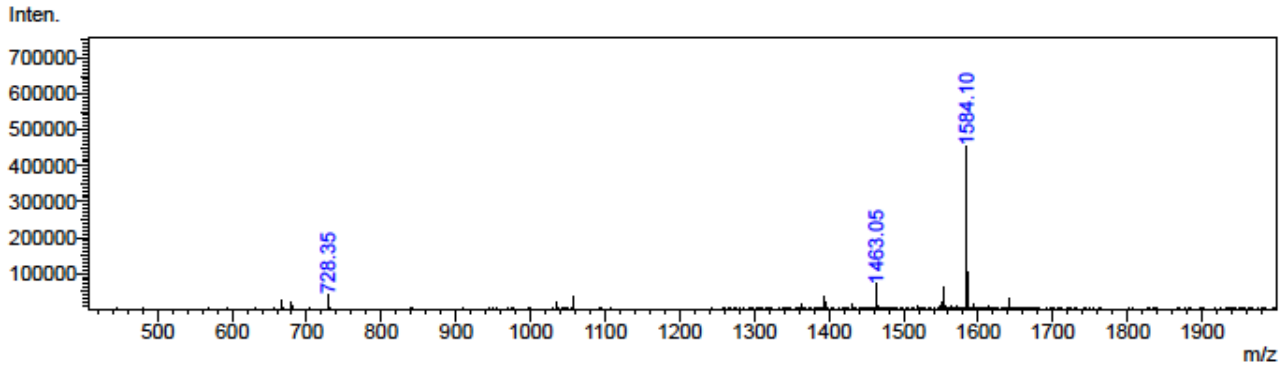


Figure 3.32 ES-MS spectrum for direct injection. Table 3.5. Entry 2

Entry 3. A freshly prepared solution of TFA:TIPS:DCM (3:5:92, 300 μL) was added and the reaction mixture was agitated at room temperature for 15 min.

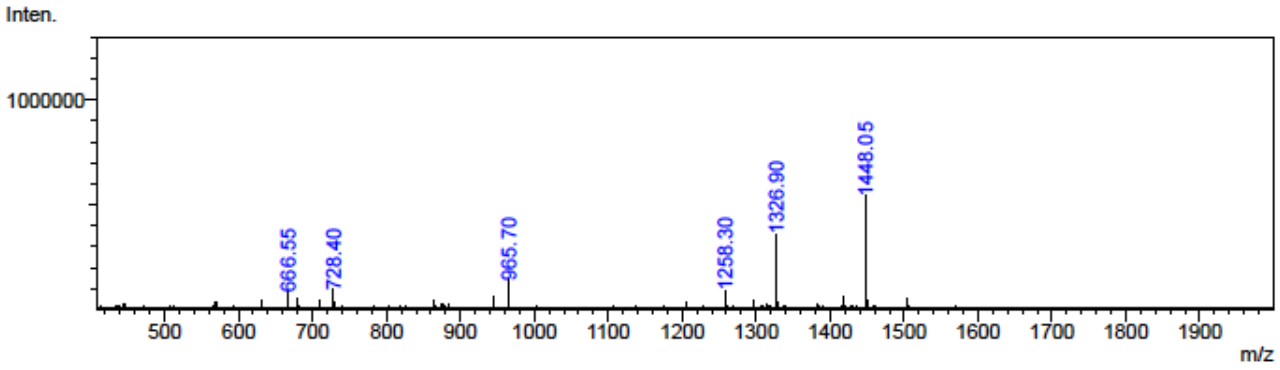


Figure 3.33 ES-MS spectrum for direct injection. Table 3.5. Entry 3

Entry 4. A freshly prepared solution of TFA:TIPS:DCM (4:5:91, 300 μL) was added and the reaction mixture was agitated at room temperature for 10 min.

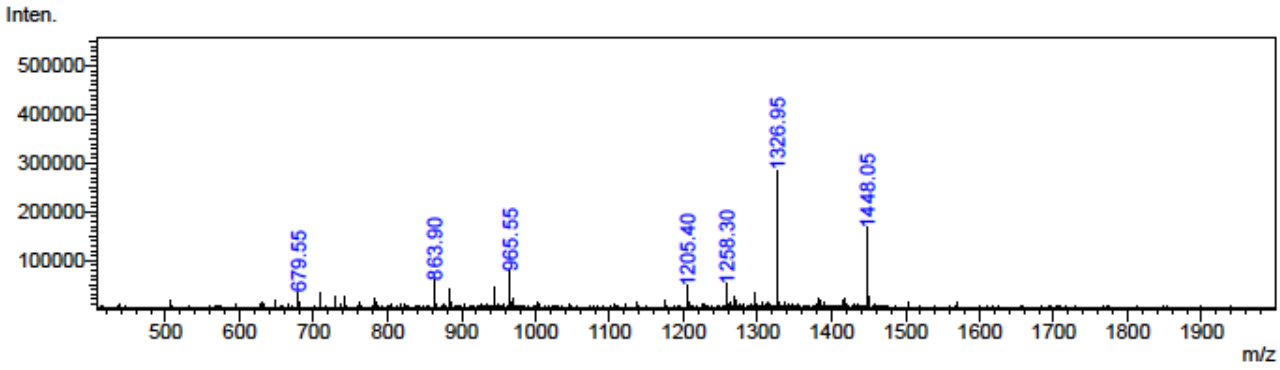


Figure 3.34 MS spectrum for direct injection. Table 3.5. Entry 4

Entry 5. A freshly prepared solution of TFA:TIPS:DCM (4:5:91, 300 μL) was added and the reaction mixture was agitated at room temperature for 5 min.

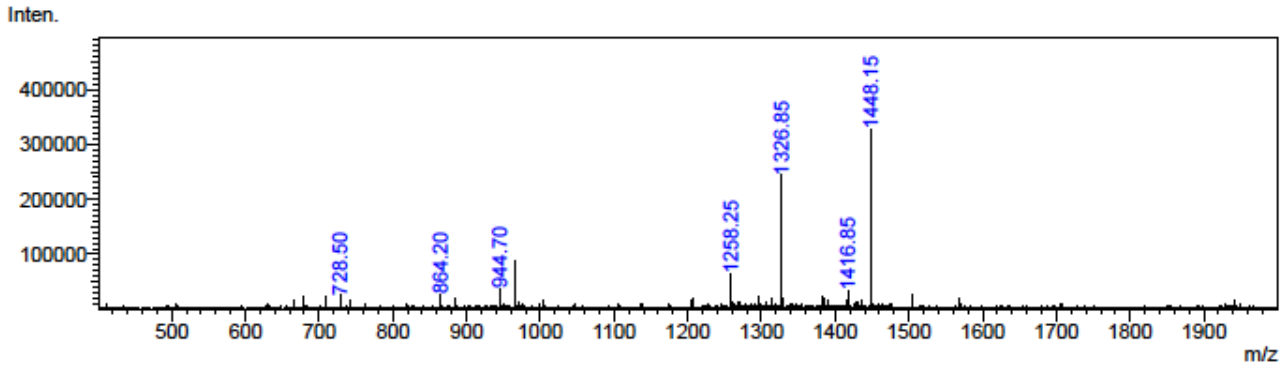


Figure 3.35 ES-MS spectrum for direct injection. Table 3.5. Entry 5

Entry 6. A freshly prepared solution of TFA:TIPS:DCM (5:5:90, 300 μL) was added and the reaction mixture was agitated at room temperature for 5 min.

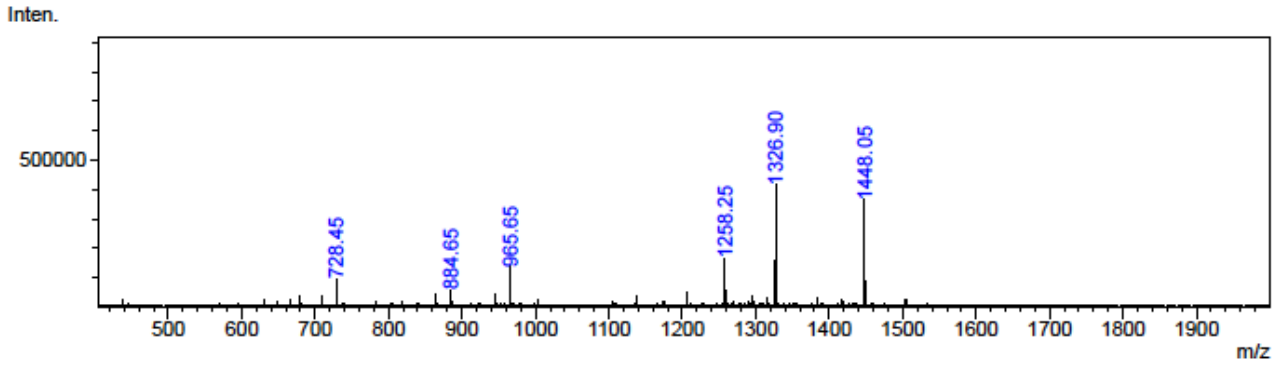
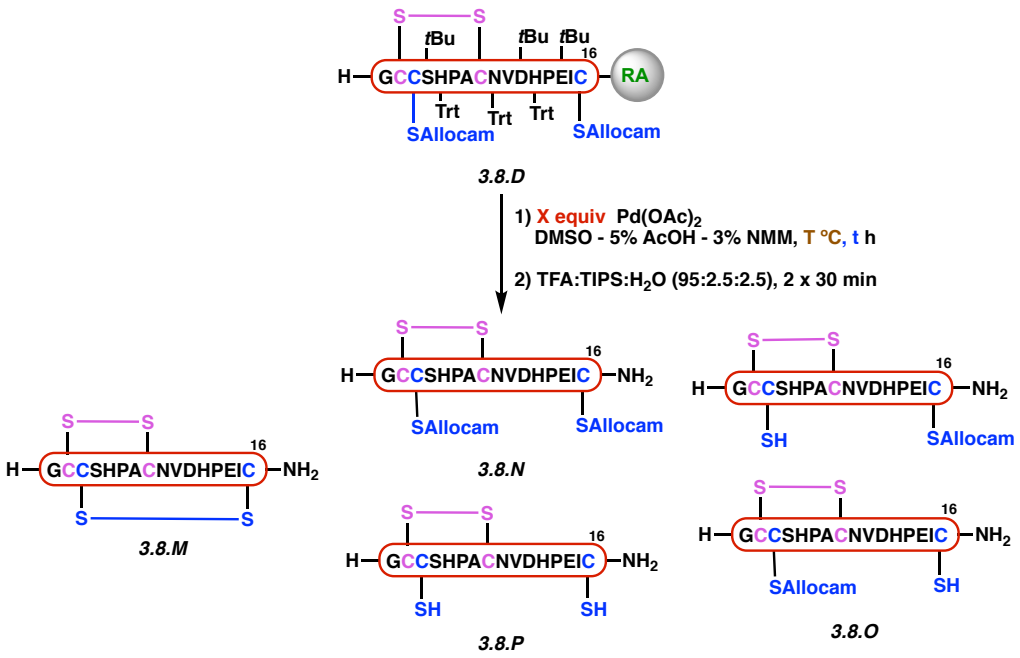


Figure 3.36 ES-MS spectrum for direct injection. Table 3.5. Entry 5



General Procedure for Reaction Optimization Table 3.6. For each of the reactions, the general procedure for Pd-DMSO cleavage-oxidation was followed. The stated amount of the resin **3.8.D** was subjected to the stated amount of $Pd(OAc)_2$, NMM and DMSO-5% AcOH. The reaction mixture was agitated in a 5 mL closed vial at room temperature for stated amount of time. After the wash, the dried resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 x 30 min. Finally, the TFA solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.N $[(C_{76}H_{114}N_{24}O_{26}S_4 + 3)/3]^+$: 637.04; observed: 637.20. m/z ES calc'd for peptide 3.8.O

$[(C_{71}H_{107}N_{23}O_{24}S_4 + 3)/3]^+$: 599.54; observed: 598.50. m/z ES calc'd for peptide 3.8.M $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.65.

Entry 1. The relevant quantities are: Peptide 3.X.A (30 mg, 9 μmol), $Pd(OAc)_2$ (3.0 mg, 13.5 μmol), NMM (34.0 μL, 68.4 μmol) and DMSO-5% AcOH (1125 μL). The reaction mixture was agitated at room temperature for 6 hours.

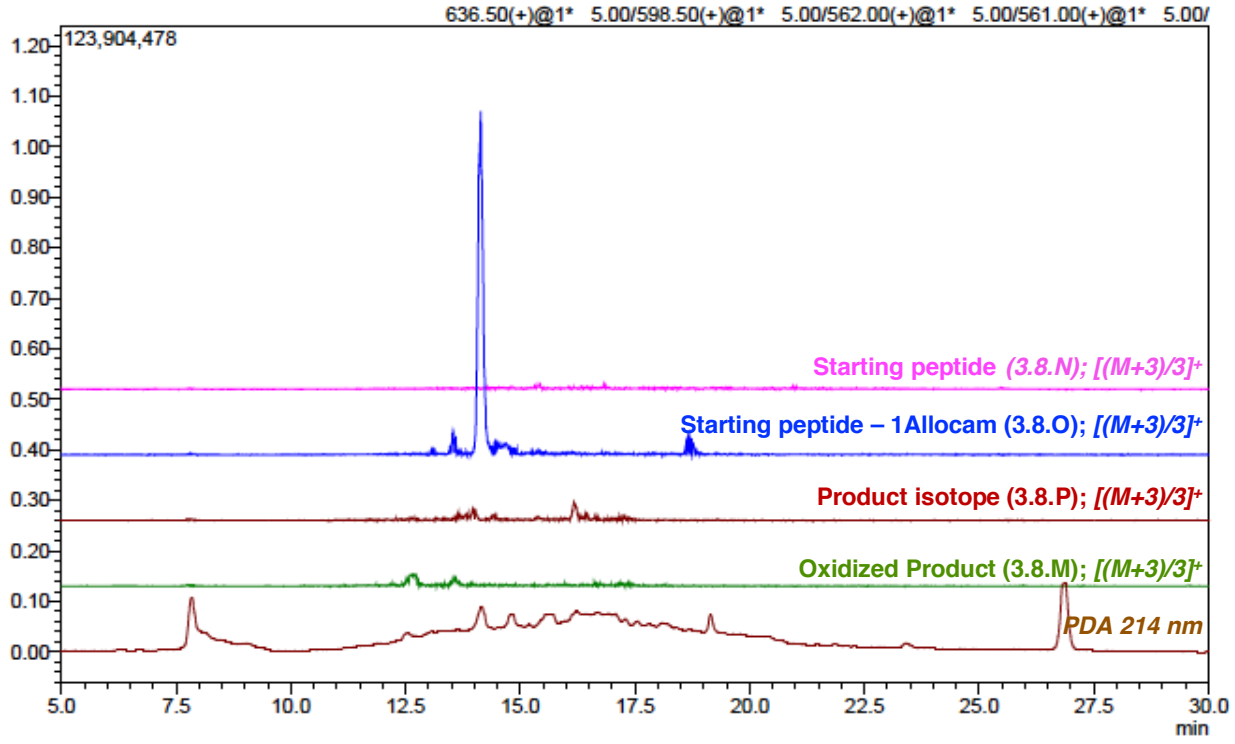


Figure 3.37 Analytical Column PDA for Table 3.6. Entry 1

Entry 2. The relevant quantities are: Peptide 3.X.A (30 mg, 9 μmol), $Pd(OAc)_2$ (3.0 mg, 13.5 μmol), NMM (34.0 μL, 68.4 μmol) and DMSO-5% AcOH (1125 μL). The reaction mixture was agitated at room temperature for 12 hours.

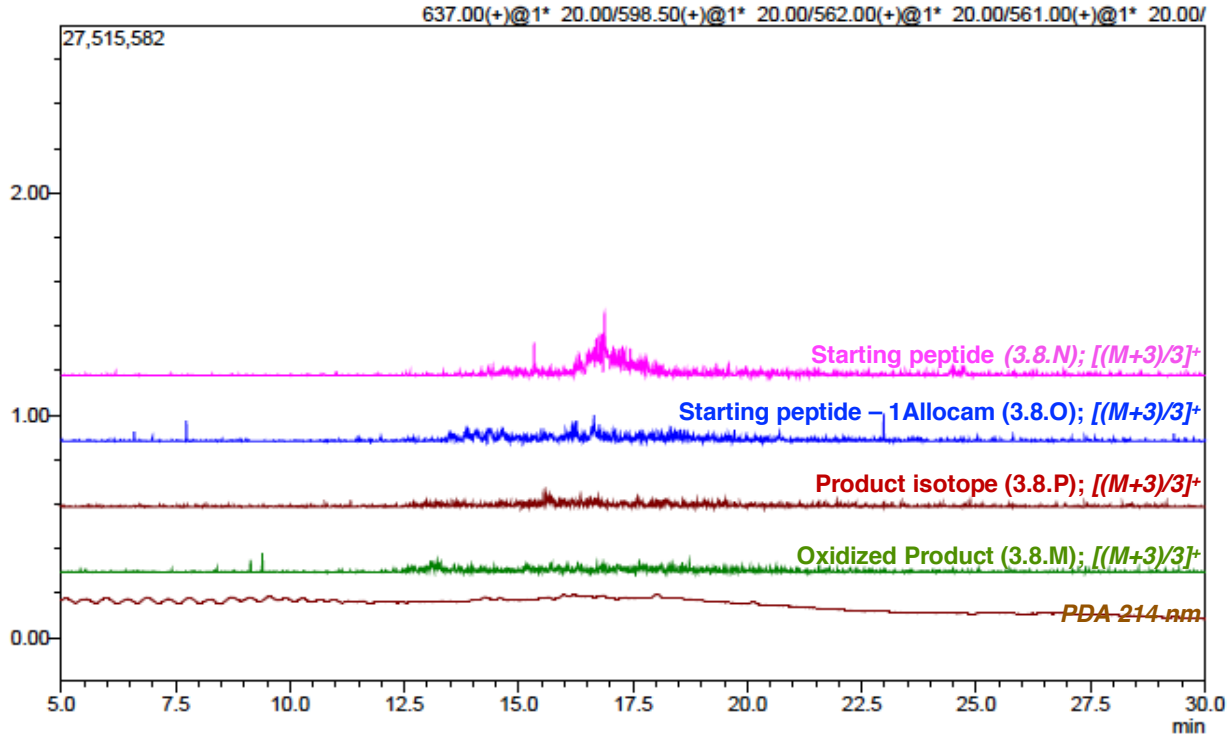


Figure 3.38 Analytical Column PDA for Table 3.6. Entry 2

Entry 3. The relevant quantities are: Peptide 3.X.A (30 mg, 9 μmol), $Pd(OAc)_2$ (6.1 mg, 27.0 μmol), NMM (34.0 μL, 68.4 μmol) and DMSO-5% AcOH (1125 μL). The reaction mixture was agitated at room temperature for 6 hours

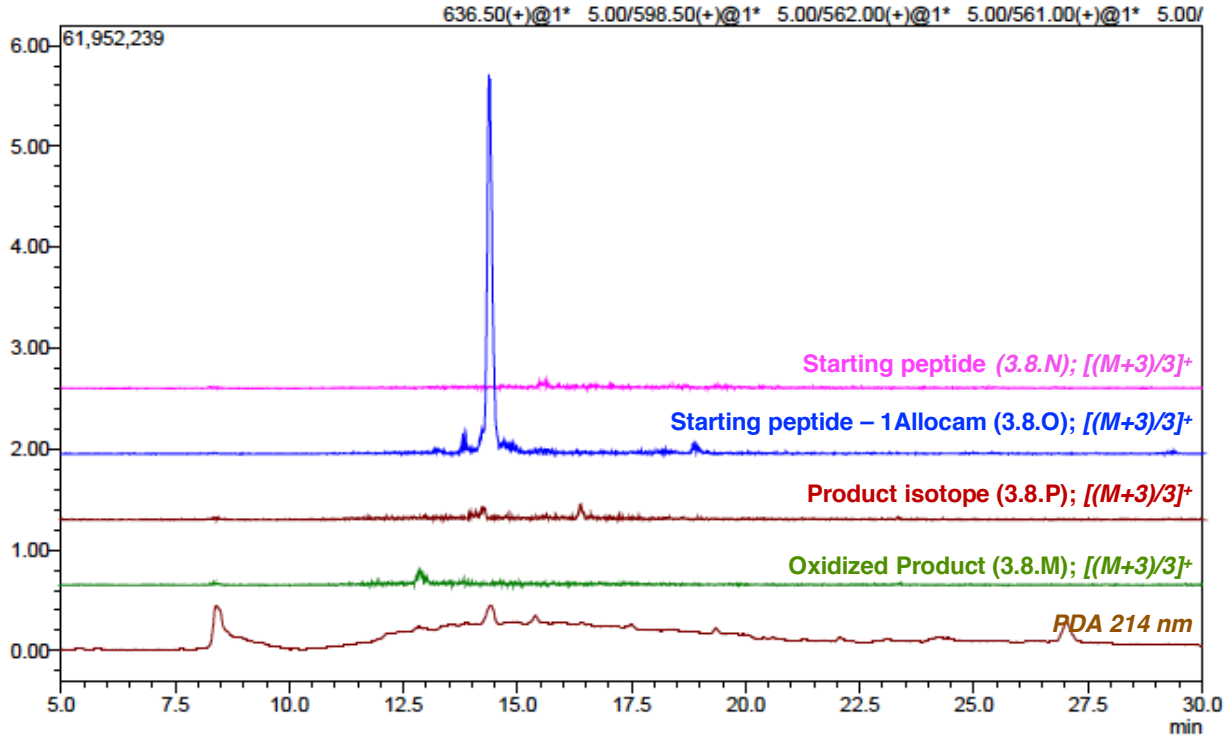


Figure 3.39 Analytical Column PDA for Table 3.6. Entry 3

Entry 4. The relevant quantities are: Peptide 3.X.A (30 mg, 9 μmol), $Pd(OAc)_2$ (14.1 mg, 63.0 μmol), NMM (34.0 μL, 68.4 μmol) and DMSO-5% AcOH (1125 μL). The reaction mixture was agitated at room temperature for 6 hours.

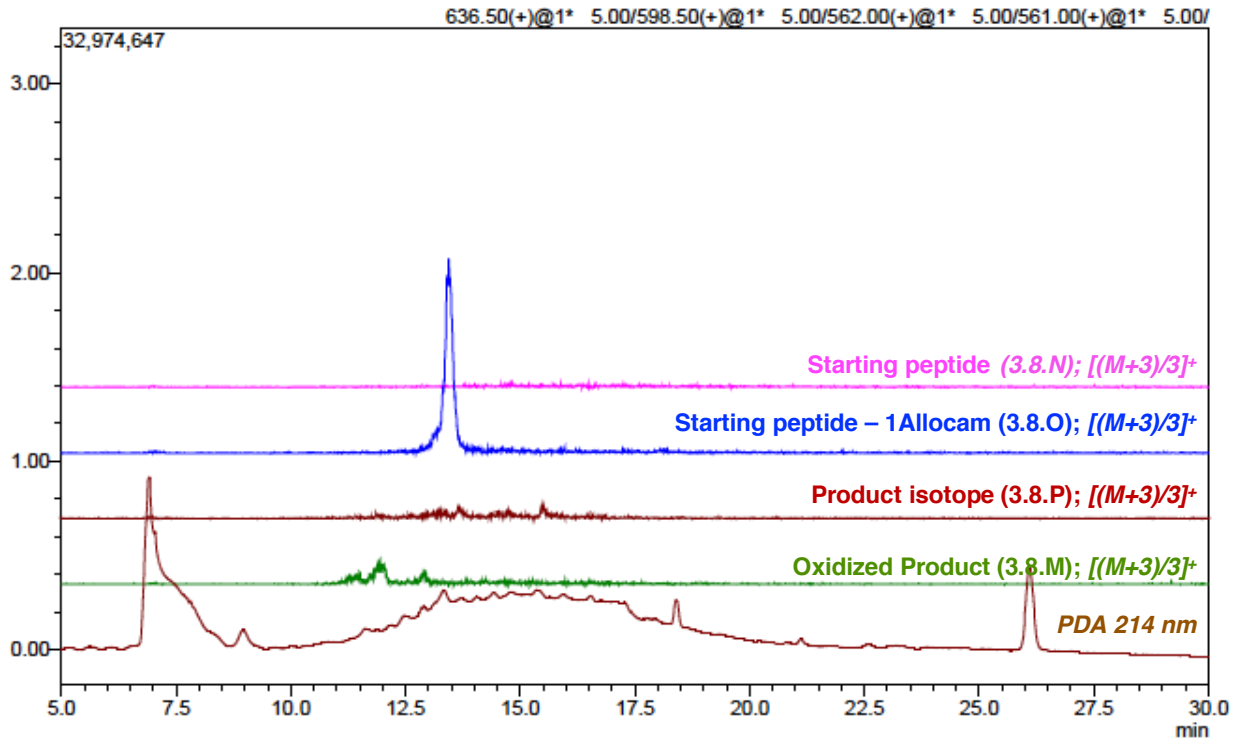


Figure 3.40 Analytical Column PDA for Table 3.6. Entry 4

Entry 5. The relevant quantities are: Peptide 3.X.A (50 mg, 15 μmol), $Pd(OAc)_2$ (10.1 mg, 45.0 μmol), NMM (56.0 μL, 513.0 μmol) and DMSO-5% AcOH (1875 μL). The reaction mixture was agitated at 60 ºC in a microwave for 6 hours.

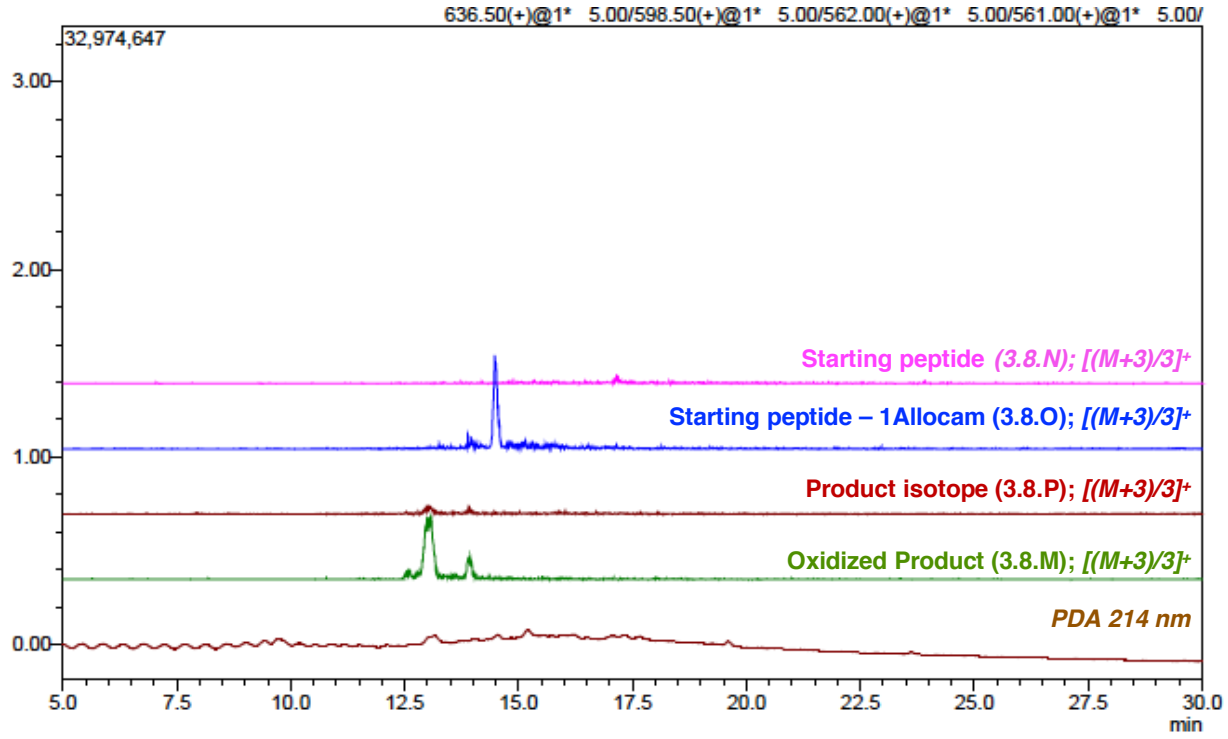


Figure 3.41 Analytical Column PDA for Table 3.6. Entry 5

Entry 6. The relevant quantities are: Peptide 3.X.A (22 mg, 6.6 μmol), $Pd(OAc)_2$ (4.4 mg, 19.8 μmol), NMM (25.0 μL, 225.72 μmol) and DMSO-5% AcOH (825 μL). The reaction mixture was agitated at 60 ºC in a microwave for 6 hours. Next, the reaction solution was then filtered and the resin was subjected again to $Pd(OAc)_2$ (2.2 mg, 9.9 μmol), NMM (25.0 μL, 225.72 μmol) and DMSO-5% AcOH (825 μL) and agitated again at 60 ºC in a microwave for 3 hours.

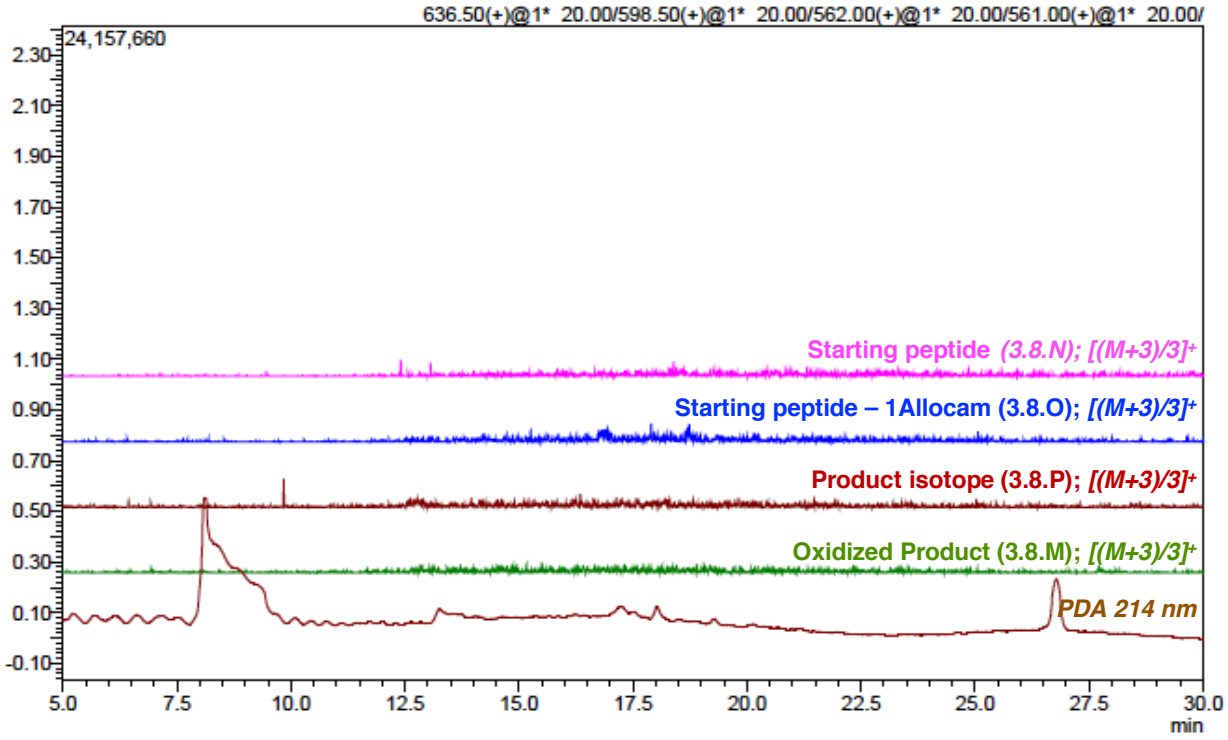


Figure 3.42 Analytical Column PDA for Table 3.6. Entry 6

Entry 7. The relevant quantities are: Peptide 3.X.A (50 mg, 15 μmol), $Pd(OAc)_2$ (10.1 mg, 45.0 μmol), NMM (56.0 μL, 513.0 μmol) and DMSO-5% AcOH (1875 μL). The reaction mixture was agitated at 60 ºC in a microwave for 7 hours.

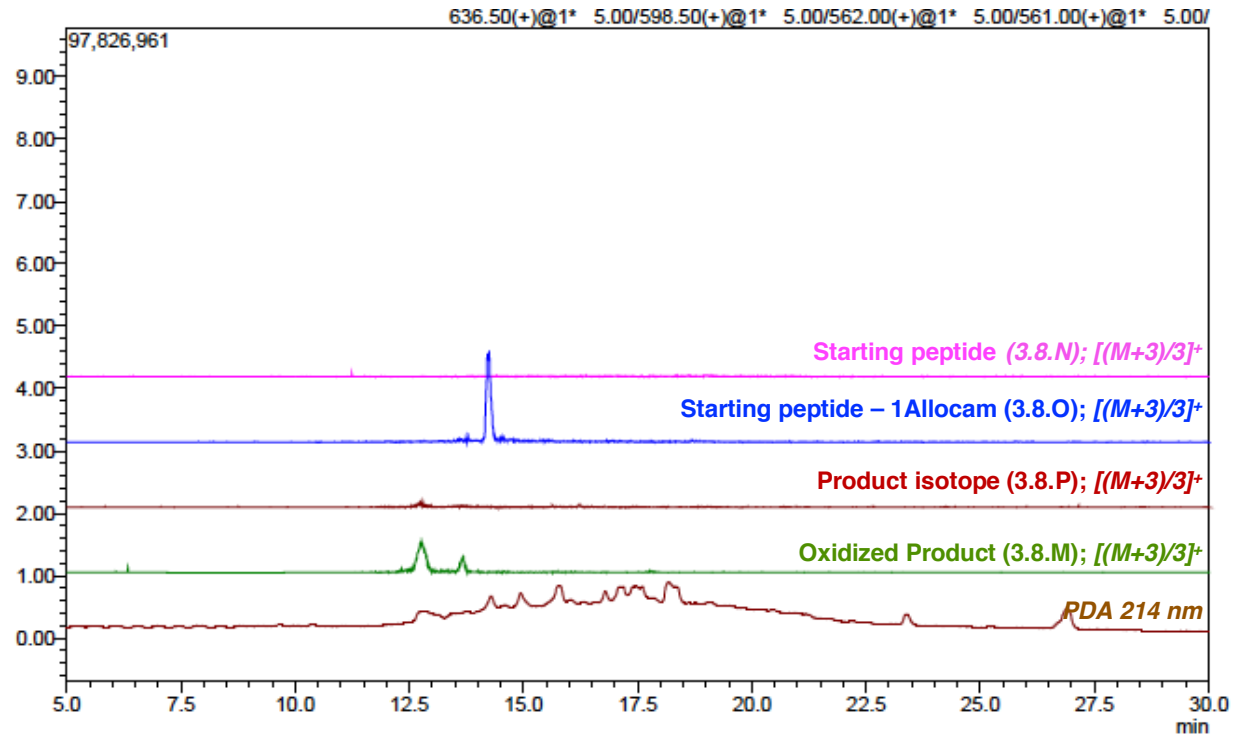


Figure 3.43 Analytical Column PDA for Table 3.6. Entry 7

Entry 8. The relevant quantities are: Resin (50 mg, 15 μmol), $Pd(OAc)_2$ (10.1 mg, 45.0 μmol), NMM (56.0 μL, 513.0 μmol) and DMSO-5% AcOH (1875 μL). The reaction mixture was agitated in a 10 mL closed vial at 60 ºC in a microwave for 6 hours.

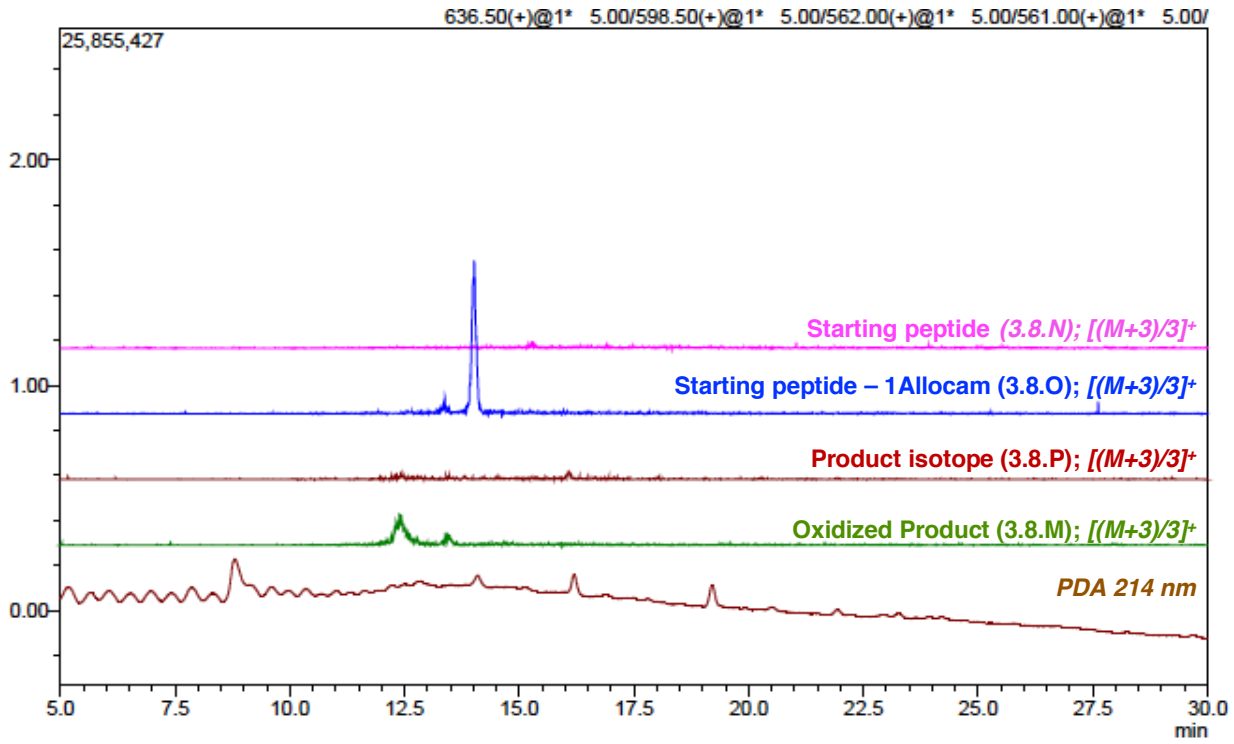


Figure 3.44 Analytical Column PDA for Table 3.6. Entry 8

Entry 9. The relevant quantities are: Peptide 3.X.A (50 mg, 15 μmol), $Pd(OAc)_2$ (16.8 mg, 75.0 μmol), NMM (56.0 μL, 513.0 μmol) and DMSO-5% AcOH (1875 μL). The reaction mixture was agitated at 60 ºC in a microwave for 6 hours.

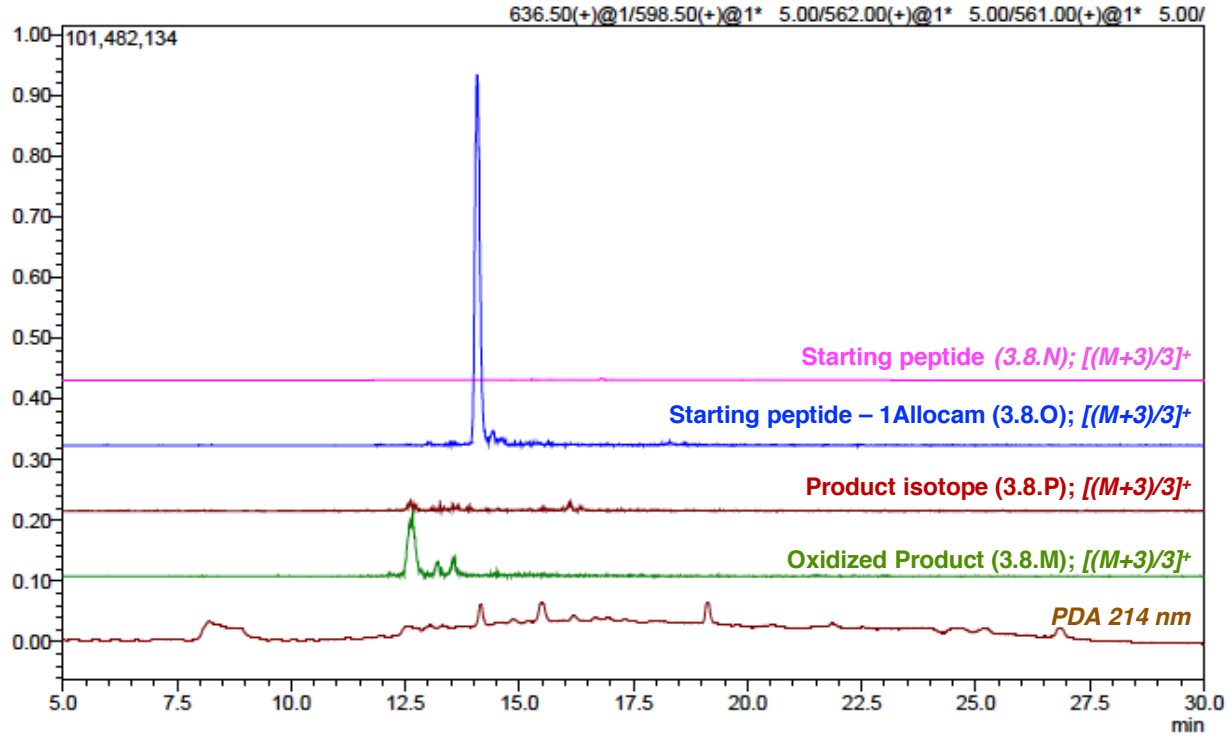
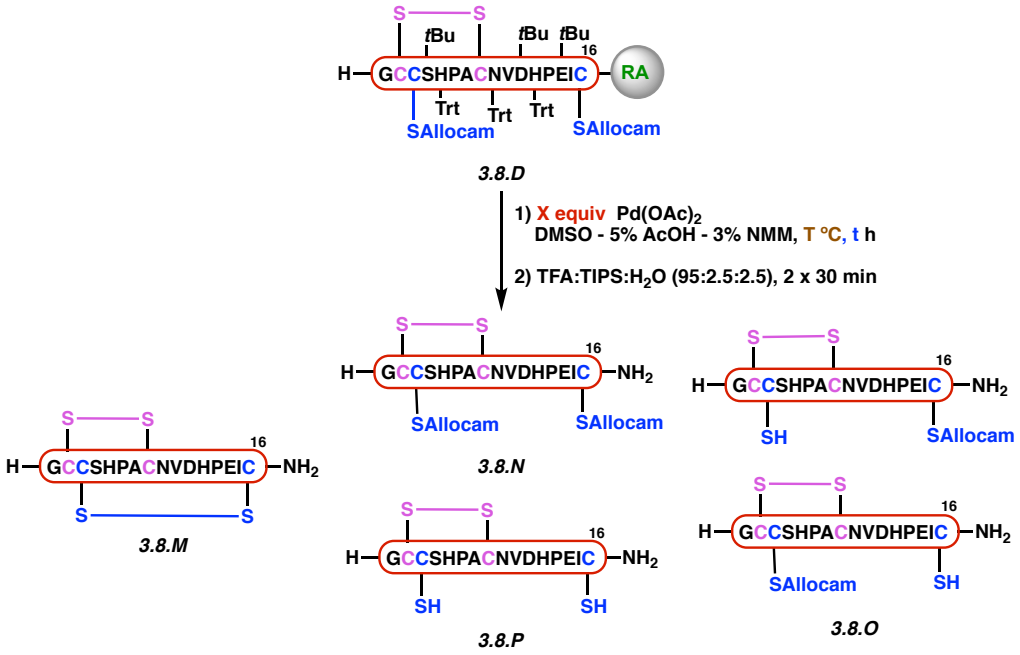


Figure 3.45 Analytical Column PDA for Table 3.6. Entry 9



General Procedure for Reaction Optimization Table 3.7. For each of the reactions, the peptide 3.8.D (20 mg, 6 μmol) was first swelled in DMF (500 μL). Then, the sated amount of NCS in DMF was added and allowed to agitate at room temperature for stated amount of time. The reaction solution was filtered and the resin was washed with DMF (5 x 1 mL) and DCM (5 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 x 30 min. Finally, the TFA solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.N $[(C_{76}H_{114}N_{24}O_{26}S_4 + 3)/3]^+$: 637.04; observed: 637.20. m/z ES calc'd for peptide 3.8.O $[(C_{71}H_{107}N_{23}O_{24}S_4 + 3)/3]^+$:

599.54; observed: 598.50. m/z ES calc'd for peptide 3.8.M $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.65.

Entry 1. A solution of NCS (4.0 mg, 30 μmol) in DMF (400 μL) was added and the reaction was allowed to agitate at room temperature for 6 hours.

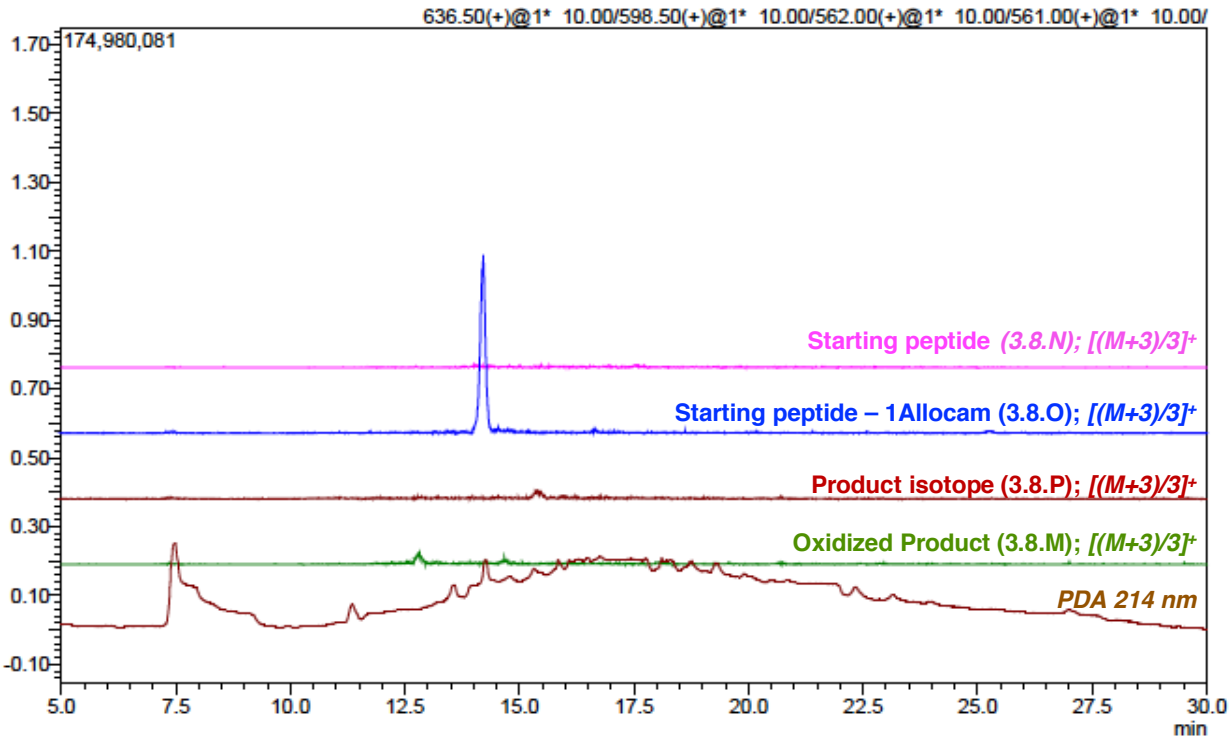


Figure 3.46 Analytical Column PDA for Table 3.7. Entry 1

Entry 2. A solution of NCS (4.0 mg, 30 μmol) in DMF (400 μL) was added and the reaction was allowed to agitate at room temperature for 10 hours.

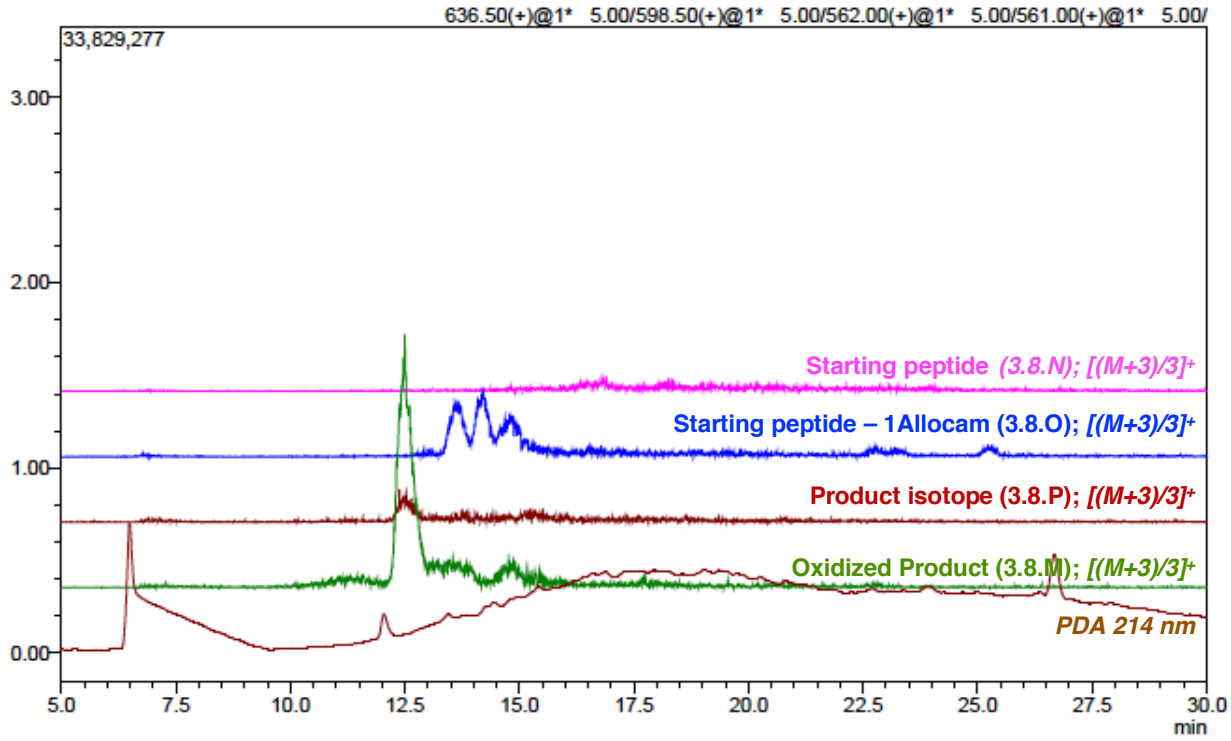


Figure 3.47 Analytical Column PDA for Table 3.7. Entry 2

Entry 3. A solution of NCS (5.6 mg, 42 μmol) in DMF (400 μL) was added and the reaction was allowed to agitate at room temperature for 6 hours.

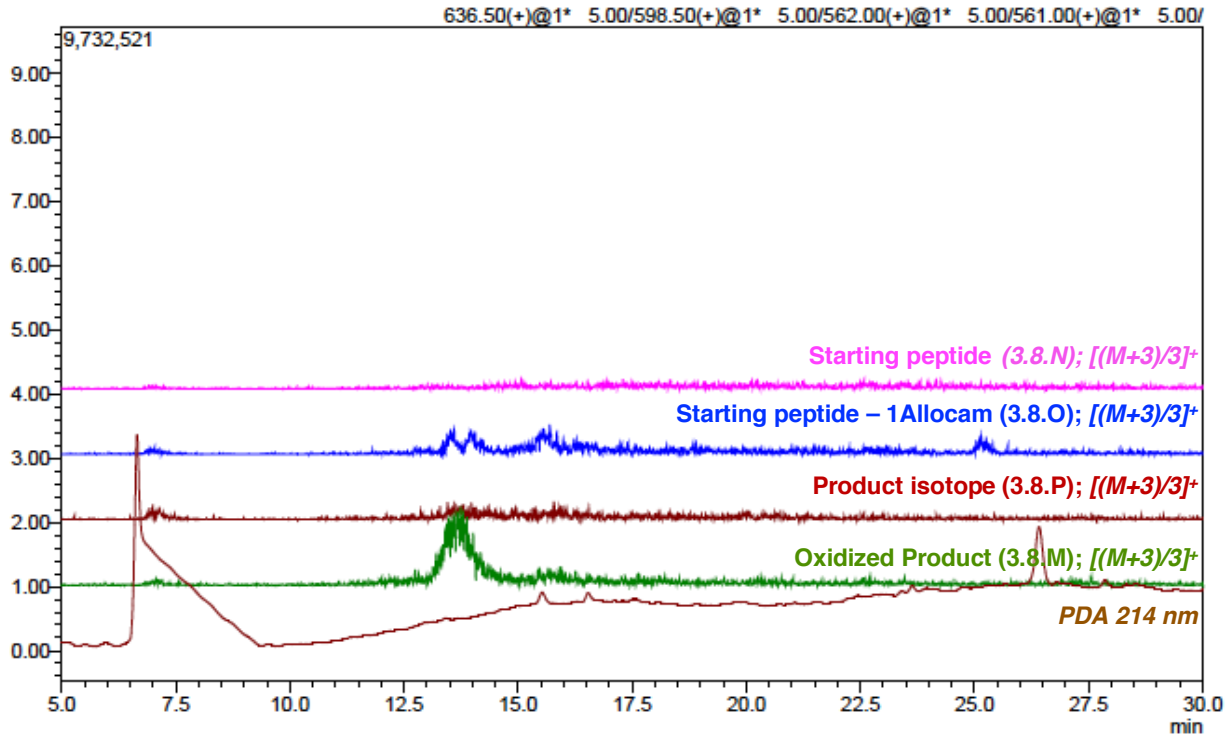
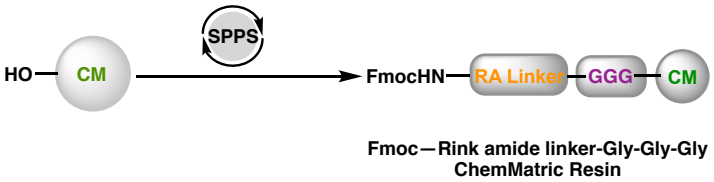
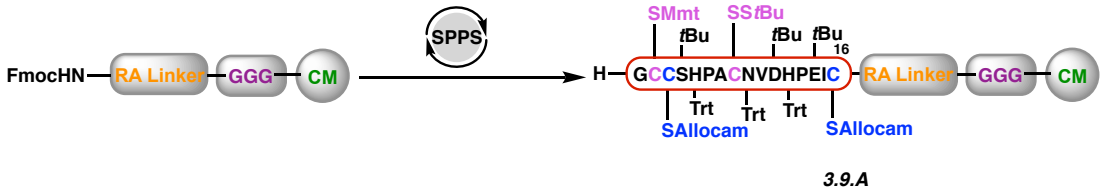


Figure 3.48 Analytical Column PDA for Table 3.7. Entry 3



Rink Amide Linker-Gly-Gly-Gly-ChemMatrix Resin 3.X.A. The standard Fmoc-SPPS protocol described was used to synthesize Rink amide linker-Gly-Gly-Gly-ChemMatrix Resin, except a special swelling method was performed to swell ChemMatrix resin before coupling the first amino acid to get efficient loading. Here, First, MeOH (3 mL) was added and agitated for 1 min at RT. The solvent was then removed and the treatment was repeated with MeOH. In a similar manner, the resin was washed with DMF (2 x 1 min), DCM (3 x 1 min), TFA-DCM (1:99) (3 x 1 min), DIEA-DCM (1:19) (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). The linker was capped after the synthesis. Loading of the linker on resin: 0.3 – 0.35 mmol/g



SPPS of Peptide 3.9.A. The standard Fmoc-SPPS protocol described was used to synthesize peptide **3.9.A** on Rink amide linker-Gly-Gly-Gly-ChemMatrix resin. The peptide was capped before the last coupling step. To analyze the final peptide, 10 mg of the resin was subjected to cleavage solution of TFA:TIPS:H_2O (95:2.5.2.5) for 30 min. Finally, the TFA solution was concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide

was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. Final loading of the resin: 0.14 mmol/g. m/z ES calc'd for peptide 3.9.A [($C_{80}H_{124}N_{24}O_{26}S_5$ + 2)/2]$^+$: 1000.15; observed: 999.80. m/z ES calc'd for [($C_{74}H_{116}N_{24}O_{26}S_5$ + 3)/3]$^+$: 667.10; observed: 666.90.

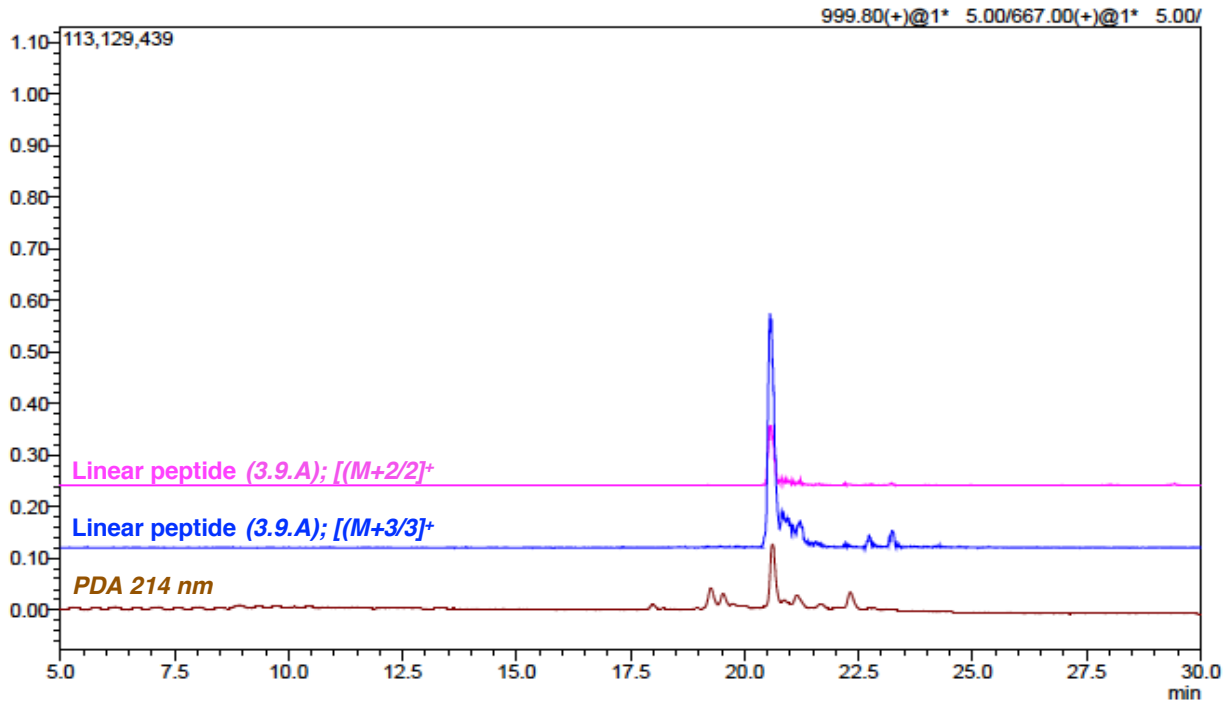


Figure 3.49 Analytical Column PDA for linear peptide **3.9.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 20.62 min

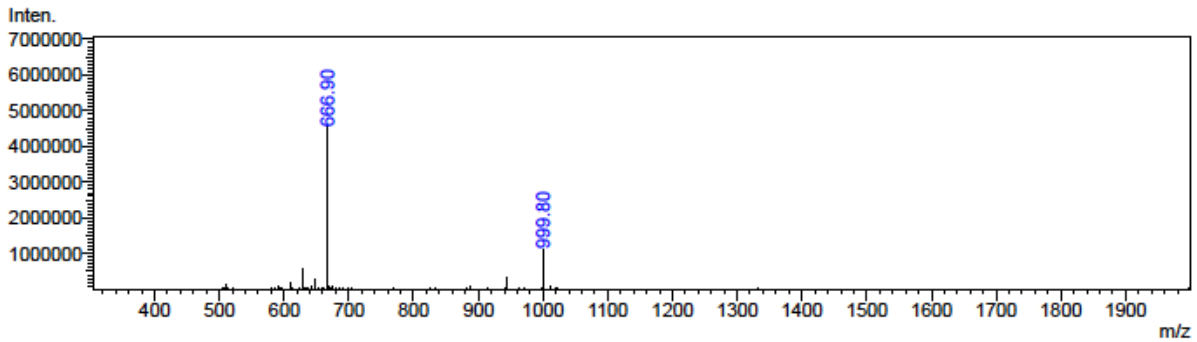
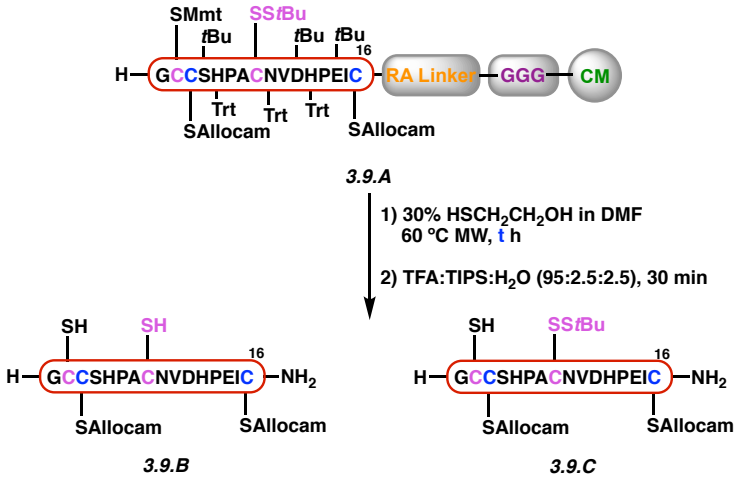


Figure 3.50 ES-MS spectrum for linear peptide **3.9.A** at retention time 20.62 min



General Procedure for Reaction Optimization Table 3.8. For each of the reactions, the peptide 3.9.A (10 mg, 3 μmol) was first swelled in DMF (500 μL). Then, a solution of 30% mercaptoethanol in DMF (1.0 mL) was added and agitated for stated amount of time. The reaction solution was filtered and the resin was washed with DMF (5 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture (95:2.5:2.5 TFA:TIPS:H_2O) for 30 min. The resulting TFA solution was collected, concentrated, and the peptide was crashed out with cold diethyl ether. The precipitate was centrifuged, ether discarded, and the pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.X.A $[(C_{80}H_{124}N_{24}O_{26}S_5 + 2)/2]^+$: 1000.15; observed: 999.80. m/z ES calc'd for $[(C_{74}H_{116}N_{24}O_{26}S_5 + 3)/3]^+$: 667.10; observed: 666.95. m/z ES calc'd for peptide 3.X.C $[(C_{76}H_{116}N_{24}O_{26}S_4 + 2)/2]^+$: 956.08; observed: 955.85. m/z ES calc'd for $[(C_{76}H_{116}N_{24}O_{26}S_4 + 3)/3]^+$: 637.72; observed: 637.60.

Entry 1. The reaction was agitated for 3 hours.

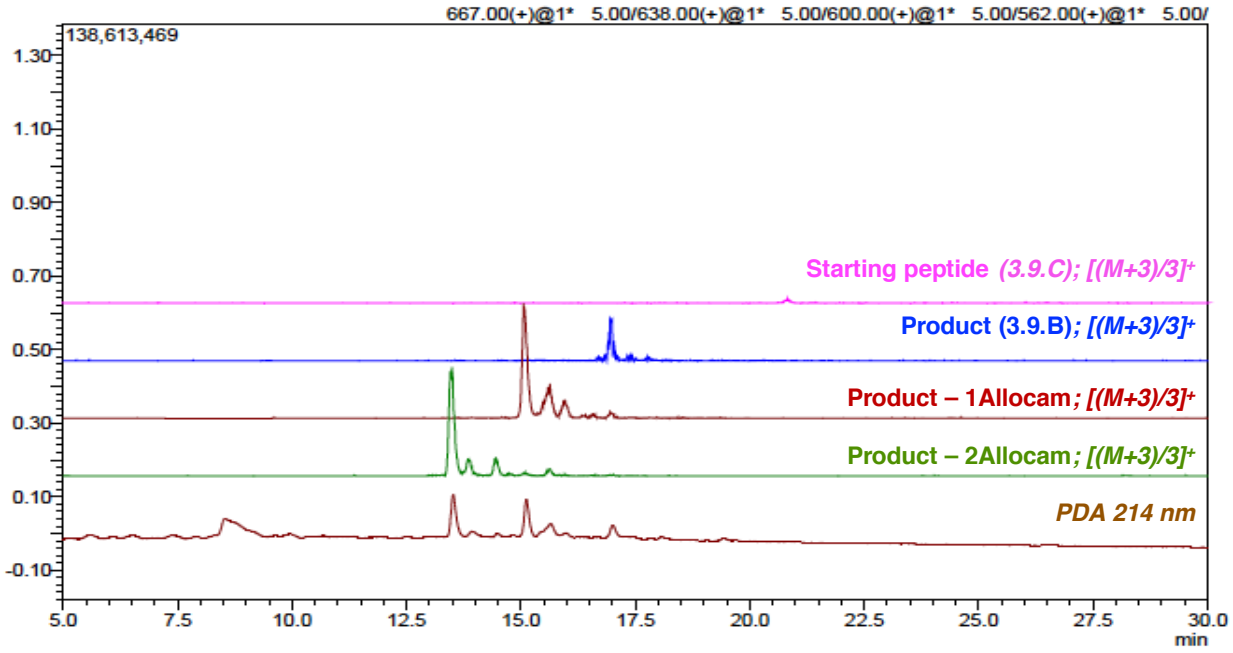


Figure 3.51. Analytical Column PDA for Table 3.8. Entry 1

Entry 2. The reaction was agitated for 1.5 hours.

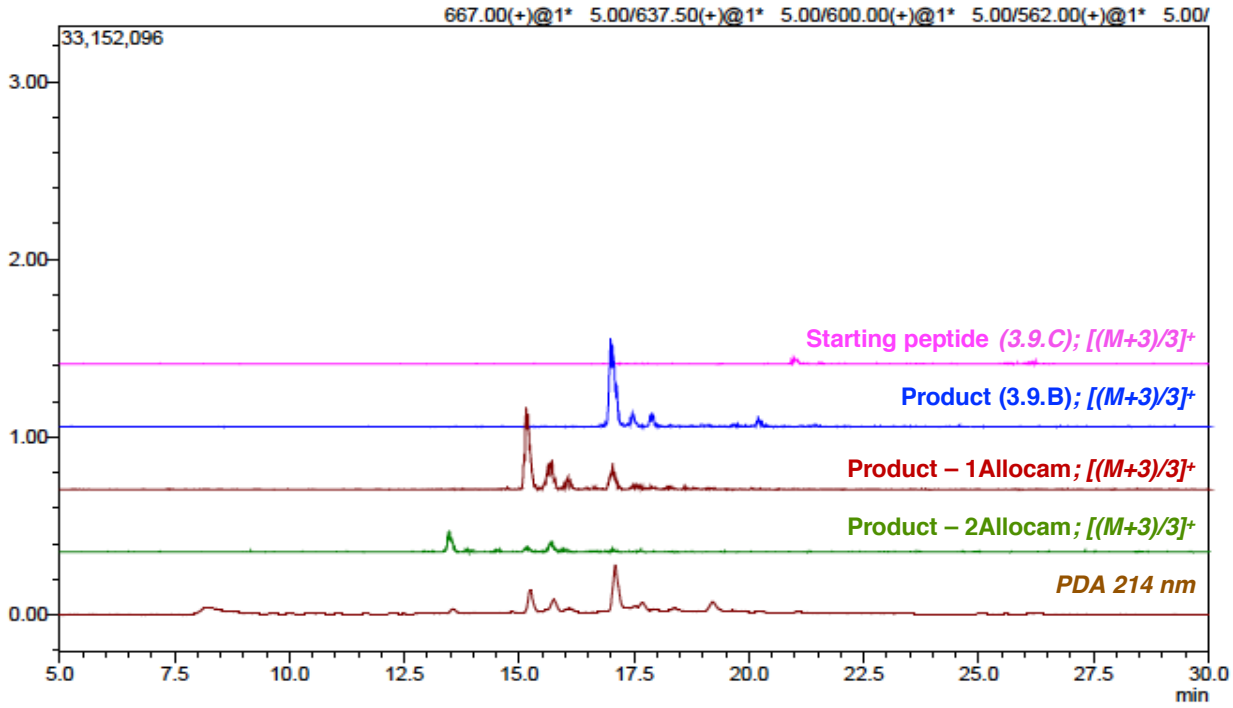


Figure 3.52 Analytical Column PDA for Table 3.8. Entry 2

Entry 3. The reaction was agitated for 1 hour.

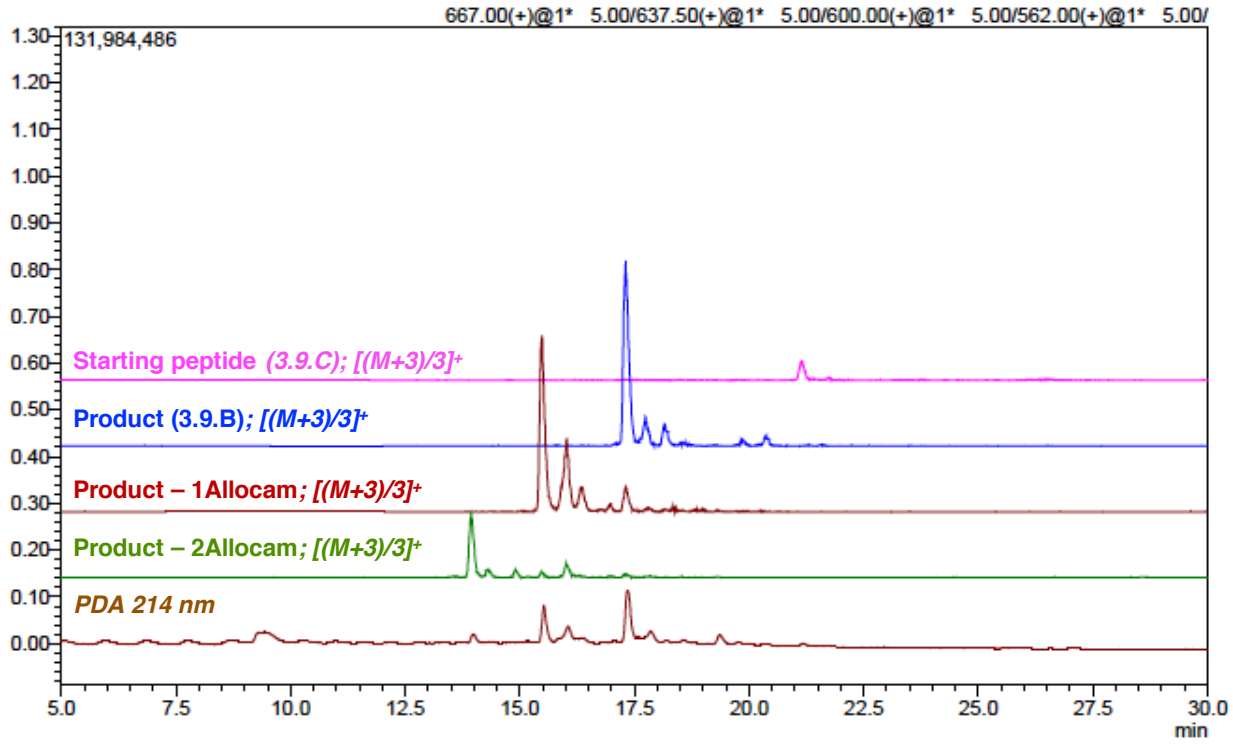
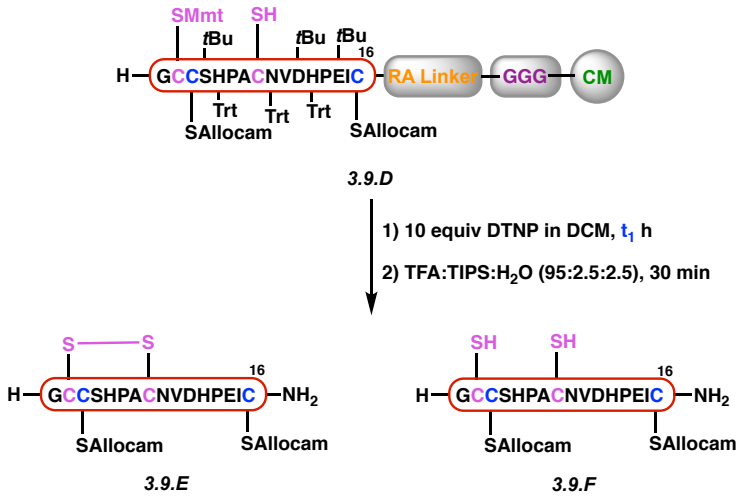


Figure 3.53 Analytical Column PDA for Table 3.8. Entry 3



General Procedure for Reaction Optimization Table 3.9. For each of the reactions, The resin **3.9.D** (10 mg, 3 μmol) was first swelled in DMF (500 μL). Then, a solution of DTNP (9.3 mg, 30 μmol) in DCM (1.0 mL) was added and allowed to stated amount of time. The reaction solution was filtered and the resin was washed with DCM (5 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture (95:2.5:2.5 TFA:TIPS:H_2O) for 30 min. The resulting TFA solution was collected, concentrated, and the peptide was crashed out with cold diethyl ether. The precipitate was centrifuged, ether discarded, and the pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.X.B $[(C_{76}H_{114}N_{24}O_{26}S_4 + 2)/2]^+$: 955.06; observed: 954.90. m/z ES calc'd for $[(C_{76}H_{114}N_{24}O_{26}S_4 + 3)/3]^+$: 637.04; observed: 636.95.

Entry 1. The reaction was agitated for 6 hour.

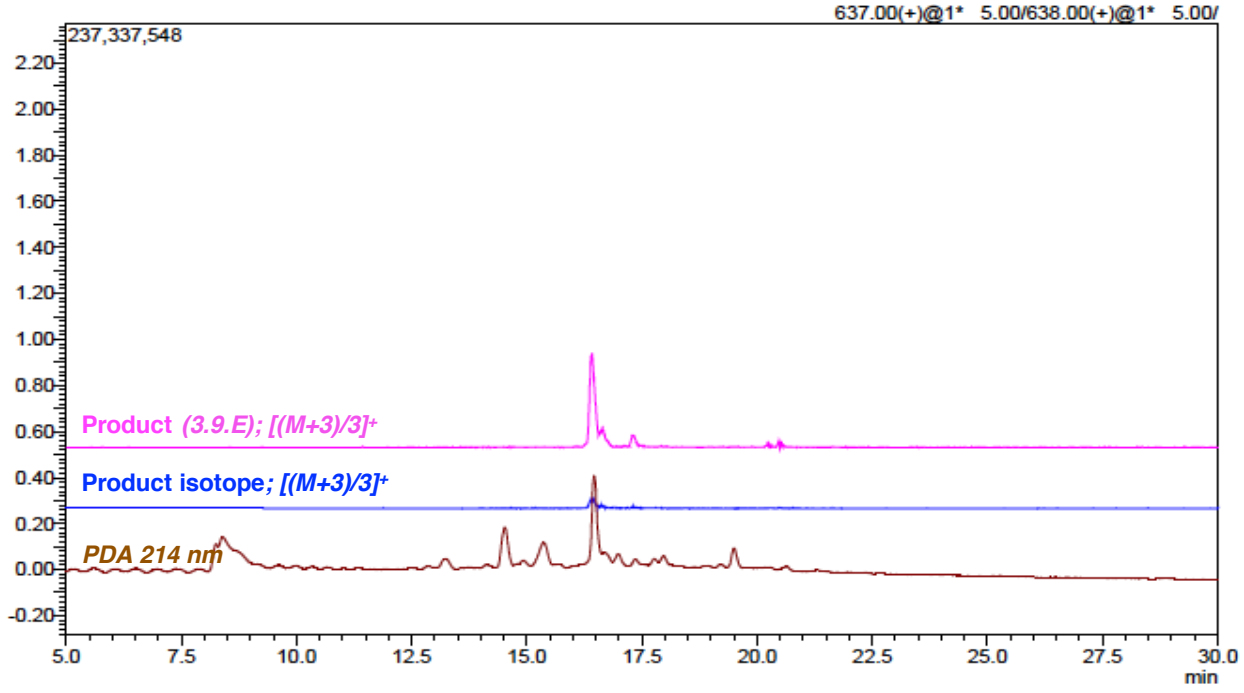


Figure 3.54 Analytical Column PDA for Table 3.9. Entry 1

Entry 2. The reaction was agitated for 3 hours.

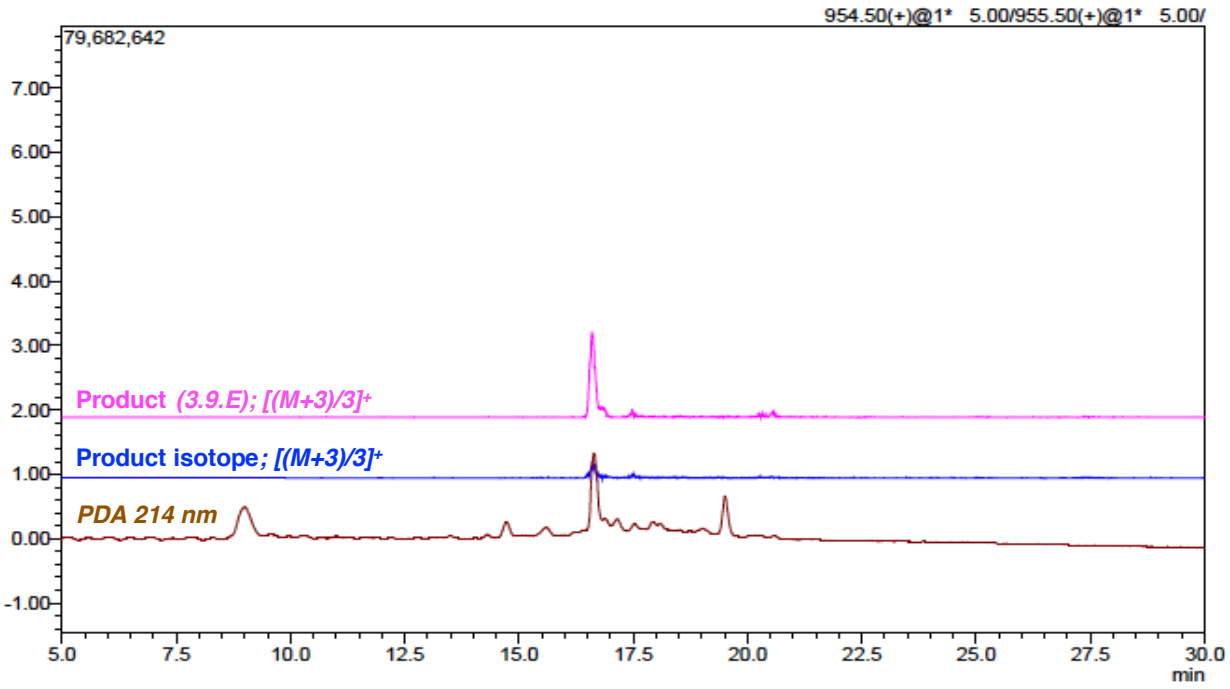


Figure 3.55 Analytical Column PDA for Table 3.9. Entry 2

Entry 3. The reaction was agitated for 1 hour.

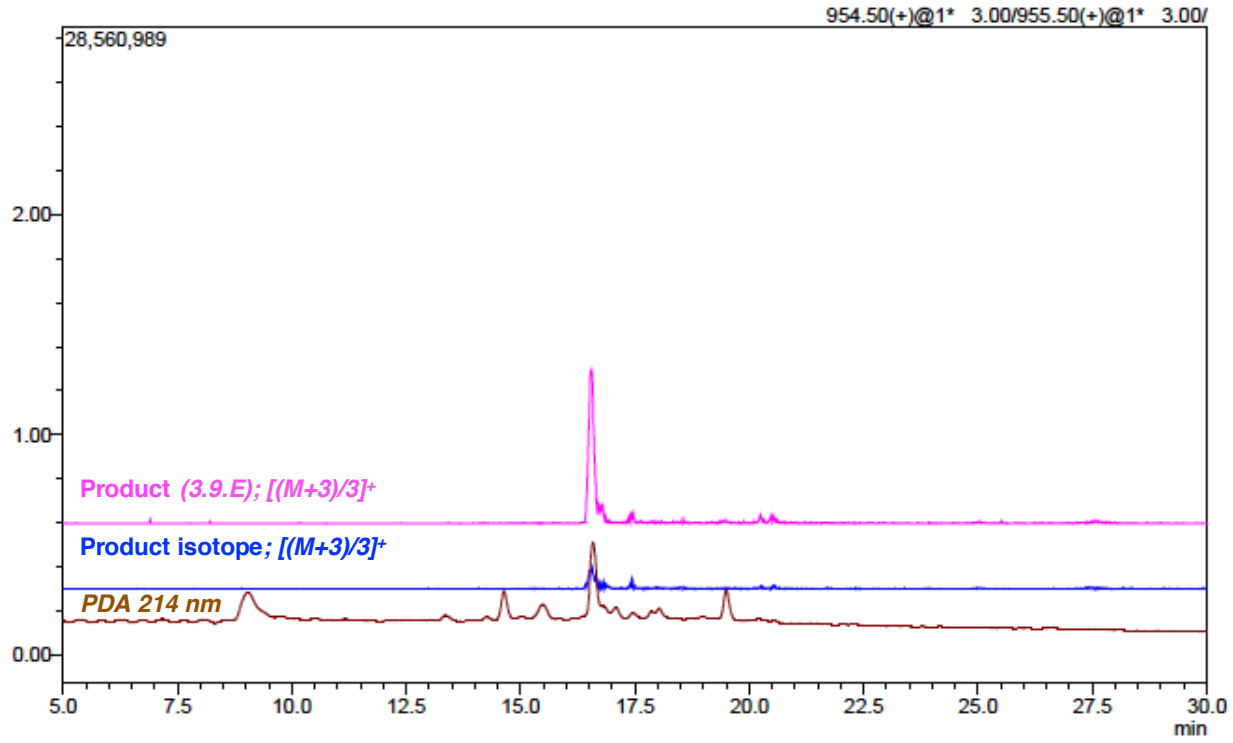
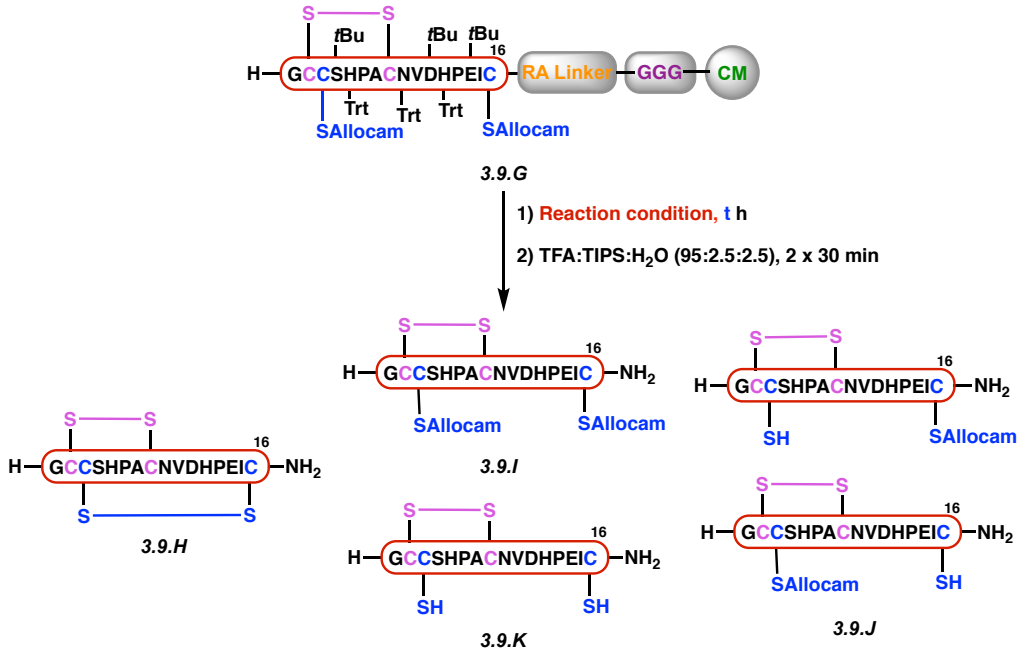


Figure 3.56 Analytical Column PDA for Table 3.9. Entry 3



Procedure for Table 3.10, Entry 1. The general procedure for Pd-DMSO cleavage-oxidation was followed. The relevant quantities are: Resin **3.9.G** (130 mg, 39 μmol), $Pd(OAc)_2$ (26.3 mg, 117.0 μmol), NMM (147.0 μL, 1333.8 μmol) and DMSO-5% AcOH (4875 μL). The reaction mixture was agitated in a 10 mL closed vial at room temperature for 6 hours. After the wash, the dried resin was subjected to 1 mL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 x 3 hours. Finally, the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.I $[(C_{76}H_{114}N_{24}O_{26}S_4 + 3)/3]^+$: 637.04; observed: 637.20. m/z ES calc'd for peptide 3.8.J

$[(C_{71}H_{107}N_{23}O_{24}S_4 + 3)/3]^+$: 599.54; observed: 598.50. m/z ES calc'd for peptide 3.8.H $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.65.

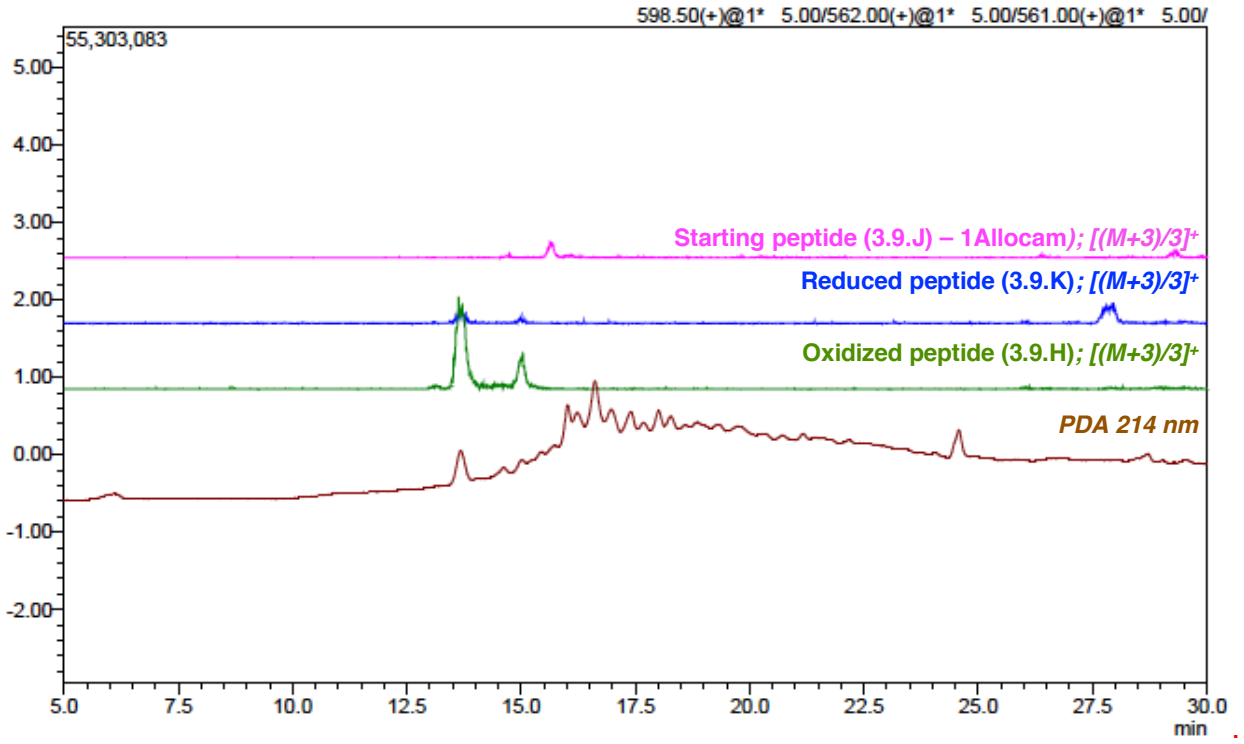


Figure 3.57 Analytical Column PDA for crude product **3.9.H**. Table 3.10. Entry 1

The crude peptide was purified via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min to afford α4/7-conotoxin LvIA in 7% yield (~1.6 mg).

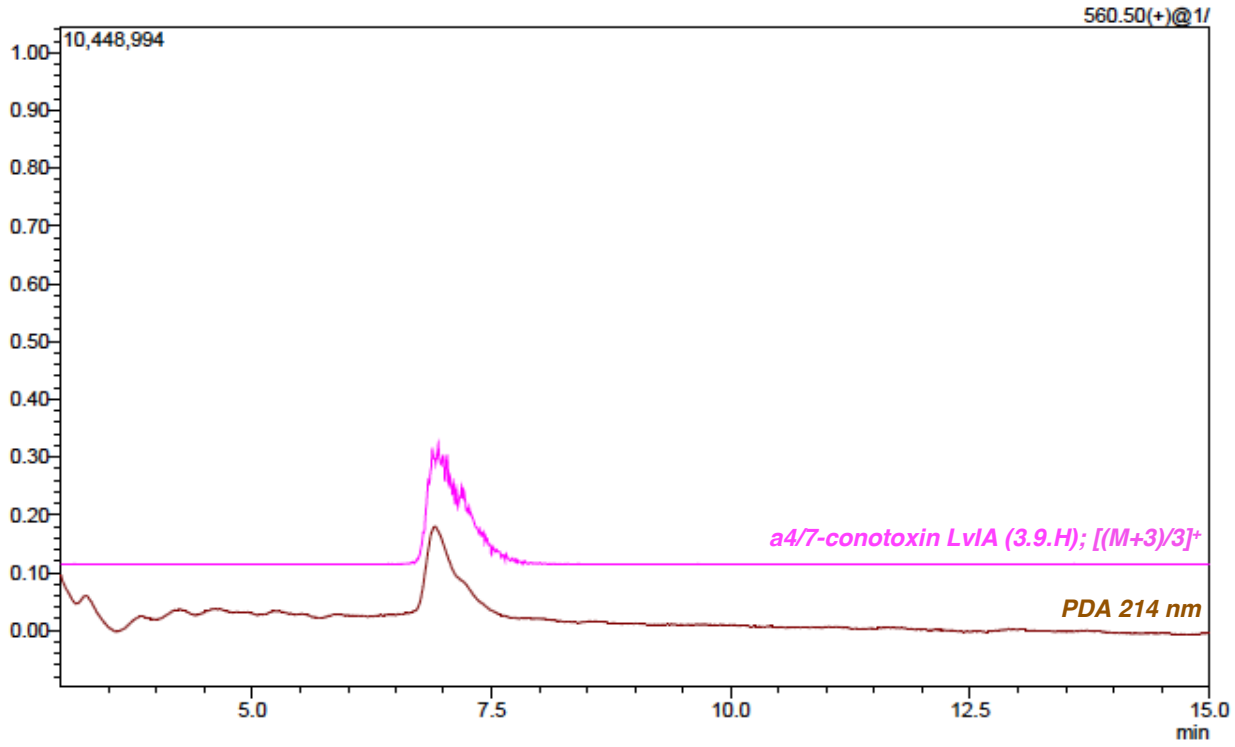


Figure 3.58 Analytical Column PDA for pure product **3.9.H**. Table 3.10. Entry 1
Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 15 min. Retention time 6.91 min

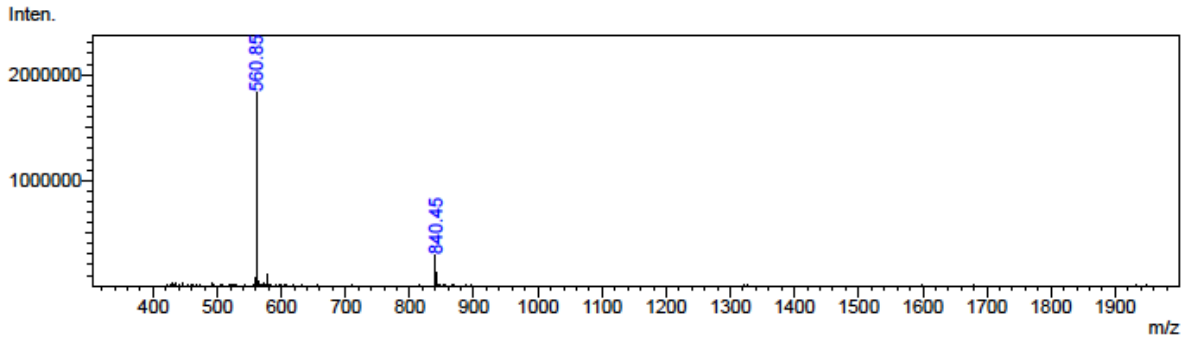


Figure 3.59 ES-MS spectrum for pure product **3.9.H** at retention time 6.91 min. Table 3.10. Entry 1

Procedure for Table 3.10, Entry 2. The resin 3.9.G (150 mg, 45 μmol) was first swelled in DMF (3 mL). Then, a solution of NCS (30.0 mg, 225 μmol) in DMF (1.0 mL) was added and allowed to agitate in a 10-mL closed vial at room temperature for 5 hours. The reaction solution was filtered and the resin was washed with DMF (5 x 3 mL) and DCM (5 x 3 mL). The resulting resin was subjected to 1 mL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 x 3 hours. Finally, the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.H $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.65.

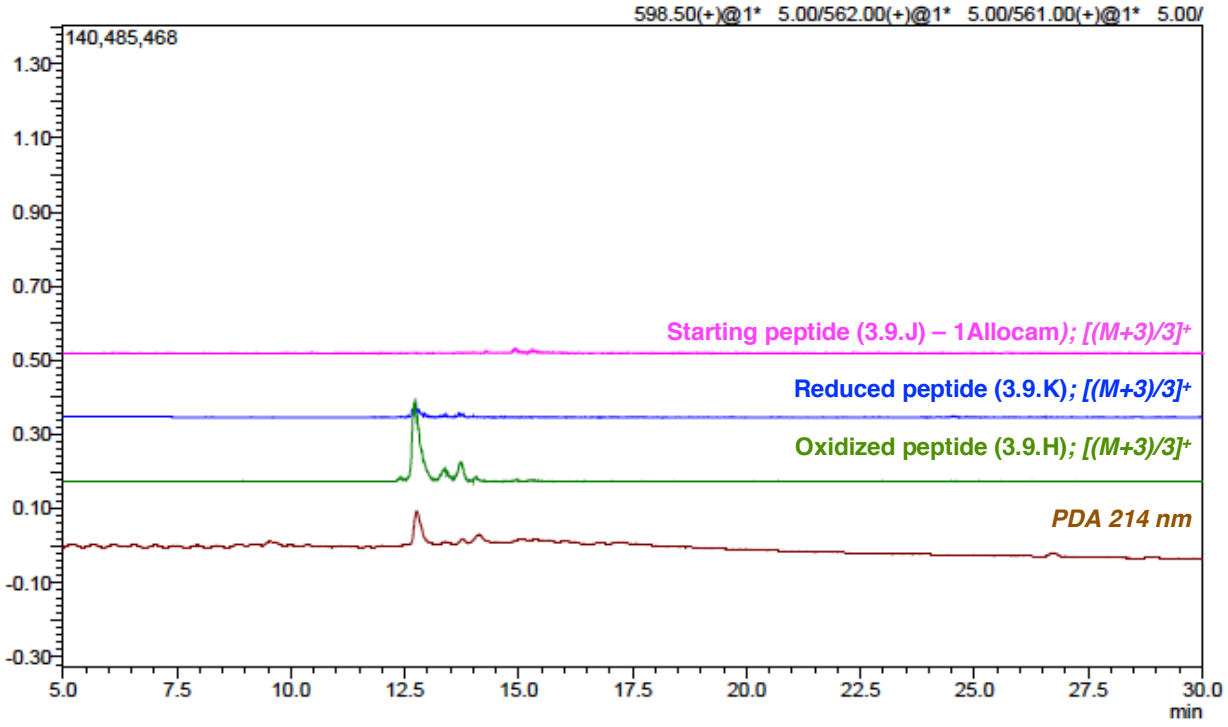


Figure 3.60 Analytical Column PDA for crude product **3.9.H**. Table 3.10. Entry 2

Procedure for Table 3.10, Entry 3. The resin 3.9.G (20 mg, 6 μmol) was first swelled in DMF (500 μL). Then, a solution of iodine (7.6 mg, 30 μmol) in DCM (400 μL) was added and allowed to agitate in a 2 mL vial at room temperature for 5 hours. The reaction solution was filtered and the resin was washed with DMF (10 x 1 mL) and DCM (10 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 x 30 min. Finally, the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.8.H $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.65. m/z ES calc'd for peptide 3.8.K $[(C_{66}H_{100}N_{22}O_{22}S_4 + 3)/3]^+$: 561.96; observed: 561.40.

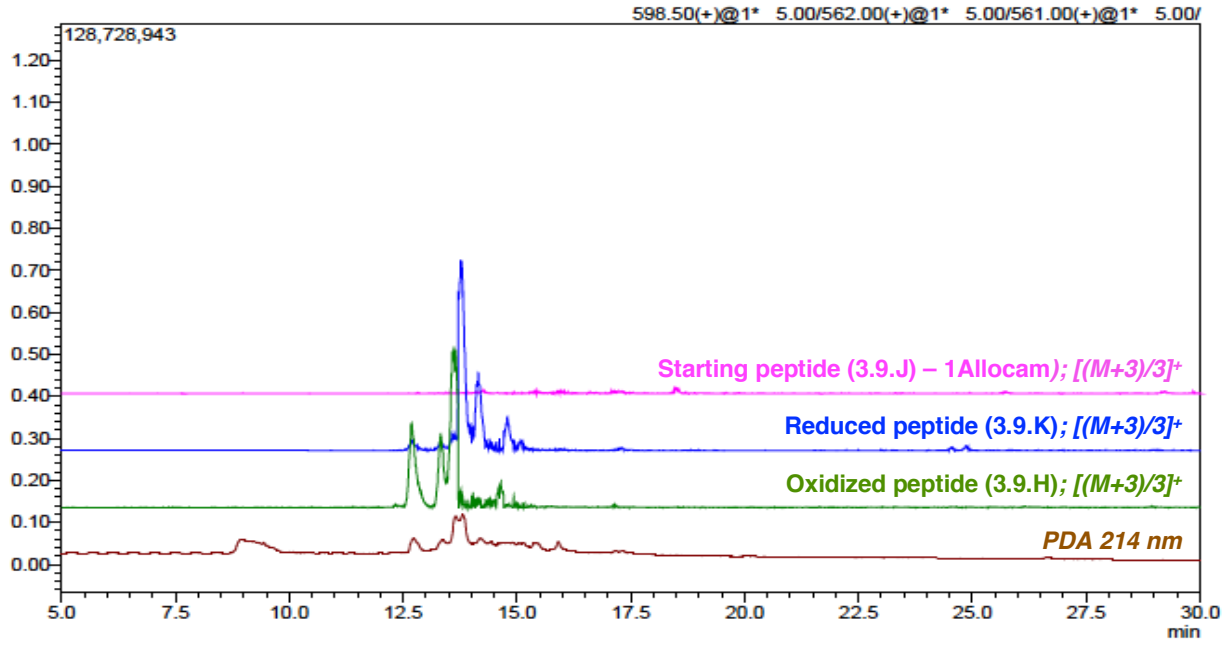


Figure 3.61 Analytical Column PDA for Table 3.10. Entry 3

Procedure for Table 3.10, Entry 4. The resin 3.9.G (150 mg, 45 μmol) was first swelled in DMF (3 mL). Then, a solution of iodine (91.3 mg, 360 μmol) in DCM (2 mL) was added and allowed to agitate in a 10-mL closed vial at room temperature for 5 hours. The reaction solution was filtered and the resin was washed with DMF (10 x 3 mL) and DCM (10 x 3 mL). The resulting resin was subjected to 1 mL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 x 3 hours. Finally, the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The peptide was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 3.X.B $[(C_{66}H_{98}N_{22}O_{22}S_4 + 2)/2]^+$: 840.94; observed: 840.70. m/z ES calc'd for $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.80.

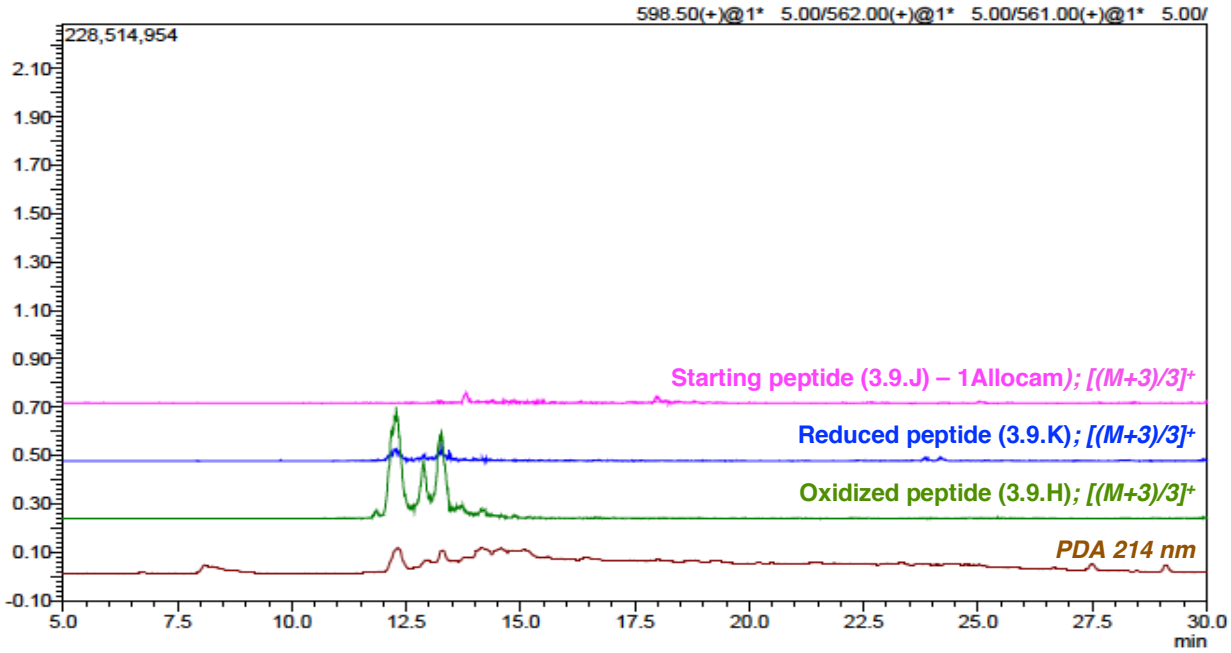


Figure 3.62 Analytical Column PDA for crude product **3.9.H**. Table 3.10. Entry 4

The crude peptide was purified via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min to afford α4/7-conotoxin LvIA in 18% yield (4.2 mg).

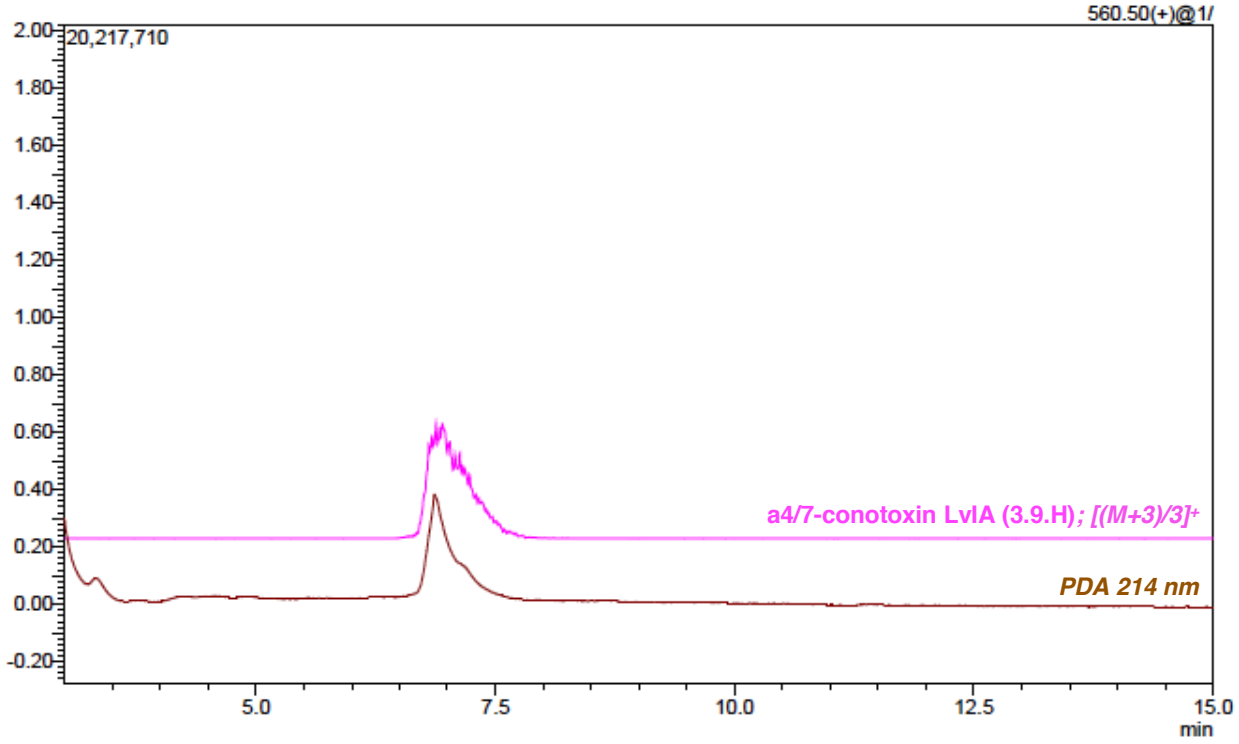


Figure 3.63 Analytical Column PDA for pure product **3.9.H**. Table 3.10. Entry 4
Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 15 min. Retention time 6.97 min

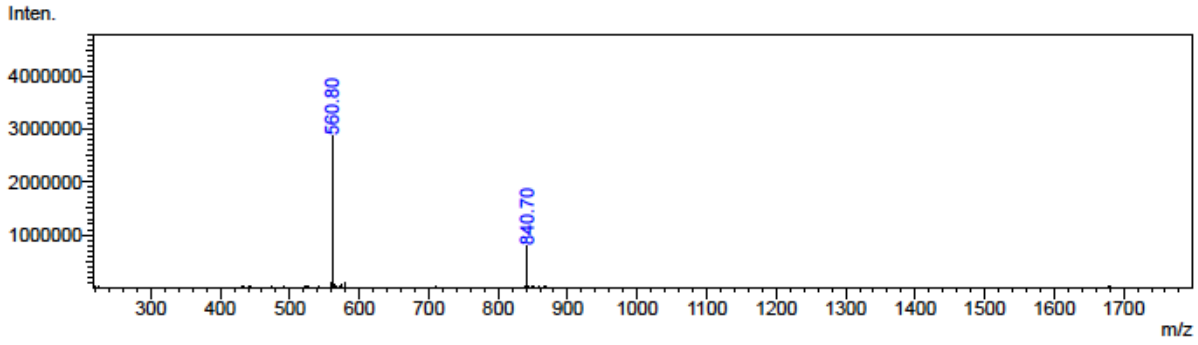


Figure 3.64 ES-MS spectrum for pure product **3.9.H** at retention time 6.97 min.

CHAPTER 4: ELLMAN'S REAGENT AS A COLORIMETRIC AND MILD OXIDIZING AGENT FOR ON-RESIN FOLDING OF CYSTEINE CONTAINING PEPTIDES

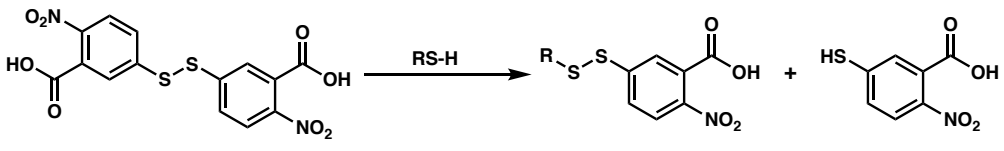
4.1 Introduction

Ellman's reagent has been the most popular reagent for spectrometric measurement of protein sulfhydryls for over four decades. It is then been adapted successfully in to a solid phase mode to mediate intramolecular disulfide bond formation in solution. However, there were no reports for its potential as a solution phase oxidizing agent to form on-resin disulfide bonds in peptides. In this chapter we are introducing a new application of Ellman's reagent, which can mediate on-resin disulfide bond formation while offering colorimetric method to monitor the reaction.

4.2 Ellman's Reagent for Free Thiol Analysis

The compound 5,5'-dithiobis-(2-nitrobenzoic acid) which is known as Ellman's reagent or DTNB was introduced in 1959 by George L. Ellman to quantify the amount and concentration of thiols groups in a sample under physiological conditions.[158,159] Since then it has been widely used in both qualitative and quantitative assays to measure protein sulfhydryls. Reaction between a thiol group and 5,5'-dithiobis-(2-nitrobenzoic acid) cleaves off the homo-disulfide bond in DTNB to give a mixed disulfide (R-S-TNB) and release one equivalent of 5-thio-2-nitrobenzoic acid (TNB) (**Scheme 4.1**).

Scheme 4.1 Reaction of Ellman's reagent with thiol groups



DTNB R-S-TNB TNB

The resulting TNB ionizes to the TNB^{2-} dianion at neutral to alkaline pH and generate a yellow color as a result of intense light absorption at 412 nm. Base on this phenomena, the standard procedure for colorimetric analysis of thiols is done using a dilute solution of Ellman's reagent in basic aqueous buffer (pH 8). The measurement of absorbance values at 412 nm allows to quantify the concentration of thiols using Beer-lambert law. The method has been used to analyze sulfhydryl group content in wide range of substrates such as cereal proteins,[160] blood samples, urine, tissues, purified proteins, etc[159]. and slight modifications in the standard procedure has been done based on stability, solubility and pH of the substrate.

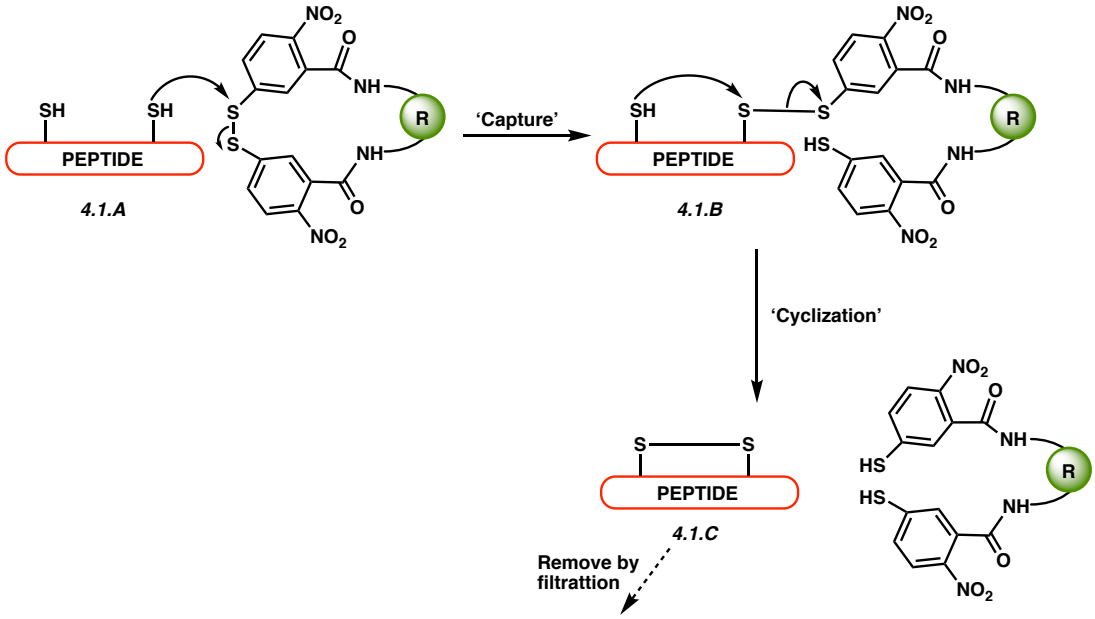
With rapid development in the solid-phase peptide synthesis, simple laboratory methods for analysis of on-resin functionality needed to be developed. Most importantly a readily accessible method for on-resin thiol group analysis. Steel and coworkers have reported their investigation of adopting Ellman's reagent to analyze resin bound thiol groups.[161] According to their findings, for highly-crosslinked polystyrene resins which are compatible with range of aqueous and non-aqueous solvent systems, standard thiol analysis conditions with aqueous solution of the Ellman's reagent failed to give reproducible results. For peptides on polystyrene based resins, Ellman's reagent in methanol, basified with DIPEA, gave excellent results. TentaGel resins, which can tolerate aqueous-organic solvent systems, react efficiently with Ellman's reagent in a THF:methanol (1:1) solvent mixture, basified with DIPEA. This is a simple and cheap method to analyze free macroporous or PEG graft resin bound thiols. Further enhancement to the procedure can be done by retreating the resin with dithiothreitol to

cleave the disulfide bond which releases a second equivalent of TNB. Colorimetric analysis of the second TNB allows to get a duplicate value for the same sample.[161]

4.3 Supported Ellman's Reagent for Cysteine Disulfide Formation

As discussed in Section 4.2, Ellman's reagent was originally developed for a colorimetric method to measure free thiol concentration under physiological conditions. It is then found to be an effective mild oxidizing agent to generate disulfides, when bound to a suitable solid support.[85] Solid supports such as polyethylene glycol-polystyrene (PEG-PS), modified Sephadex supports, controlled-pore glass (CPG) and Cross-Linked Ethoxylate Acrylate Resin (CLEAR) can be used to attach Ellman's reagent through two sites and promote cysteine disulfide bond formation in solution.[86] In the oxidation process (**Scheme 4.2**), first, one of the peptidyl-thiol groups **4.1.A** react with the solid-supported oxidant to generate solid-bound activated intermediate **4.1.B**.

Scheme 4.2 Proposed mechanism for resin-bound Ellman's reagent mediated intramolecular disulfide formation

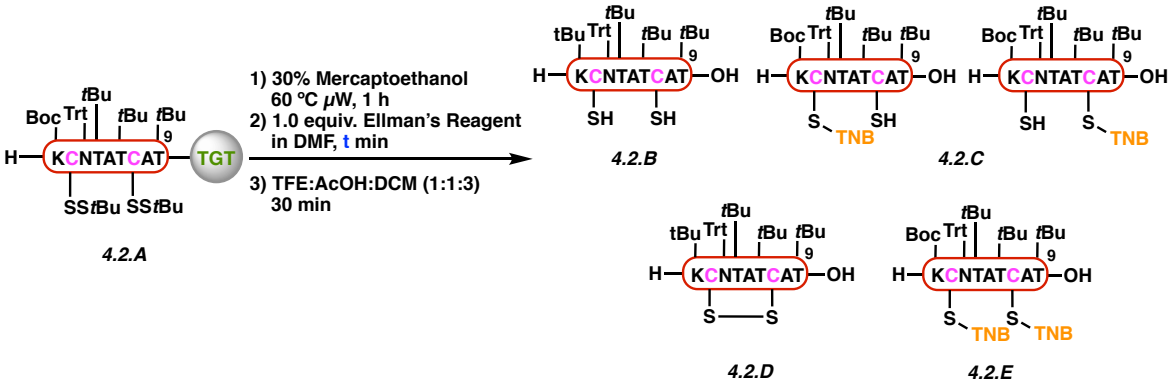


Then, the intermediate **4.1.B** undergoes intramolecular nucleophilic attack by the other peptidyl-thiol group to form the desired disulfide linkage, detached from the solid support and release the oxidized product **4.1.C** back in to the solution. Simple filtration allows the isolation of desired disulfide products and the solid support reagent can be recycled and reused.[162] Resin-bound Ellman's oxidation offers a quick and straightforward tool to obtain disulfide bonds in peptides. Most significantly, it can function at a wide range of pH values and give relatively higher yields and purity over other solution phase oxidation methods such as Glutathione-based oxidation and DMSO mediated oxidation.[162] Furthermore, it can be used in orthogonal synthesis schemes (see Chapter 1) and offers high selectivity.[163] The major drawback of this system is covalent binding of the substrate to the solid support, which diminish the overall yield of the product obtained in solution. However, the byproduct, the covalently bound peptide can be separated from the product easily by filtration at the end of oxidation, along with the resin-bound reduced reagent. The covalently bound peptide can be then released from the solid support by treatment with dithiothreitol to give the reduced from of the peptide.[162,164]

4.4 Potential of Ellman's Reagent as Mild Oxidizing Agent for On-Resin Cysteine Disulfide Formation

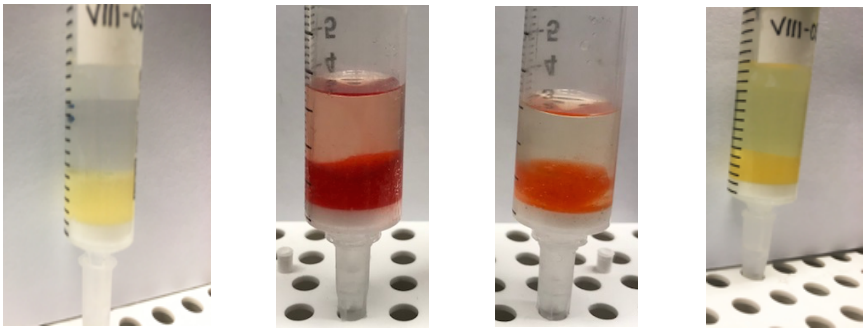
After understanding the potential of resin bound Ellman's reagent as an oxidant to facilitate cysteine disulfide formation in solution, we were curious to examine its oxidation potential to form on-resin cysteine disulfide bonds when Ellman's reagent is used as a solution. Preliminary test reactions were done using the model peptide $IAPP_{1-9}$ (**Table 4.1**).

Table 4.1 On-resin disulfide formation on model peptide $IAPP_{1-9}$ using Ellman's reagent



Entry	Rxn Time (min)	Resin color	*4.2.B*	*4.2.C*	*4.2.D*	*4.2.E*
1.	10	Dark peach	*45*	00	65	00
2.	50	Light peach	<5	00	>65	>25
3.	60	Yellow	0	00	70	30

Figure 4.1 Color change of the resin during on-resin Ellman's reagent-mediated oxidation in DMF



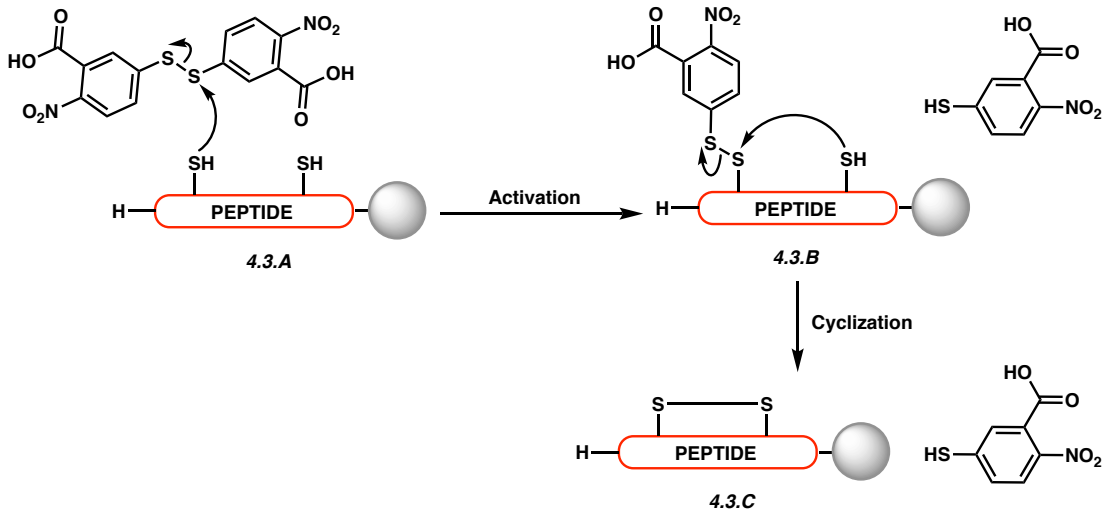
A t = 0 min | **B** t = 5-10 min | **C** t = 50 min | **D** t = 60 min

For these experiments, linear synthesis of $IAPP_{1-9}$ **4.2.A** was done on TGT resin using S*t*Bu as the cysteine protecting group. First, Deprotection of Cys(S*t*Bu) was done under microwave conditions at 60 °C with 30% mercaptoethanol for an hour to get $IAPP_{1-9}$ with reduced cysteines. Next, 1.0 equivalence of Ellman's reagent in DMF was added to the on-resin reduced peptide and allowed to agitate in an open vial at room temperature. An interesting color changing effect was observed during this reaction. At the beginning when the resin was treated with Ellman's reagent, the resin color turned to dark peach-orange

color (**B. Figure 4.1**). With time, intensity of the peach-orange color decreased (**C. Figure 4.1**) and eventually the resin color turned to yellow (**D. Figure 4.1**). For our curiosity, the peptide was cleaved from the resin at different time interval related to each color change and the peptide was analyzed by RP-HPLC-MS to evaluate the status of the reaction at each time interval (**Table 4.1**). After 10 min, when the resin color is still dark peach-orange, about 45% starting peptide **4.2.A** was observed with 65% oxidized product **4.2.D** (entry 1). At 50 min, when the initial resin color get faded to light peach color, hardly any starting peptide **4.2.A** was observed and formation of a *S*-Sthionitrobenzoic double adduct on peptide **4.2.E** was observed along with oxidized peptide **4.2.D** (entry 2). When the resin color changed to yellow, at 60 min reaction time, fully oxidized product **4.2.D** was observed as the major product with 30% of the peptide adduct **4.2.E** (entry 3). with *S*-Sthionitrobenzoic double adduct. This is an interesting observation which demonstrate the colorimetric behavior of Ellman's reagent to indicate on-resin cysteine disulfide bond formation while acting as the mild oxidant for the reaction.

The mechanism of Ellman's reagent-mediated on-resin disulfide formation can be related to that of solid-bound Ellman's reagent-mediated solution phase oxidation described in Section 4.3 (**Scheme 4.3**). In this method the peptide containing reduced cysteines is attached to a resin instead of the oxidant been attached to a solid support. When the resin-bound peptide **4.3.A** is treated with a solution of Ellman's reagent in DMF, one of the peptidyl-thiol groups react with Ellman's reagent and break the homo S-S bond to generate solid-bound activated intermediate **4.3.B**.

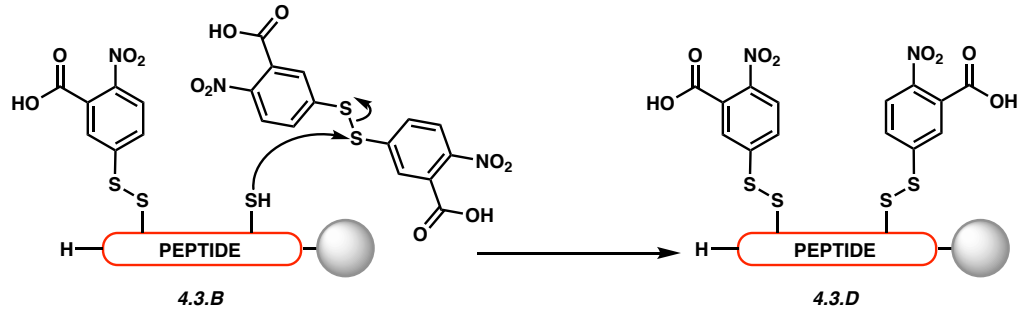
Scheme 4.3 Suggested mechanism for on-resin intramolecular disulfide formation via Ellman's reagent



This result release of one equivalent of 5-thio-2-nitrobenzoic acid, which gives a yellow color to the reaction system. Formation of the intermediate **4.3.B** may be indicated by the intense peach-orange color during the reaction, which diminishes the yellow coloration of 5-thio-2-nitrobenzoic acid in the reaction solution. As the peptide is attached to a resin, due the pseudo-dilution effect, an intramolecular disulfide bond formation is favored by the nucleophilic attack of other peptidyl-thiol group. The intramolecular reaction breaks the hetero-disulfide bond in the on-resin activated intermediate **4.3.B**, which may results the disappearance of initial intense peach-orange color. The intramolecular reaction facilitates release of another 5-thio-2-nitrobenzoic acid. Hence, when the reaction goes to completion, the yellow color of 5-thio-2-nitrobenzoic acid becomes the dominant color in the reaction. Beside an intramolecular reaction, the second free thiol group of solid-bound activated intermediate can react with another molecule of Ellman's reagent to form *S*-Sthionitrobenzoic double adduct on peptide (**Scheme 4.4**). This competitive intermolecular reaction forms a byproduct **4.3.D** which can diminish the yield of the final folded product. Therefore, this Ellman's reagent-

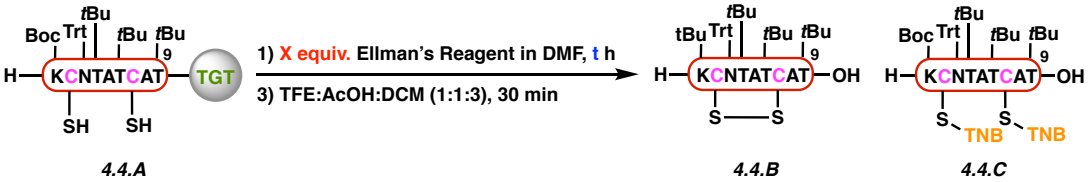
mediated on-resin disulfide formation reaction needed to be optimized for minimum intermolecular byproduct formation.

Scheme 4.4 Proposed mechanism for formation of *S*-Sthionitrobenzoic double adduct on peptide



In order to minimize the byproduct formation, a test experiment was performed using reduced $IAPP_{1-9}$ **4.4.A** with different loadings of Ellman's reagent in DMF (**Table 4.2**).

Table 4.2 Optimization for Ellman's reagent loading to minimize byproduct formation

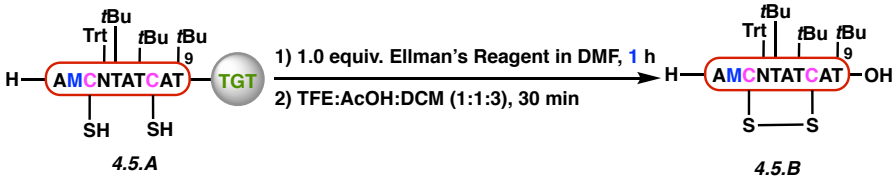


Entry	X equiv. of Ellman's R.	Time for color change (h)	*4.4.B*	: *4.4.C*
1.	1.00	1.0	60	40
2.	0.50	2.5	90	10
3.	0.25	3.0	100	00

Each test reaction was run until the color of the resin changed from dark peach-orange to yellow. A relationship was observed between the loading of Ellman's reagent and the amount of byproduct **4.4.C** formed. As the loading of Ellman's reagent is lowered a decrement in the byproduct **4.4.C** formation was observed along with an increment in the reaction time (entry 1 – entry 3). For $IAPP_{1-9}$ **4.4.A**, 0.25 equivalent of Ellman's reagent gave the oxidized product with zero byproduct in 3 hours (entry 3).

Sensitive amino acid side chains of Met, Typ and Tyr can undergo various modifications in presence of an oxidant.[164,165,166] Therefore a test reaction to determine the stability of methionine towards Ellman's reagent oxidation was evaluated. For the test reaction, methionine incorporated model peptide $IAPP_{1-9}$ **4.5.A** was used as the substrate (**Scheme 4.5**). The resin-bound peptide **4.5.A** was subjected to Ellman's reagent in DMF and the reaction was run until the color of the resin changes from dark peach-orange to yellow. The reaction was analyzed by analytical RP-HPLC and neither sulfoxide nor sulfone byproducts was observed. The desired oxidized product **4.5.B** was observed with clean analytical trace.

Scheme 4.5 Determination of Met stability toward Ellman's reagent oxidation condition



The stability of Typ and Tyr amino acids towards Ellman's oxidation were also evaluated and the results will be discussed later in this chapter.

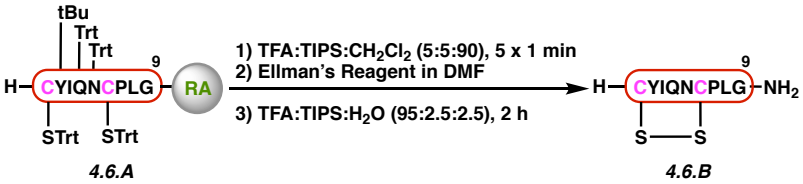
4.5 On-Resin Disulfide Bond Formation in Peptides using Ellman's Reagent in DMF

4.5.1 On-Resin Synthesis of Oxytocin

After evaluating the potential of Ellman's reagent to oxidize resin-bound free thiols in a model peptide, we wanted to apply this method to a real system. One of the common targets, oxytocin, was chosen and the peptide folding was achieved using Ellman's reagent in DMF on rink amide resin (**Scheme 4.6**). The linear synthesis of the peptide **4.6.A** was done on rink amide resin via Fmoc-SPPS and it was subjected to 5% TFA in DCM in presence of TIPS to deprotect Cys(Trt) group. The resulting reduced peptide was

then treated with 0.25 equivalent of Ellman's reagent to access the disulfide bond in oxytocin **4.6.B**. The color change of the resin was used to monitor the reaction.

Scheme 4.6 On-resin synthesis of oxytocin

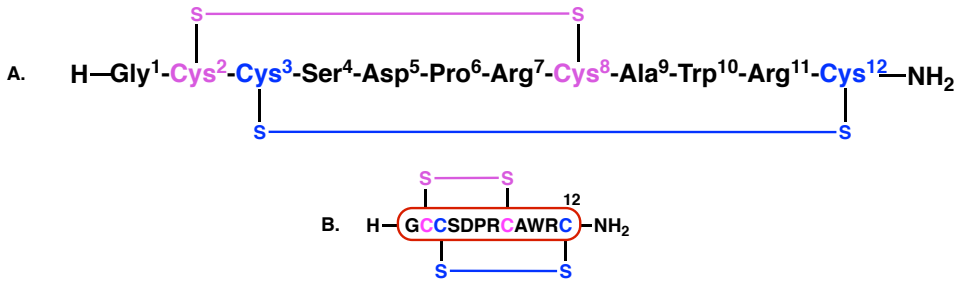


No byproduct of *S*-Sthionitrobenzoic double adduct on peptide was observed during the reaction. Moreover, the Tyr group has not been affected by Ellman's reagent-mediated oxidation condition. The reaction was clean according to the analytical RP-HPLC-MS data.

4.5.2 On-Resin Synthesis of α-Conotoxin ImI

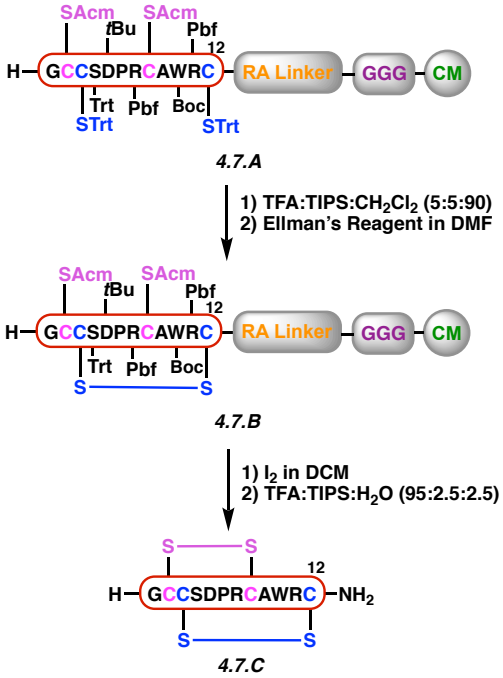
The success in the synthesis of oxytocin, which contains single disulfide linkage, led us to evaluate the Ellman's reagent – mediated oxidation method for more advanced systems. One of such systems we selected was the C-terminal carboxamide α-conotoxin ImI, a sub-type selective nicotinic acetylcholine receptor antagonist[167,168,169] isolated from the venom of *Conus imperialis* marine snails.[170,171,172] It has two disulfide linkages lie between Cys[2]-Cys[8] and Cys[3]-Cys[12] (**Figure 4.3**)[173].

Figure 4.2 A. Structure of α-conotoxin ImI **B.** Graphical represtation of α-conotoxin ImI structure



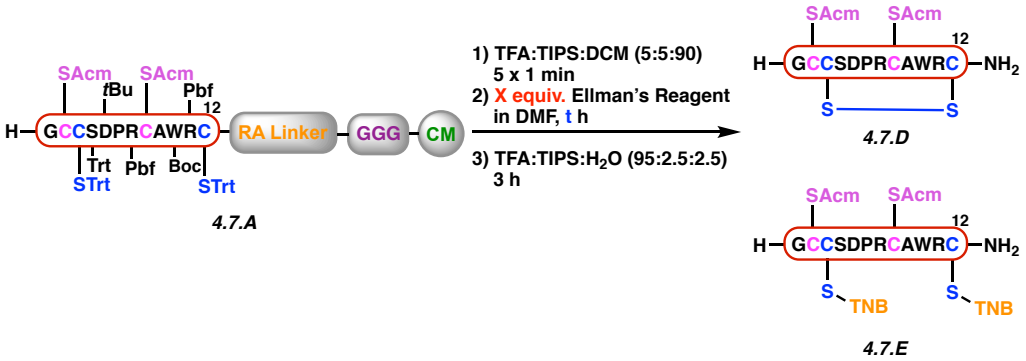
The on-resin synthetic pathway for α-conotoxin ImI was designed in two steps using Cys(Acm) and Cys(Trt) groups (**Scheme 4.7**). The linear synthesis of the starting peptide **4.7.A** was done on rink amide linker-Gly-Gly-Gly-ChemMatric resin. The synthesis protocol for α-conotoxin ImI was designed such that the larger loop between Cys^{3}-Cys^{12} is made first followed by the small loop between Cys^{2}-Cys^{8}. To achieve the larger loop, first, Cys(Trt) deprotection can be done with 5% TFA in DCM in presence of TIPS as the scavenger. The resulting free thiol can then be oxidized using Ellman's reagent in DMF.

Scheme 4.7 Synthetic design for on-resin synthesis of α-Conotoxin ImI



Before start the synthesis, the loading of Ellman's reagent was optimized to get zero byproduct during reaction (**Table 4.3**).

Table 4.3 Optimization of the loading of Ellman's reagent for minimum byproduct formation



Entry	X equiv. of Ellman's R.	Time for color change (h)	*4.7.D*	: *4.7.E*
1.	1.000	3	70	30
2.	0.250	12	90	10
3.	0.125	24	100	00

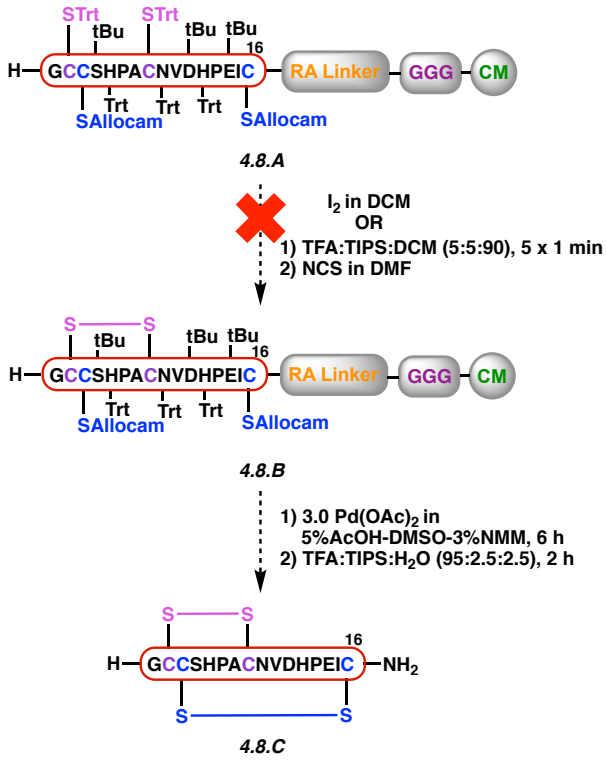
The loadings of Ellman's reagent was varied from 1.0 equivalent to 0.125 equivalent and each of the reactions were run until the color of the resin changes from dark peach-orange to yellow. With 1.0 equivalent of Ellman's reagent, 70% conversion of the desired product **4.7.D** was observed in 3 hours with 30% conversion of the byproduct **4.7.E** (entry 1). About 90% conversion of the desired oxidized product was observed in 12 hours when the loading of Ellman's reagent was 0.25 equivalent (entry 2). For the larger loop of α-conotoxin ImI, zero byproduct formation can be achieved with 0.125 equivalent of Ellman's reagent in DMF in 24 hours (entry 3). Hence, the first step of the synthesis was scaled up using the optimized condition for Ellman's reagent in DMF. Then, the resulting mono cyclized peptide **4.7.B** was treated with 8 equivalent of iodine in DCM for 2 hours to remove Acm group and oxidize the free thiol groups, which result the fully folded peptide **4.7.C** on resin. The α-conotoxin ImI has Trp amino acid residue and no side chain modification was observed during the Ellman's reagent-mediated oxidation process. Only

the fully folded product with desired disulfide bond pattern was observed in 59% overall yield.

4.5.3 On-Resin Synthesis of α4/7-conotoxin LvIA

One of the suggested synthetic routes to access α4/7-conotoxin LvIA (see Chapter 03) was use Cys(Trt) to form small loop first, either by iodine oxidation or NCS oxidation, followed by Cys(Allocam) deprotection and oxidation with Pd-DMSO based cleavage-oxidation condition to fold the larger loop (**Scheme 4.8**). Sensitivity of the Allocam group towards both iodine oxidation and NCS oxidation failed this synthetic path (**4.8.A – 4.8.B**) and we had to select another method to access first disulfide bond formation, which described in chapter 03.

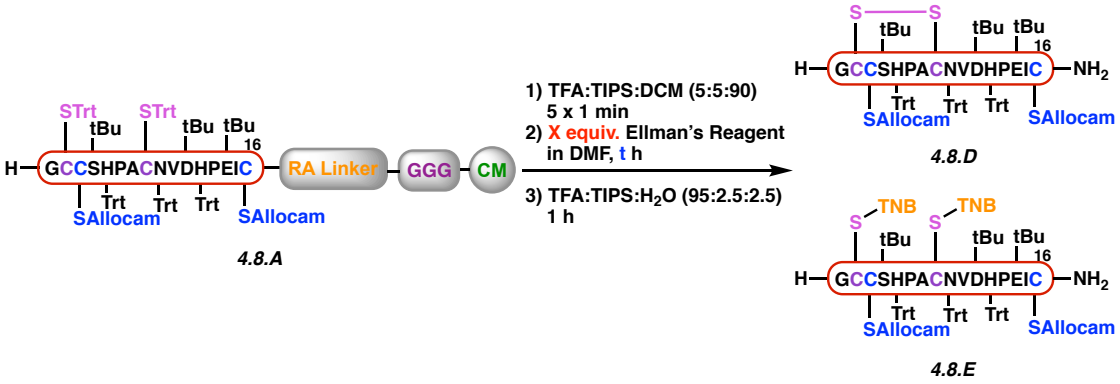
Scheme 4.8 Suggested on-resin synthesis of α4/7-Conotoxin LvIA with Cys(Trt) and Cys(Allocam)



After the discovery of Ellman's reagent as a mild oxidant for on-resin cysteine disulfide bond formation, we wanted to revisit the synthesis of α4/7-conotoxin LvIA using Ellman's reagent to form the first S-S bond.

After the linear synthesis of the peptide **4.8.A**, it was subjected to screening the best loading condition for Ellman's reagent to get 100% conversion of the desired mono cyclic product **4.8.B**. with zero byproduct formation (**Table 4.4**).

Table 4.4 Optimization of the loading of Ellman's reagent for minimum byproduct formation

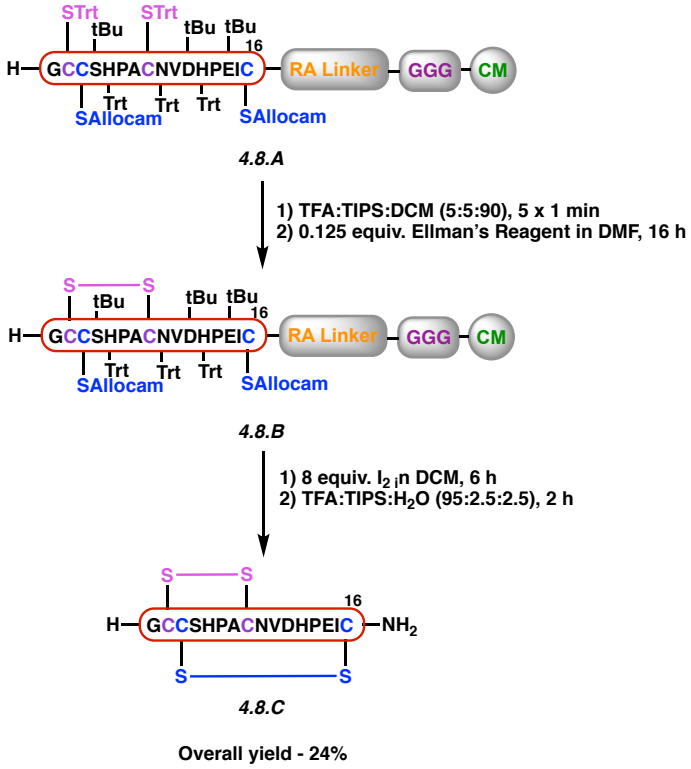


Entry	X equiv. of Ellman's R.	Time for color change (h)	*4.8.D*	:	*4.8.E*
1.	1.000	2	95		05
2.	0.250	10	97		03
3.	0.125	16	100		00

From the optimization data, it was found that 0.125 equivalent of Ellman's reagent is the best condition for this target peptide to form its first disulfide linkage without any byproduct made during the oxidation step (entry 3). With the optimized data in hand, the reaction was scaled up. Next, the resulting mono cyclic peptide **4.8.B** was subjected to I_2 oxidation for simultaneous Allocam removal and oxidize to get the bicyclic peptide **4.8.C** with desired disulfide pattern (**Scheme 4.9**). From chapter 03, it was identified that iodine oxidation gives better conversion with good yield compared to Pd-DMSO based Allocam

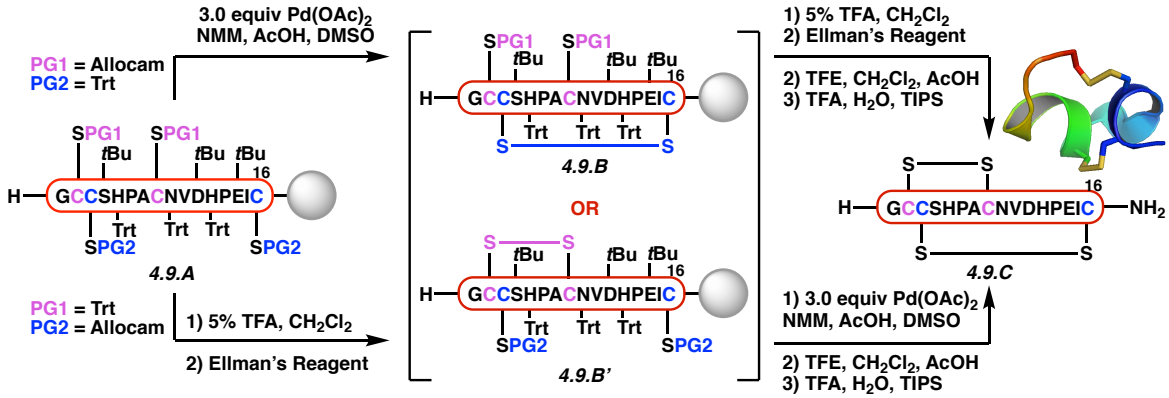
removal and oxidation condition. Therefore, in this synthesis protocol, iodine oxidation was chosen over the Pd-DMSO based Allocam removal and oxidation condition to make the second disulfide bond. From this method, we were able to synthesize α4/7-conotoxin LvIA in 24% overall yield.

Scheme 4.9 On-resin synthesis of α4/7-Conotoxin LvIA with Cys(Trt) and Cys(Allocam)



Interestingly, Ellman's reagent-mediated oxidation of on-resin free thiols give access to combine Allocam with Trt as the cysteine protecting group to generate disulfides in peptides in either order, which was not possible before. This permits the formation of disulfides on-resin in either order from a single linear sequence/substrate **4.9.A** (**Scheme 4.10**). This strategic flexibility can be applied to any peptide containing two disulfide linkages.

Scheme 4.10 Strategic flexibility in the controlled folding of α4/7-Conotoxin LVIA.



4.6 On-Resin Folding of Peptides using Ellman's Reagent as the Only Oxidizing Agent

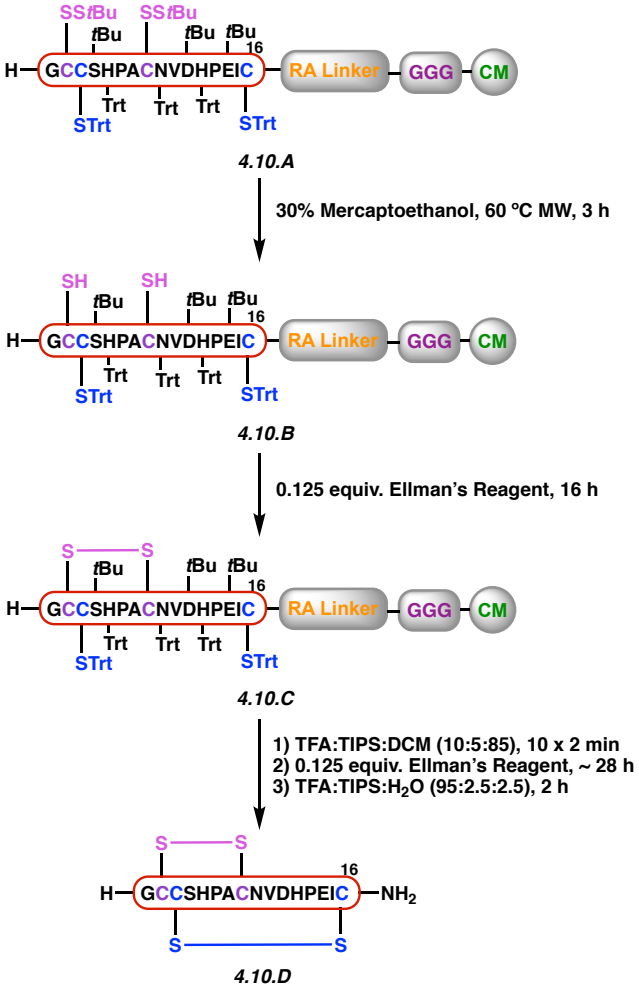
After investigating the potential of Ellman's reagent as a colorimetric and mild oxidizing agent, we were curious to take advantage of its potential to form all the disulfide bonds in a peptide selectively using Ellman's reagent as the only oxidizing agent. As we have seen in previous synthetic procedures, Ellman's reagent react on free thiols and oxidize them to S-S bonds. Therefore, in order to perform oxidation with Ellman's reagent, the cysteine pairs in the target peptide needed to be protected with protecting groups which have deprotecting condition to get free thiol groups. Examples for such protecting groups are S*t*Bu, which can be cleaved with mercaptoethanol to get free thiol, Trt and Mmt, which result the reduced cysteine with mild TFA conditions. To evaluate the method, α4/7-conotoxin LvIA was chosen as the target.

4.6.1 Synthesis of α4/7-Conotoxin LvIA using Ellman's Reagent in DMF

For the synthesis of α4/7-conotoxin LvIA using Ellman's reagent as the only oxidant, S*t*Bu and Trt were chosen to protect each pair of cysteines in the peptide. The linear synthesis of the peptide **4.10.A** was first done on rink amide linker-Gly-Gly-Gly-

Chemmatrix resin using Cys(S*t*Bu) and Cys(Trt). Selective folding methods were then proceeded on the linear peptide to get the desired folding pattern (**Scheme 4.11**). The linear peptide was first treated with 30% mercaptoethanol for 3 hours under microwave heating at 60 °C to remove S*t*Bu groups.

Scheme 4.11 On-resin formation of disulfide bonds in α4/7-Conotoxin LvIA using Ellman's Reagent in DMF



The resulting free thiols in the peptide **4.10.B** was then oxidized using previously optimized condition (see Table 4.4) for Ellman's reagent – mediated oxidation. The reaction was run until the color of the resin changes from dark peach-orange to yellow. This step formed the small loop selectively. Next, Cys(Trt) deprotection of the mono cyclic

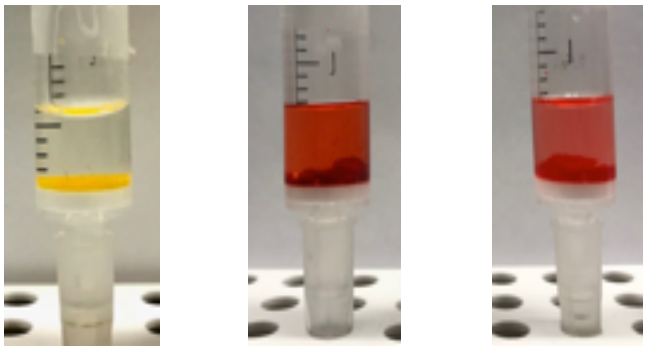
peptide **4.10.C** was done using 5% TFA in DCM in presence of TIPS for 5 x 1 min. The resulting free thiol was then oxidized again with Ellman's reagent in DMF and the reaction was monitored by the color change of the resin. It took about 28 hours to form the larger loop of the peptide in DMF. The 5% TFA treatment was done several times followed by addition of Ellman's reagent during the reaction in order to ensure completion of the reaction (see experimental procedures for more details). When no color change was observed after addition of Ellman's reagent, the reaction was terminated by filtering off the solution of Ellman's reagent followed by several DMF wash. The total deprotection and resin cleavage was done to collect the resulting peptide and analytical RP-HPLC was performed to confirm the product **4.10.D** formation. Surprisingly in the analytical HPLC trace the fully folded product **4.10.D** was observed in very minor amount whereas the mono-cyclic peptide **4.10.C** was observed as the major product. The last step of the synthesis was revisited with longer TFA treatment with 10% TFA in DCM for 10 x 2 min, followed by Ellman's reagent – mediated oxidation. The resulting peptide was cleaved after color change of the resin and analyzed again via analytical RP-HPLC. This slight modification in the last step of the synthesis facilitated formation of the desired fully folded product **4.10.D.** This experiment illustrates that removal of Trt group from the mono cyclic system is difficult and longer exposer time or higher TFA concentration is needed to improve the synthesis.

4.6.2 Synthesis of α4/7-Conotoxin LvIA using Ellman's Reagent in NMP

When considering all the synthesis protocols we performed using Ellman's reagent in DMF to form disulfide bonds, the only drawback is longer reaction time. In addition to

DMF as the solvent, the reaction was done with 1.0 equivalent of Ellman's reagent in methanol, DMSO and NMP to evaluate their colorimetric behavior. The test experiments were done using reduced $IAPP_{1-9}$ as the model peptide. The color of the resin changed to intense yellow color with the addition of Ellman's reagent in methanol (**A. Figure 4.3**) whereas with DMSO (**B. Figure 4.3**) and NMP (**C. Figure 4.3**), the color of the resin changed to dark peach-orange color similar to that in DMF. The reactions were then eft for few hours to observe any other changes. Even after two hours, no color change was observed in the reactions with methanol and DMSO. Interestingly, in the reaction with NMP, the color of the resin changed from dark peach-orange to yellow in 20 min instead of an hour with DMF. Analytical RP-HPLC-MS analysis were performed for all of the peptides and it confirmed disulfide bond formation during the reaction. In the reaction with NMP, no *S*-Sthionitrobenzoic double adduct on peptide was observed, which was significant in the reaction with DMF.

Figure 4.3 Color of the resin with Ellman's reagent in different solvents



A
Ellman's in MeOH

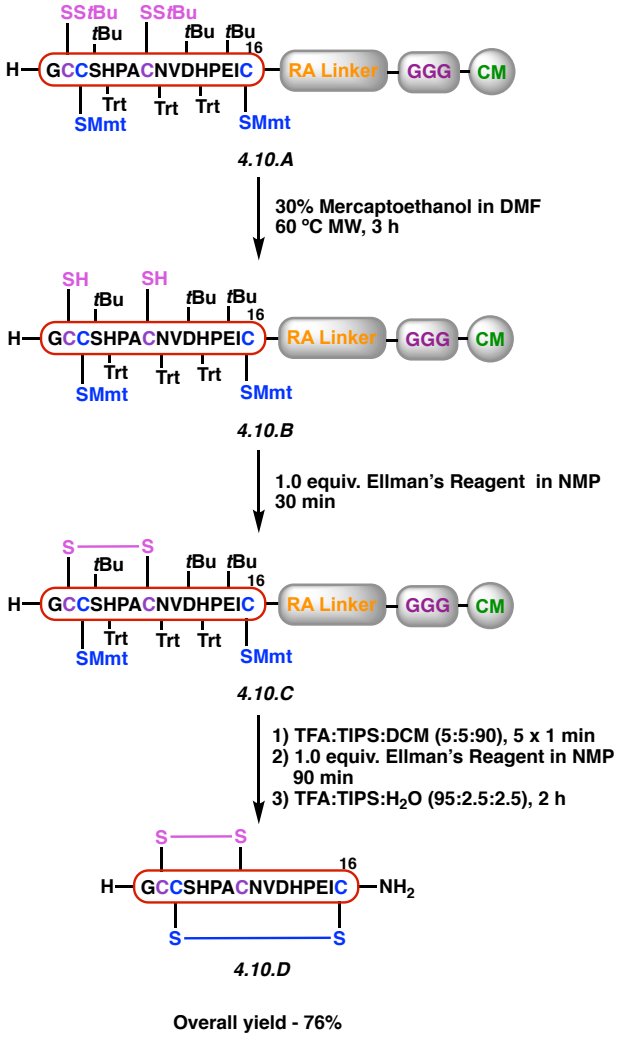
B
Ellman's in DMSO

C
Ellman's in NMP

After investigation of faster reaction rates in NMP solvent, the method described in section 4.6.2 was revisited with Cys(S*t*Bu) and Cys(Mmt) to synthesize α4/7-conotoxin LvIA using

Ellman's reagent in NMP (**Scheme 4.12**). First, the linear peptide **4.10.A** was subjected to 30% mercaptoethanol for 3 hours under microwave heating at 60 ºC to deprotect Cys(S*t*Bu). Then, the resulting reduced peptide **4.10.B** was treated with Ellman's reagent in NMP.

Scheme 4.12 On-resin formation of disulfide bonds in α4/7-Conotoxin LvIA using Ellman's Reagent in NMP



Overall yield - 76%

Completion of the first disulfide bond formation was monitored by color change of the resin and it required only 30 min. The resulting mono cyclic peptide **4.10.C** was treated with 5% TFA in DCM in presence of TIPS as a scavenger for 5 x 1 min to ensure Mmt group removal in the system. Another solution of Ellman's reagent in NMP was added to

the resin with free thiols at Cys^3 and Cys^{16} to form the second disulfide bond. Color changed from dark orange to yellow in 90 min for this step. Finally, Ellman's reagent was filtered off and the resin was washed thoroughly with NMP. Analytical RP-HPLC-MS was performed to the cleaved peptide and ES-MS confirmed the fully folded peptide **4.2.D**. After purification, α4/7-conotoxin LvIA was isolated in 76% overall yield. Disulfide bond formation using Ellman's reagent in NMP shows greater improvement in reaction rates compared to that of in DMF.

4.7 Summary

We demonstrated the potential of Ellman's reagent as a colorimetric and mild oxidizing agent to form on-resin disulfide bonds in peptides. The reaction procedure is simple, no complex time consuming reaction set up is needed. Most importantly, the reaction can be monitored easily by observing the color change of the resin, which is advantageous over time consuming lengthy reaction analysis protocols. The colorimetric behavior of Ellman's reagent can be seen both in DMF and NMP. Ellman's reagent – mediated disulfide bond formation in DMF is relatively slower and the yield-diminishing intermolecular side reaction involving formation of *S*-Sthionitrobenzoic double adduct on peptide can be addressed by optimizing the loading of Ellman's reagent for each substrate. Therefore, DMF is best in formation of smaller disulfide linkages in peptides. In contrast, Ellman's reagent – mediated disulfide bond formation in NMP has faster reaction rates and no *S*-Sthionitrobenzoic double adduct on peptide was observed even with 1.0 equivalent of Ellman's reagent. The insight of colorimetric behavior of Ellman's reagent in disulfide bond formation process needed to be further investigated. However,

on-resin disulfide bond formation using Ellman's reagent introduces a simple and an efficient synthetic route to access disulfide rich bioactive peptides.

4.8 Methods and Material

4.8.1 General Information

Unless otherwise specified, all commercially available reagents were purchased from Sigma-Aldrich and used without further purification. Anhydrous Et_2O, MeCN, DMF, DMSO, CH_2Cl_2 were purchased from Fisher. These were passed through a commercial solvent purification system (2 columns of alumina) and used without further drying. Hünig's base was distilled over CaH_2 immediately prior to use. All amino acids were purchased from Chem-Impex Int'l. Inc. unless otherwise noted. HATU were purchased from Chem-Impex Int'l. Inc. Unless otherwise noted, all reactions were performed in 2 mL, 5 mL and 10 mL Biotage reactor vials with PTFE frit (depend on reaction volume) at room temperature. All yields refer to chromatographically and spectroscopically pure products. All HPLC analyses and purifications were performed on a Custom Reverse Phase Shimadzu Liquid Chromatograph Mass Spectrometer (LCMS-2020), which can toggle between analytical and semi-preparative columns. This instrument has a photodiode array (PDA) detector (D2 & W lamp), which collects a range of wavelengths, in place of a traditional single channel UV detector. RP-HPLC-MS mobile phases (MeCN and H_2O) contained 0.1% Formic Acid. Analytical HPLC was performed on a Phenomenex Kinetex C18 column (5 μm, 250 x 4.6 mm) and a Thermo Scientific Hypersil Gold C8 column (5 μm, 250 x 4.6 mm). Semi-Preparative HPLC was conducted using a Thermo Scientific

Hypersil Gold C8 column (5 μm, 150 x 10 mm). All peptide yields are calculated based on the final loading.

SPPS General Information. Solid-phase peptide synthesis was executed on a Biotage Isolera+ semi-automated synthesizer with microwave heating.

- *Reactor Vials [Vial size (Volume range allowed)]:* 2 mL reactor vial (0.8-1.1 mL), 5 mL reactor vial (1.6-3.2 mL), and 10 mL reactor vial (3.2-6.4 mL)
- *Swelling + Heat:* DMF was added and vortexed at 1200 RPM for 20 m at 70 ºC. The solvent was then removed over 1 m followed by two DMF washes (DMF was added and the suspension was vortexed at 600 RPM for 45 s, followed by the removal of solvent (over 2 m)).
- *Coupling:* A solution of Fmoc-aa-OH (3 equiv), HATU (3 equiv), and DIPEA (6 equiv) in DMF was made immediately prior to addition to the reaction vial containing the resin. Once the solution was added, the suspension was heated to 75 ºC (except for Fmoc-Cys-OH which was heated to 50 ºC) for 5 minutes with a vortex rate of 1200 RPM. After the reaction, the solution was removed (over 2 m) and the resin was rinsed with DMF 4 times (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).
- *Fmoc Removal (Deprotection):* The reactor vial was filled with 20% piperidine in DMF. The suspension was vortexed at 1200 RPM for 3 m at RT. The solvent is removed followed by addition of 20% piperidine in DMF. The suspension is vortexed again at 1200 RPM for 10 m at RT. The solvent was removed over 2 m, followed by 4 DMF

washes (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).

- *Wash*: DMF was added to the reaction vial and agitated at a vortex rate of 1200 RPM for 1 m. The solvent was removed over 1 m and repeated for a total of 4 times.
- *Final Wash:* Resin was rinsed with CH_2Cl_2 (3 x 1 mL) and MeOH (3 x 1 mL).
- *Drying for Storage/Weighing:* After the final wash, the resin was placed on the lyophilizer overnight for drying.

General Procedure for Capping - First, 25% acetic anhydride in DMF (3 mL) was added and agitated for 5 min. Then,1.5 eqiuv. of DIPEA was added to the reaction and agitated for 30 min. Finally, the reaction solution was filtered and the resin was washed with DMF (5 x 3 mL).

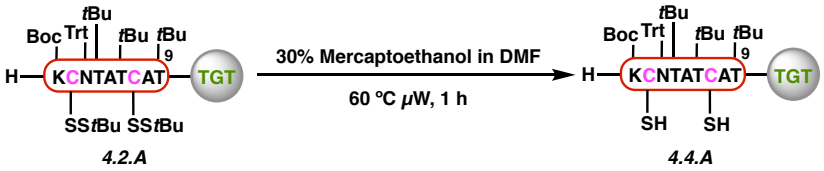
Procedure for Resin Loading Analysis. Initial and final loading of resin was analyzed based on amount of Fmoc group.

Fmoc Deprotection – 10 mg of the resin was weighed out and swelled in DMF in the peptide synthesizer. Then, 1.00 ml of 20% piperidine in DMF was added and it was allowed to vortex in the peptide synthesizer at room temperature for 3 min. After 3 min, the filtrate was collected in to a separate tube, another 1.00 ml of 20% piperidine in DMF was added to it and allowed to vortex in the peptide synthesizer at room temperature for 10 min. The resulting filtrate was collected to the same tube and the deprotected resin was washed with DMF (4 x 1.75 ml).

Sample preparation for UV analysis – From the collected ”deprotection solution” above, 100 μL was transferred in to an eppendorf tube and it was diluted with 900 μl of DMF.

The resulting diluted sample was used to get the absorbance at 301 nm versus a DMF blank using a UV spectrometer with 1 cm cuvette.

4.8.2 Experimental Procedures and Spectroscopic Data



Reduced IAPP$_{1-9}$ 4.4.A. The resin **4.2.A** (60 mg, 12 μmol) was first swelled in DMF (500 μL). Then, a solution of 30% mercaptoethanol in DMF (1.0 mL) was added and agitated at 60 °C under microwave condition for 1 hour. The reaction solution was filtered and the resin was washed with DMF (5 x 1 mL). To analyze the reaction 5 mg of the resulting resin was subjected to 300 μL of the cleavage mixture (1:1:3 TFE:AcOH:DCM) for 30 min. The resulting solution was filtered, concentrated and analyzed via MS, direct injections at a flow rate of 0.4 mL/min with 50% MeCN/H_2O for 1.5 minutes. m/z ES calc'd for peptide 4.4.A $[(C_{70}H_{107}N_{11}O_{16}S_2)+1]^+$: 1422.81; observed: 1423.55; m/z ES calc'd for $[(C_{70}H_{107}N_{11}O_{16}S_2 + 2)/2]^+$: 712.41; observed: 712.00.

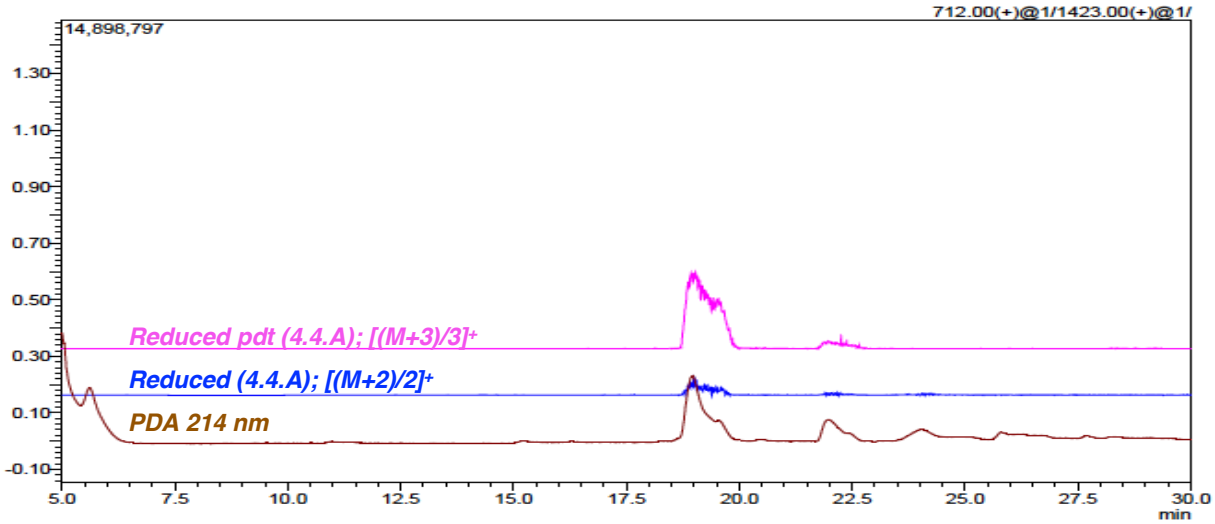
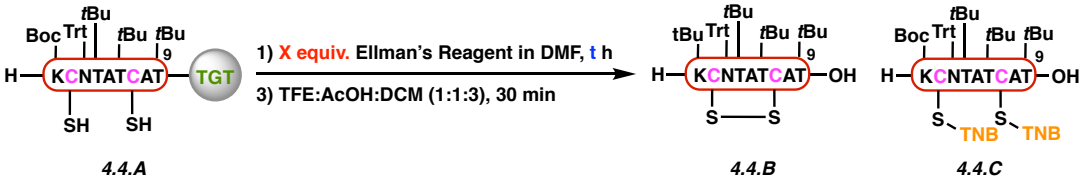


Figure 4.4 Analytical Column PDA for reduced IAPP$_{1-9}$ **4.4.A**



4.4.A *4.4.B* *4.4.C*

General Procedure for Table 4.2. For each of the test reactions, the resin **4.4.A** (10 mg, 2 μmol) was first swelled in DMF (500 μL). Then, the stated amount of Ellman's reagent in DMF (1.0 mL) was added and agitated for the stated amount of time. The reaction solution was filtered and the resin was washed with DMF (5 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture (1:1:3 TFE:AcOH:DCM) for 30 min. The resulting solution was filtered, concentrated and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 4.4.B $[C_{70}H_{107}N_{11}O_{16}S_2 + 1]^+$: 1420.79; observed: 1421.85. m/z ES calc'd for $[(C_{70}H_{107}N_{11}O_{16}S_2 + 2)/2]^+$: 711.39; observed: 711.20. m/z ES calc'd for peptide 4.4.C $[C_{84}H_{113}N_{13}O_{24}S_4 + 1]^+$: 1817.14; observed: 1817.15. m/z ES calc'd for $[(C_{84}H_{113}N_{13}O_{24}S_4 + 2)/2]^+$: 909.57; observed: 909.30.

Entry 1. A solution of Ellman's reagent (0.8 mg, 2 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 1 hour.

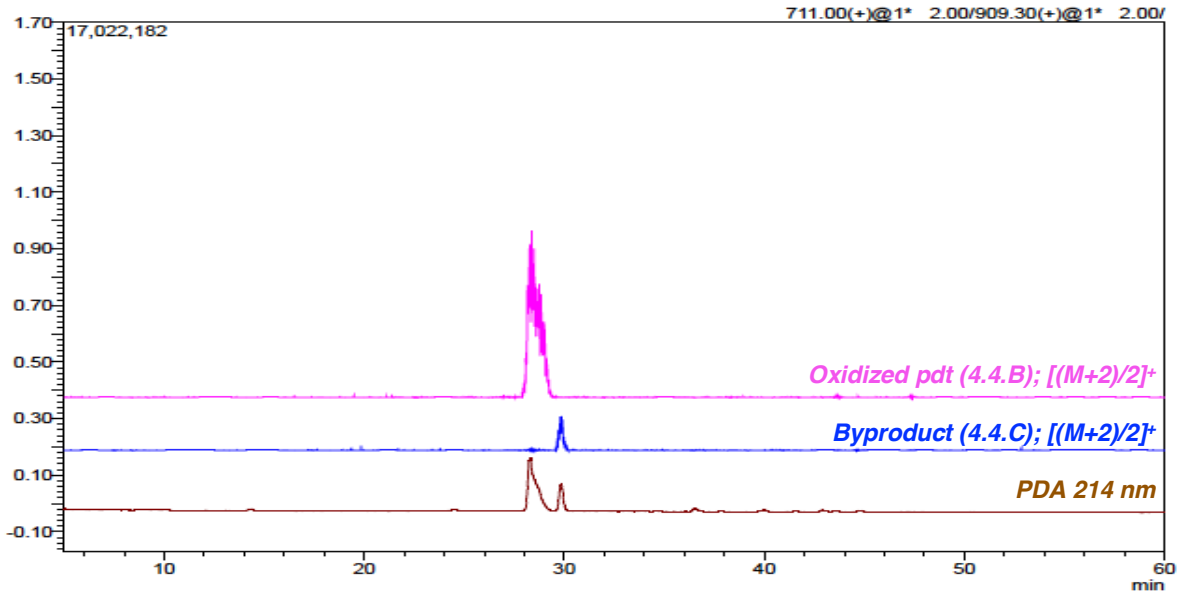


Figure 4.5 Analytical Column PDA for Table 4.2 – Entry 1

Entry 2. A solution of Ellman's reagent (0.4 mg, 1 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 2.5 hours.

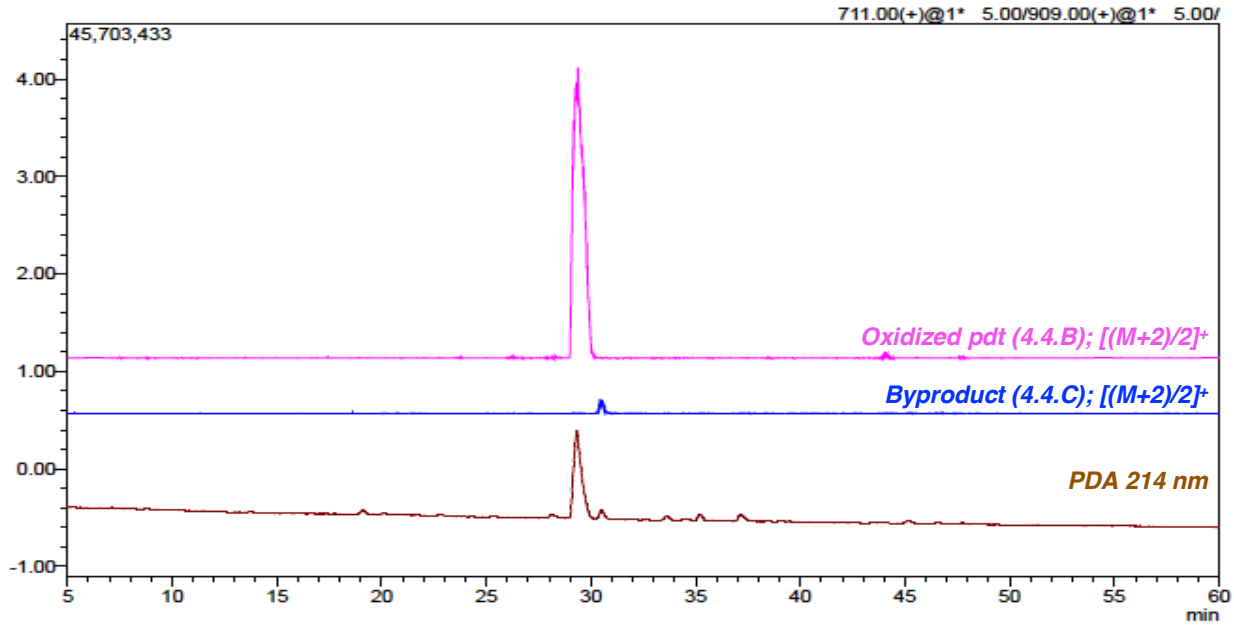


Figure 4.6 Analytical Column PDA for Table 4.2 – Entry 2

Entry 3. A solution of Ellman's reagent (0.2 mg, 0.5 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 3 hours.

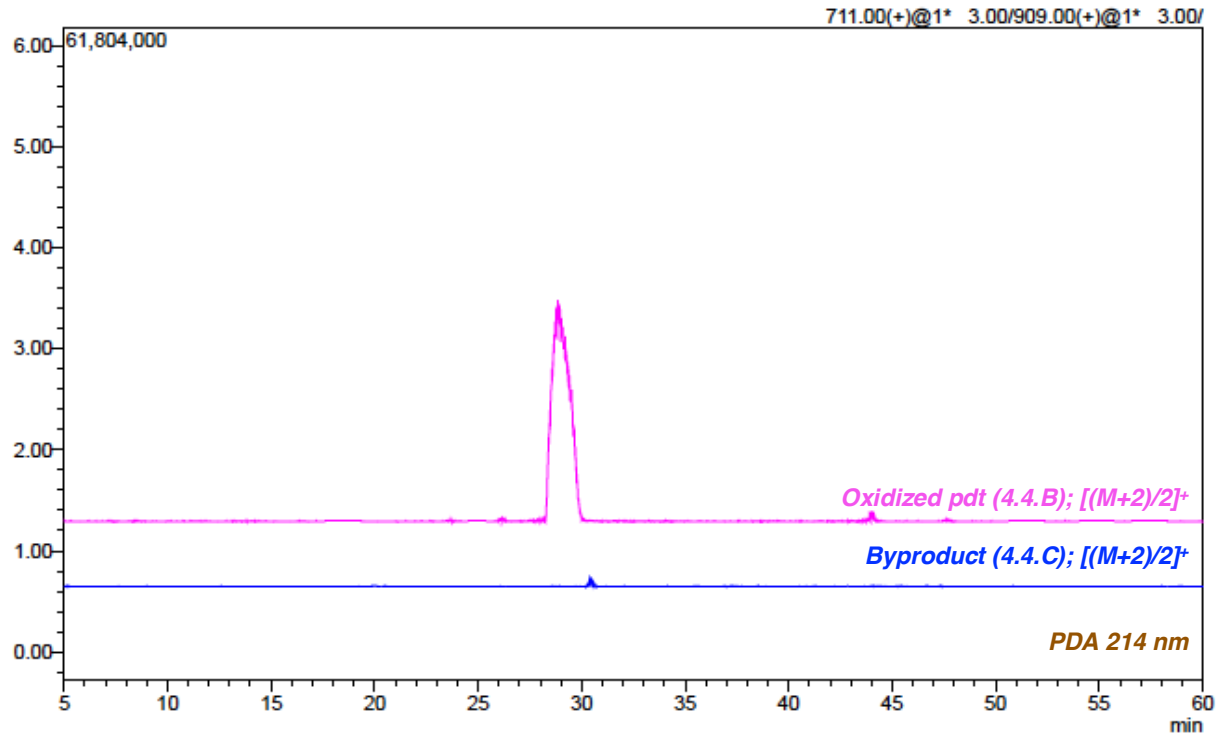
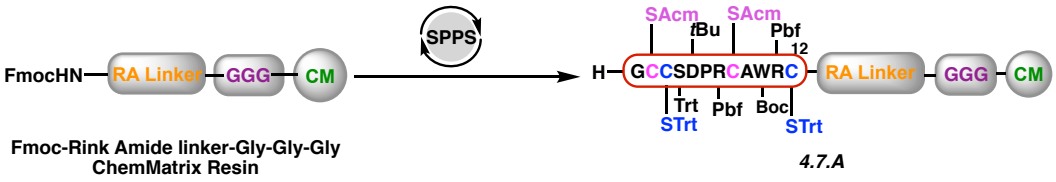


Figure 4.7. Analytical Column PDA for Table 4.2 – Entry 3



SPPS of Linear Peptide 4.7.A. The standard Fmoc-SPPS protocol described was used to synthesize peptide 4.7.A on Rink Amide linker-Gly-Gly-Gly-ChemMatrix resin. The peptide was capped before the last coupling step. To analyze the peptide, 10 mg of the resin was subjected general ammonia cleavage protocol and the resulting crude product was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via Rp-

HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. Final loading of the resin: 0.15 mmol/g. m/z ES calc'd for peptide 4.X.B [$(C_{58}H_{92}N_{22}O_{17}S_4 + 2)/2]^+$: 749.87; observed: 746.60. m/z ES calc'd for [$(C_{58}H_{92}N_{22}O_{17}S_4 + 3)/3]^+$: 500.25; observed: 500.05.

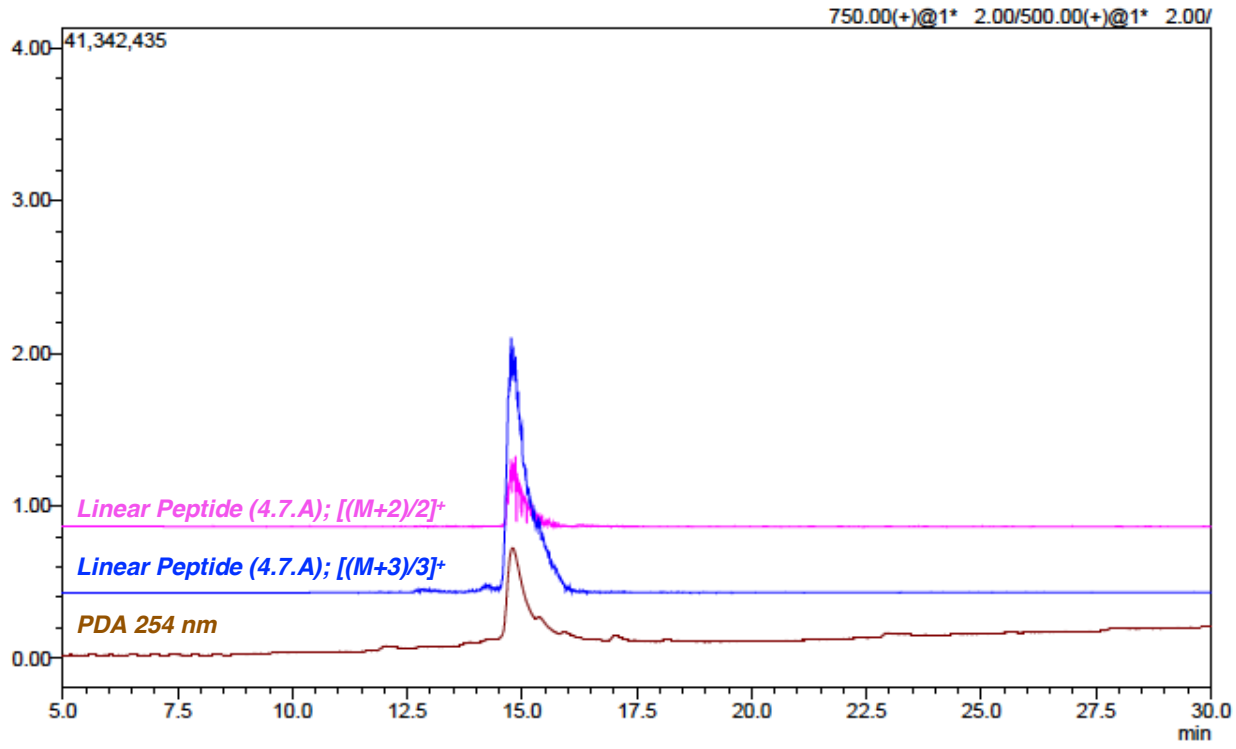


Figure 4.8 Analytical Column PDA for linear peptide **4.7.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 14.02 min

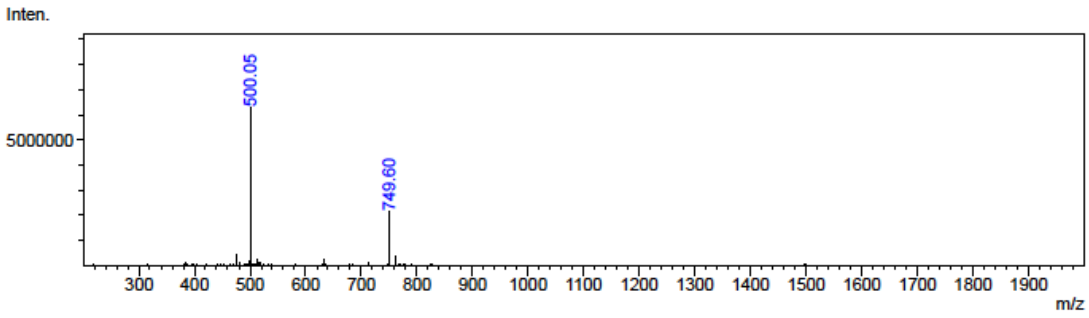
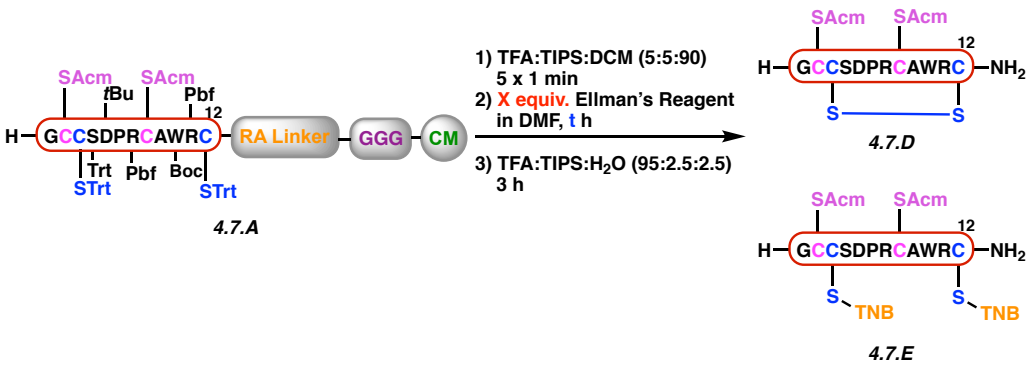


Figure 4.9 ES-MS spectrum for linear peptide **4.7.A** at retention time 14.02 min



General Procedure for Table 4.3. For each of the test reactions, the resin **4.7.A** (10 mg, 4 μmol) was first swelled in DMF (500 μL). Then, a freshly prepared solution of TFA:TIPS:DCM (5:5:90) was added and the reaction mixture was agitated at room temperature for 5 x 2 min. About 1 – 2 drops of more TIPS were added during the reaction until the orange-yellow color disappeared in the reaction mixture. The resulting TFA solution was filtered and the resin was washed with DCM (3 x 1 mL). After that, the resin was subjected to the stated amount of Ellman's Reagent in DMF for the stated amount of time until the resin color changed from peach to yellow. Finally, the reaction solution was filtered and the resin was washed with DMF (6 x 1 mL) and DCM (6 x 1 mL). In order to confirmed the total Trt group removal, the resulting peptide was retreated with a solution of TFA:TIPS:DCM (5:5:90) for 5 x 1 min and re-subjected to the stated amount of Ellman's Reagent in DMF. If the resin color changes to peach-orange color upon addition of Ellman's reagent, the reaction was agitated further until the resin color changed from peach to yellow. This procedure was repeated until no color change was observed. When no color change was observed, the solution of Ellman's reagent added was filtered and the resin was washed with DMF (6 x 1 mL) and DCM (6 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 3 hours. The

resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 4.7.D $[(C_{58}H_{90}N_{22}O_{17}S_4 + 2)/2]^+$: 748.87; observed: 748.75. m/z ES calc'd for $[(C_{58}H_{90}N_{22}O_{17}S_4 + 3)/3]^+$: 499.58; observed: 499.40. m/z ES calc'd for peptide 4.7.E $[(C_{72}H_{98}N_{24}O_{25}S_6+ 2)/2]^+$: 947.04; observed: 946.75. m/z ES calc'd for $[(C_{72}H_{98}N_{24}O_{25}S_6+ 3)/3]^+$: 631.69; observed: 631.50

Entry 1. A solution of Ellman's reagent (1.6 mg, 4 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 3 hours.

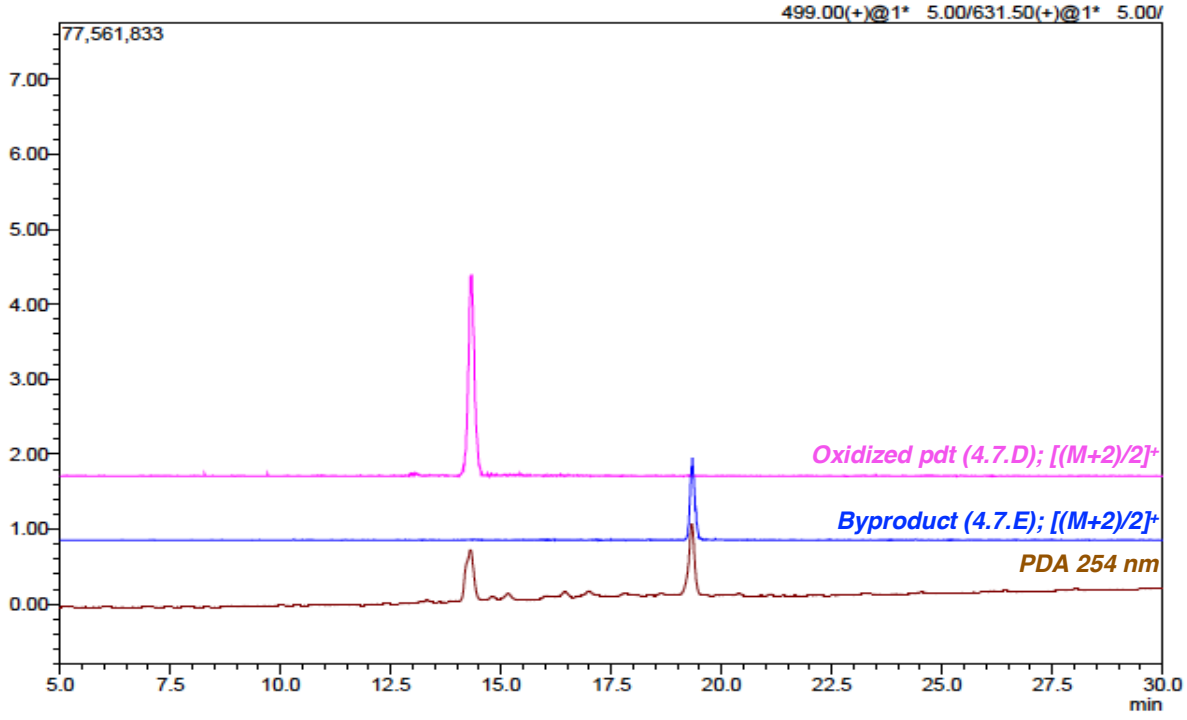


Figure 4.10 Analytical Column PDA for reaction optimization; Table 4.3 – Entry 1

Entry 2. A solution of Ellman's reagent (0.4 mg, 1 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 12 hours

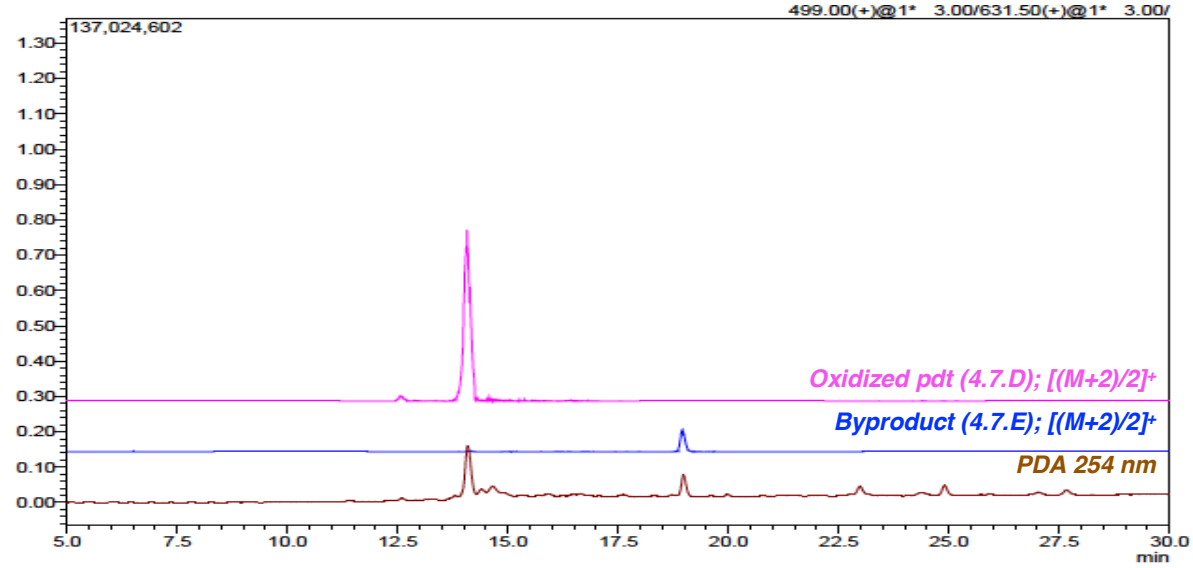


Figure 4.11 Analytical Column PDA for reaction optimization; Table 4.3 – Entry 2

Entry 3. A solution of Ellman's reagent (0.2 mg, 0.5 μmol) in DMF (1.0 mL) was added and th reaction was agitated for 24 hours.

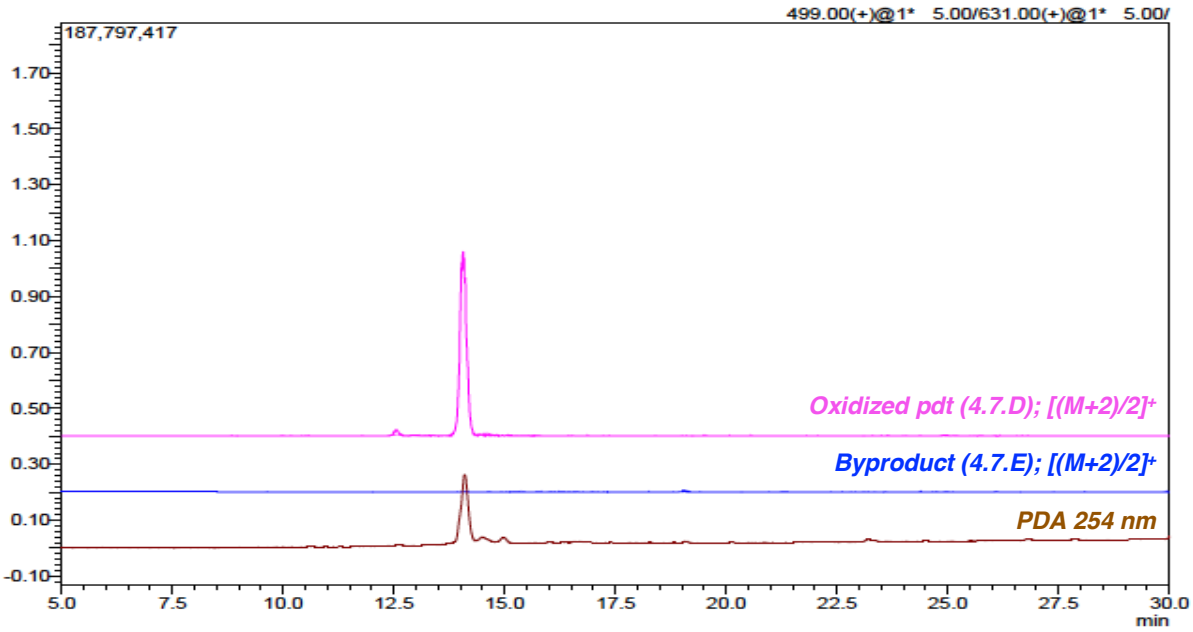
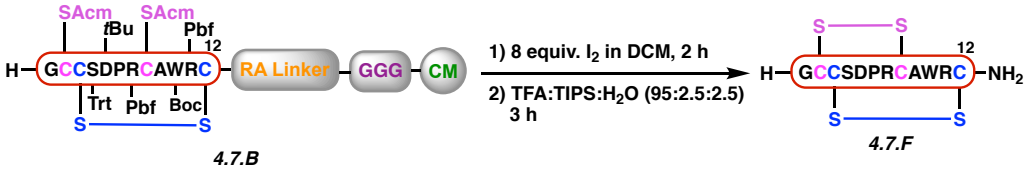


Figure 4.12 Analytical Column PDA for reaction optimization; Table 4.3 – Entry 3



Synthesis of α-conotoxin lml 4.7.F. The peptide **4.7.B** (100 mg, 40 μmol) was first swelled in DCM (3 mL). Then, a solution of iodine (81.2 mg, 320 μmol) in DCM (3 mL) was added and allowed to agitate in a 10 mL vial at room temperature for 2 hours. The reaction solution was filtered and the resin was washed with DMF (10 x 1 mL) and DCM (10 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 3 hours. Finally, the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA and purified via RP-HPLC-MS using a Semi-Preparative Thermo Scientific Hypersil Gold C8 column at a flow rate of 5 mL/min with 5–40% MeCN/H_2O gradient for 15 minutes to yield the pure peptide in 59% overall yield (11.9 mg). m/z ES calc'd for peptide 4.7.F $[(C_{52}H_{78}N_{20}O_{15}S_4 + 2)/2]^+$: 676.78; observed: 676.60. m/z ES calc'd for $[(C_{52}H_{78}N_{20}O_{15}S_4 + 3)/3]^+$: 451.52; observed: 451.35.

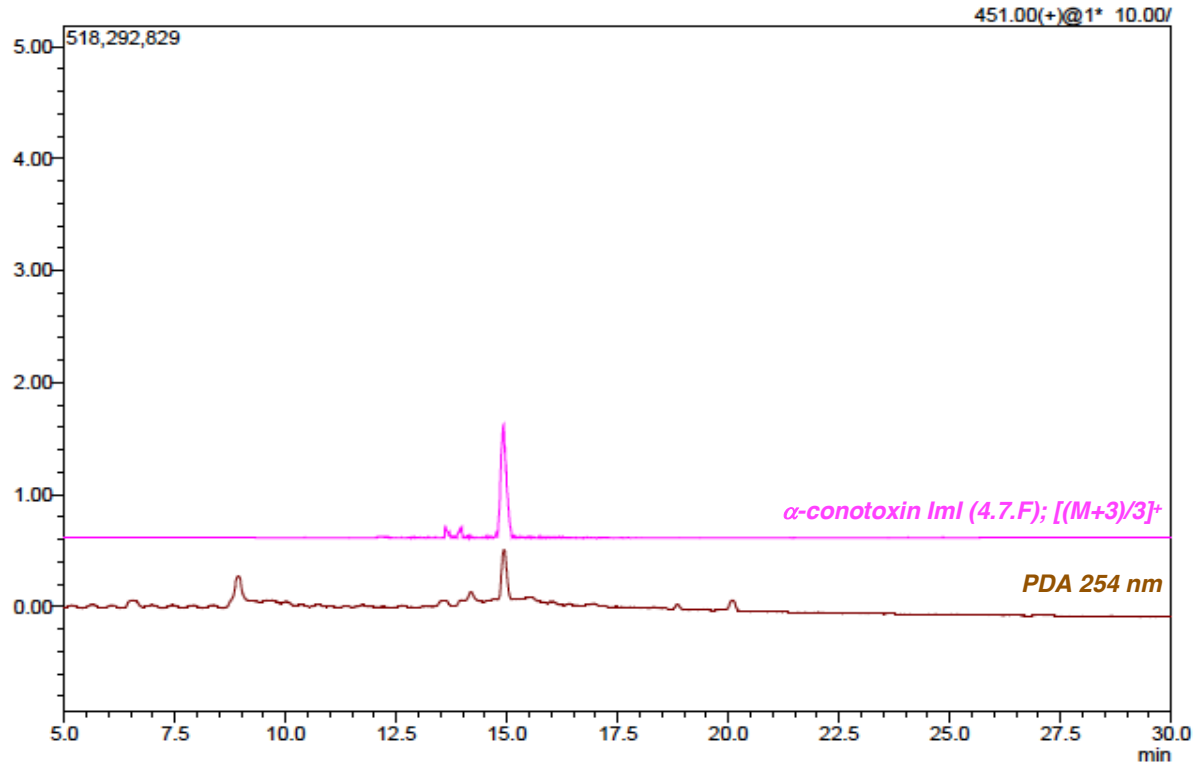


Figure 4.13 Analytical Column PDA of crude α-conotoxin ImI **4.7.F**. Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min.

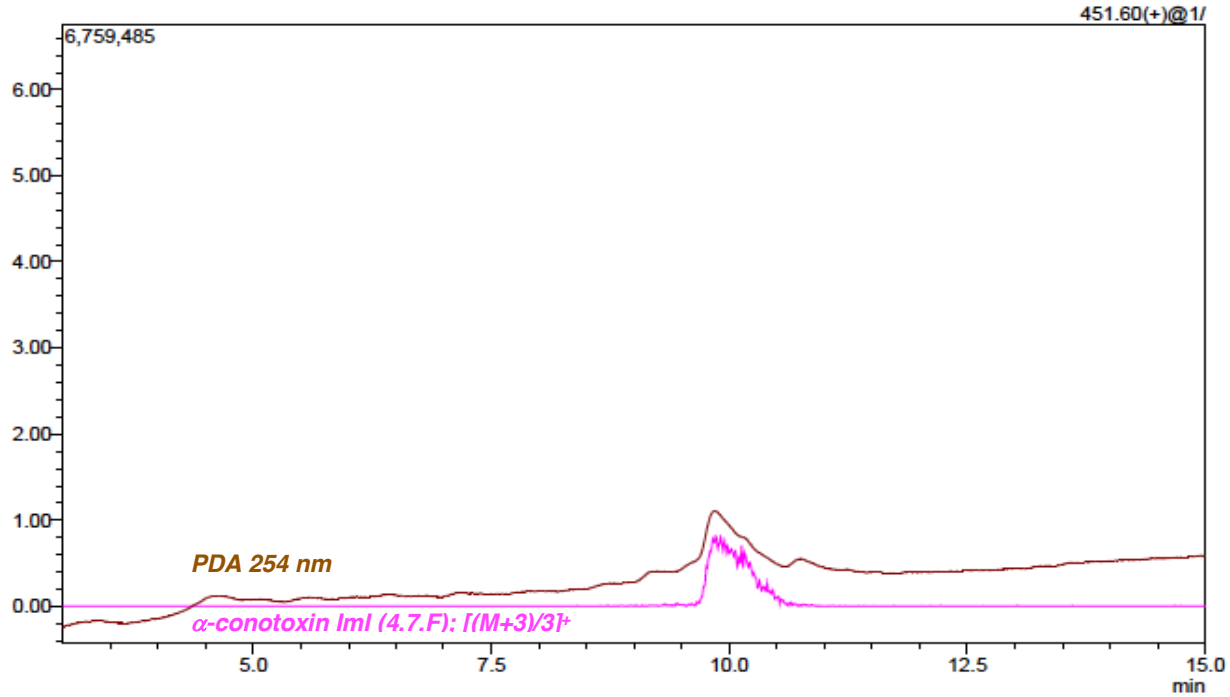


Figure 4.14 Analytical Column PDA of pure α-conotoxin ImI **4.7.F**. Gradient: 5-40% MeCN/H_2O + 1% HCOOH over 15 min. Retention time 10.01 min

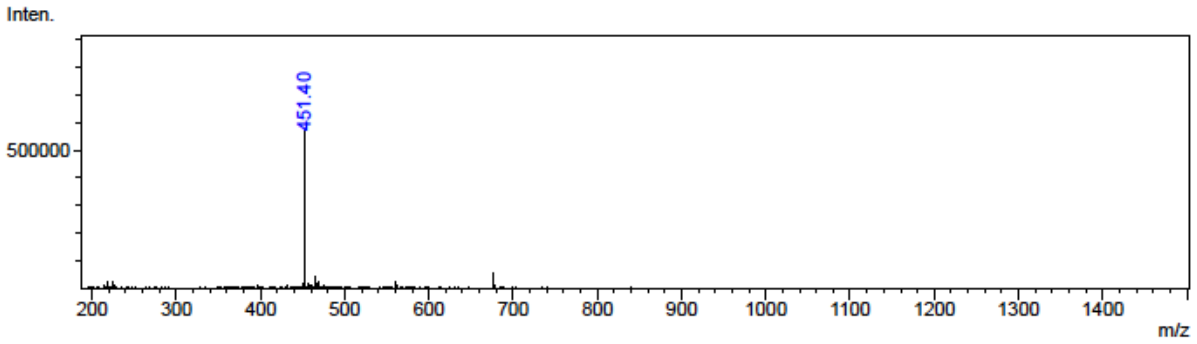
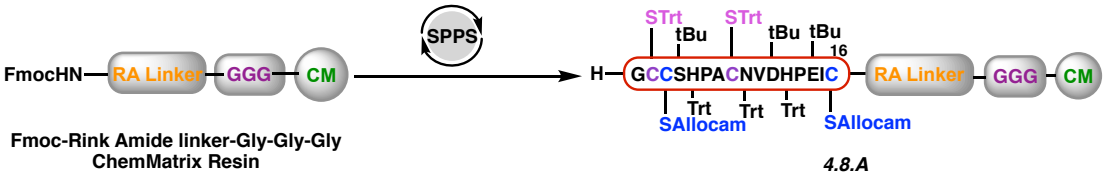


Figure 4.15 ES-MS spectrum for pure α-conotoxin ImI **4.7.F** at retention time 10.01 min



SPPS of Linear Peptide 4.8.A. The standard Fmoc-SPPS protocol described was used to synthesize peptide **4.8.A** on Rink Amide linker-Gly-Gly-Gly-ChemMatrix resin. The peptide was capped before the last coupling step. To analyze the peptide, 10 mg of the resin was subjected general ammonia cleavage protocol and the resulting crude product was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via Rp-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. Final loading of the resin: 0.10 mmol/g. m/z ES calc'd for peptide 4.8.B $[(C_{76}H_{116}N_{24}O_{26}S_4 + 2)/2]^+$: 956.07;

observed: 955.80. m/z ES calc'd for $[(C_{76}H_{116}N_{24}O_{26}S_4 + 3)/3]^+$: 637.72; observed: 637.60.

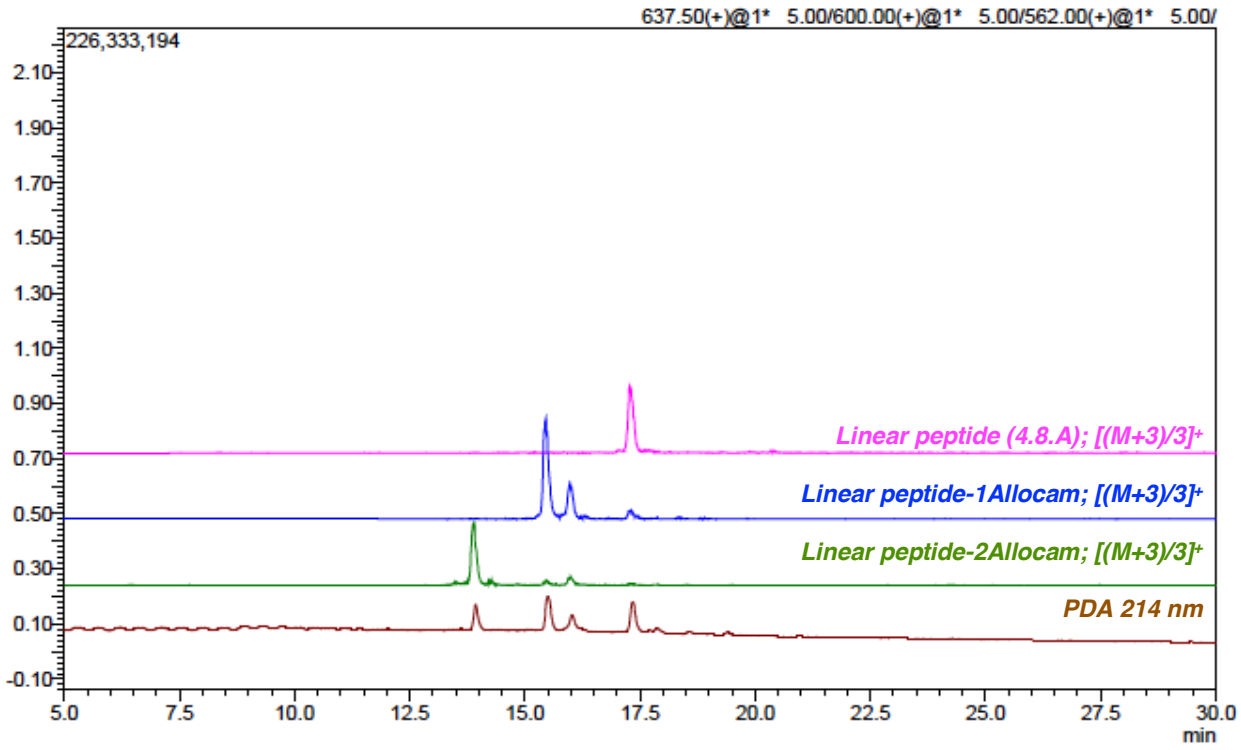


Figure 4.16 Analytical Column PDA for linear peptide **4.8.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 17.35 min

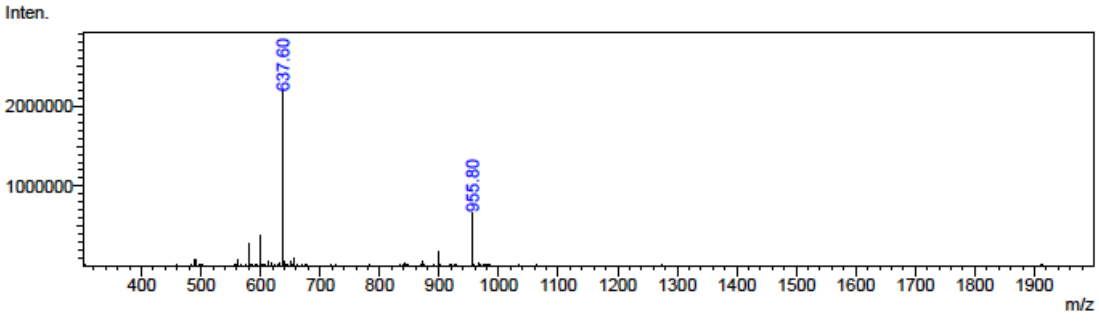
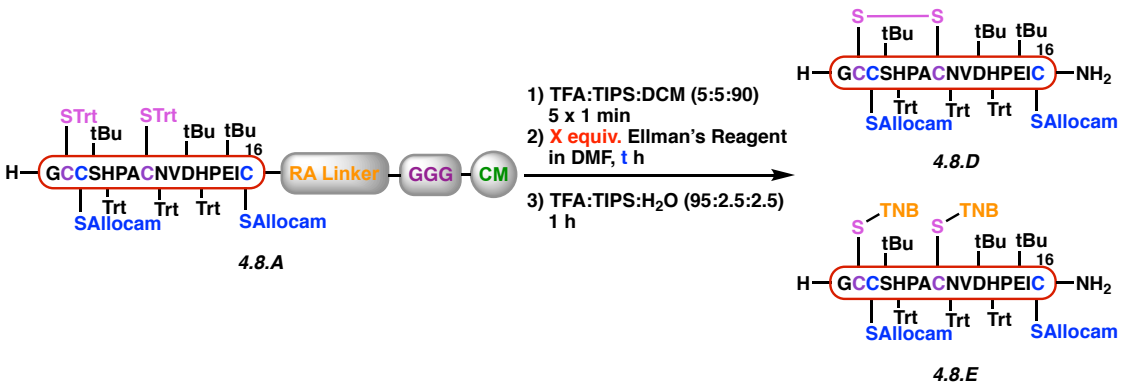


Figure 4.17 ES-MS spectrum for linear peptide **4.8.A** at retention time 17.35 min



General Procedure for Table 4.4. For each of the test reactions, the resin **4.8.A** (10 mg, 3 μmol) was first swelled in DMF (500 μL). Then, a freshly prepared solution of TFA:TIPS:DCM (5:5:90) was added and the reaction mixture was agitated at room temperature for 5 x 1 min. About 1 – 2 drops of more TIPS were added during the reaction until the orange-yellow color disappeared in the reaction mixture. The resulting TFA solution was filtered and the resin was washed with DCM (3 x 1 mL). After that, the resin was subjected to the stated amount of Ellman's Reagent in DMF for the stated amount of time until the resin color changed from peach to yellow. Finally, the reaction solution was filtered and the resin was washed with DMF (6 x 1 mL) and DCM (6 x 1 mL). In order to confirmed the total Trt group removal, the resulting peptide was retreated with a solution of TFA:TIPS:DCM (5:5:90) for 5 x 1 min and re-subjected to the stated amount of Ellman's Reagent in DMF. If the resin color changes to peach-orange color upon addition of Ellman's reagent, the reaction was agitated further until the resin color changed from peach to yellow. This procedure was repeated until no color change was observed. When no color change was observed, the solution of Ellman's reagent added was filtered and the resin was washed with DMF (6 x 1 mL) and DCM (6 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 1 hour. The

resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. . m/z ES calc'd for peptide 4.8.D $[(C_{76}H_{114}N_{24}O_{26}S_4 + 2)/2]^+$: 955.06; observed: 954.85. m/z ES calc'd for $[(C_{76}H_{114}N_{24}O_{26}S_4 + 3)/3]^+$: 637.04; observed: 636.95. m/z ES calc'd for peptide 4.8.E $[(C_{90}H_{122}N_{26}O_{34}S_6 + 3)/3]^+$: 769.16; observed: 769.00.

Entry 1. A solution of Ellman's reagent (1.2 mg, 3 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 2 hours

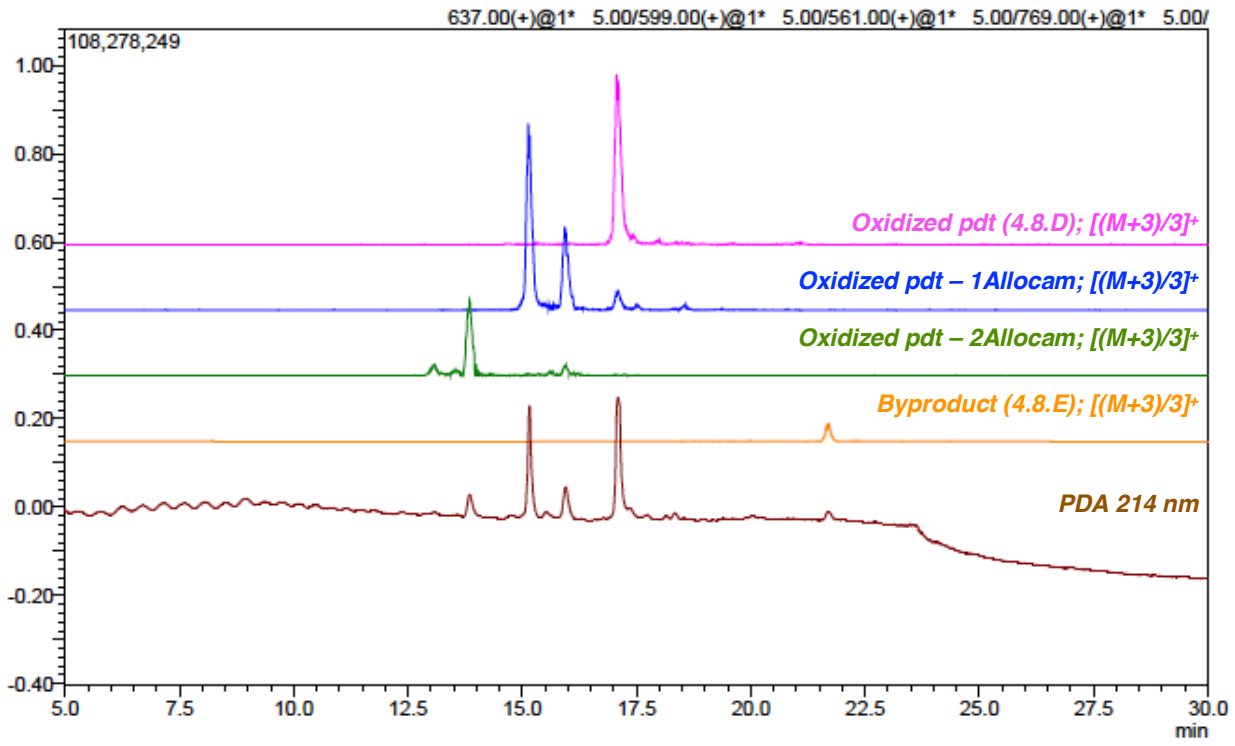


Figure 4.18 Analytical Column PDA for reaction optimization; Table 4.4 – Entry 1

Entry 2. A solution of Ellman's reagent (0.3 mg, 0.75 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 10 hours.

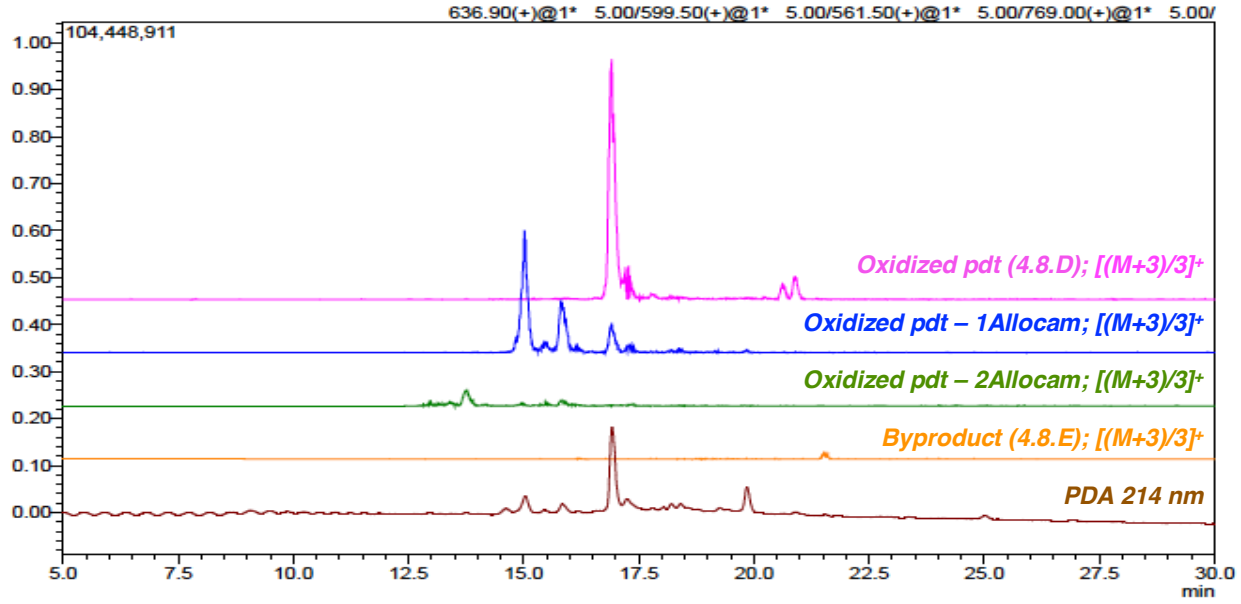


Figure 4.19 Analytical Column PDA for reaction optimization; Table 4.4 – Entry 2

Entry 3. A solution of Ellman's reagent (0.15 mg, 0.375 μmol) in DMF (1.0 mL) was added and the reaction was agitated for 16 hours.

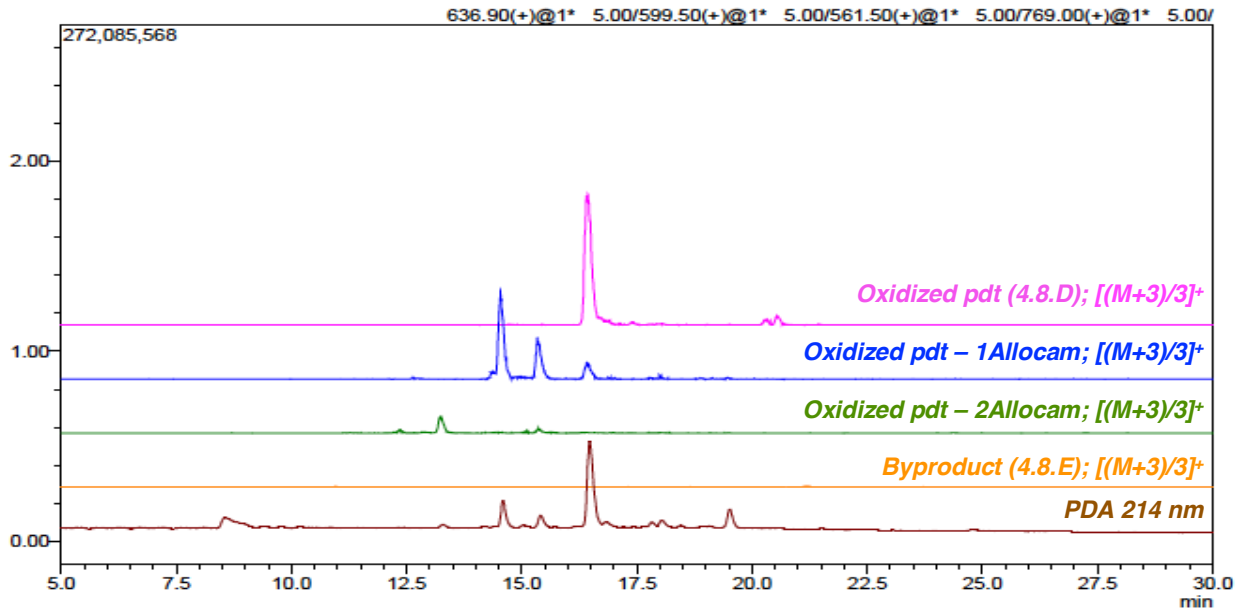
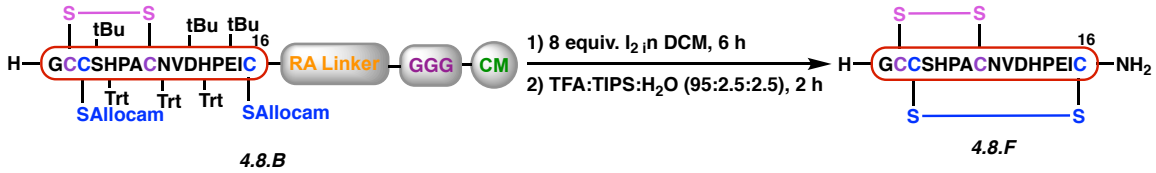


Figure 4.20 Analytical Column PDA for reaction optimization; Table 4.4 – Entry 3



Synthesis of α4/7-conotoxin LvIA 4.8.F. The resin 4.8.B (100 mg, 30 μmol) was first swelled in DCM (3 mL). Then, a solution of iodine (60.9 mg, 240 μmol) in DCM (400 μL) was added and allowed to agitate in a 10 mL vial at room temperature for 6 hours. The reaction solution was filtered and the resin was washed with DMF (10 x 1 mL) and DCM (10 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 hours. Finally, the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA and purified via RP- HPLC-MS using a Semi-Preparative Thermo Scientific Hypersil Gold C8 column at a flow rate of 5 mL/min with 10–50% MeCN/H_2O gradient for 15 minutes to yield the pure peptide in 24% overall yield (5.6 mg). m/z ES calc'd for α4/7-conotoxin LvIA $[(C_{66}H_{98}N_{22}O_{22}S_4 + 2)/2]^+$: 840.94; observed: 840.80; m/z ES calc'd for $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.85

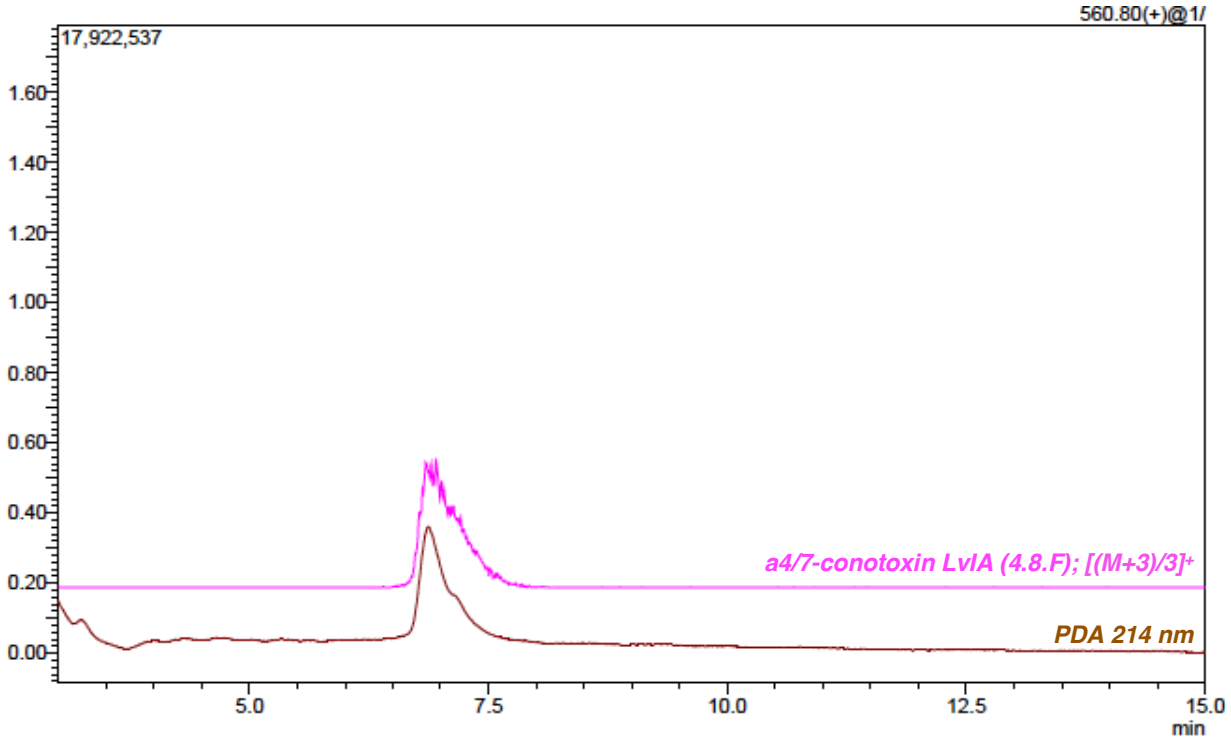


Figure 4.21 Analytical Column PDA of pure α-conotoxin LvIA **4.8.F**. Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 15 min. Retention time 6.97 min

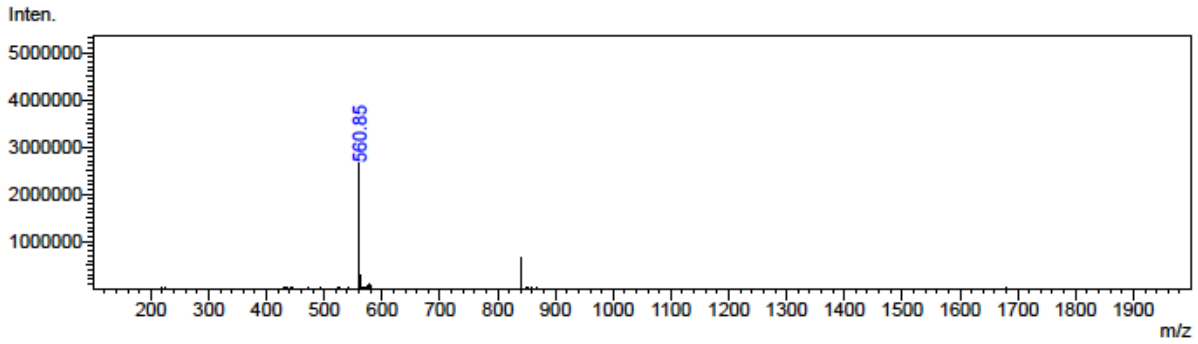
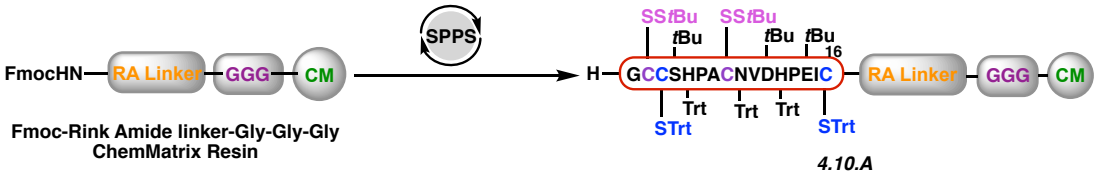


Figure 4.22 ES-MS spectrum for pure α-conotoxin LvIA **4.8.F** at retention time 6.97 min



SPPS of Linear Peptide 4.10.A. The standard Fmoc-SPPS protocol described was used to synthesize resin **4.10.A** on Rink Amide linker-Gly-Gly-Gly-ChemMatrix resin. The peptide was capped before the last coupling step. To analyze the peptide, 10 mg of the resin was subjected general ammonia cleavage protocol and the resulting crude product was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via Rp-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. Final loading of the resin: 0.13 mmol/g. m/z ES calc'd for peptide 4.X.B $[(C_{74}H_{118}N_{22}O_{22}S_6 + 2)/2]^+$: 931.13; observed: 930.85. m/z ES calc'd for $[(C_{74}H_{118}N_{22}O_{22}S_6 + 3)/3]^+$: 621.08; observed: 620.95.

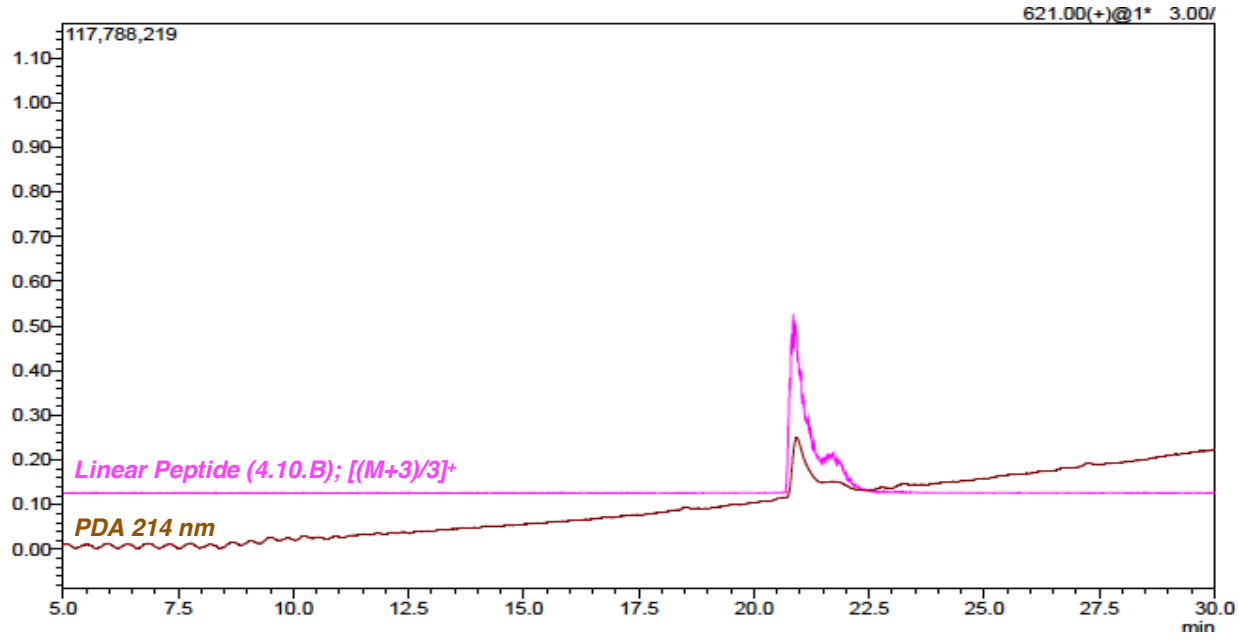


Figure 4.23 Analytical Column PDA for linear peptide **4.10.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 21.01 min

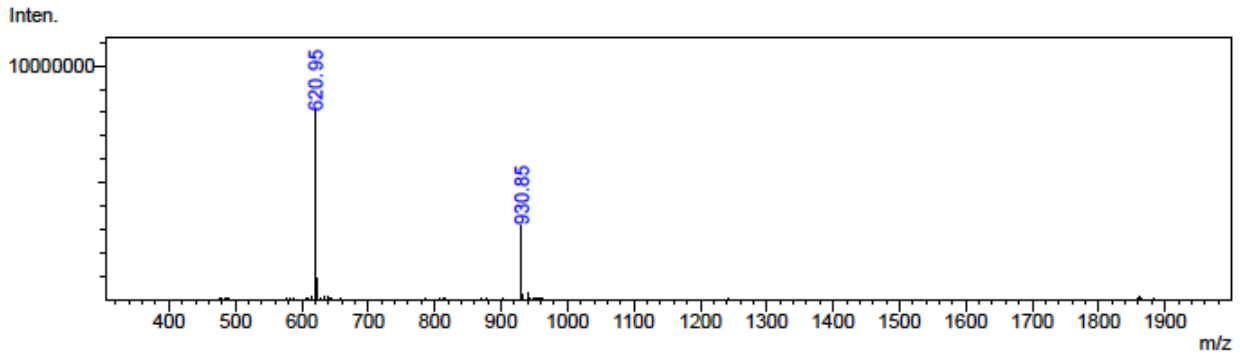
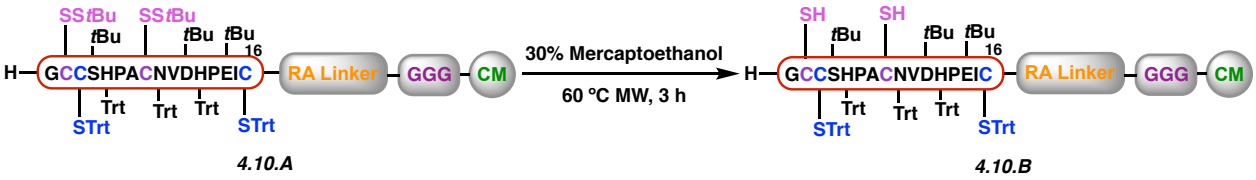


Figure 4.24 ES-MS spectrum for linear peptide **4.10.A** at retention time 21.01 min



4.10.A *4.10.B*

Synthesis of Peptide 4.10.B. The resin **4.10.A** (60 mg, 18 μmol) was first swelled in DMF (500 μL). Then, a solution of 30% mercaptoethanol in DMF (1.0 mL) was added and agitated at 60 ºC under microwave condition for 3 hour. The reaction solution was filtered and the resin was washed with DMF (5 x 1 mL). To analyze the reaction 10 mg of the resulting resin was subjected to 300 μL of the cleavage mixture (95:2.5:2.5 TFA:TIPS:H_2O) for 1 hour. The resulting TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10–50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 4.10.B

$[(C_{66}H_{102}N_{22}O_{22}S_4 + 2)/2]^+$: 842.96; observed: 842.60. m/z ES calc'd for $[(C_{66}H_{102}N_{22}O_{22}S_4 + 3)/3]^+$: 561.97; observed: 562.05.

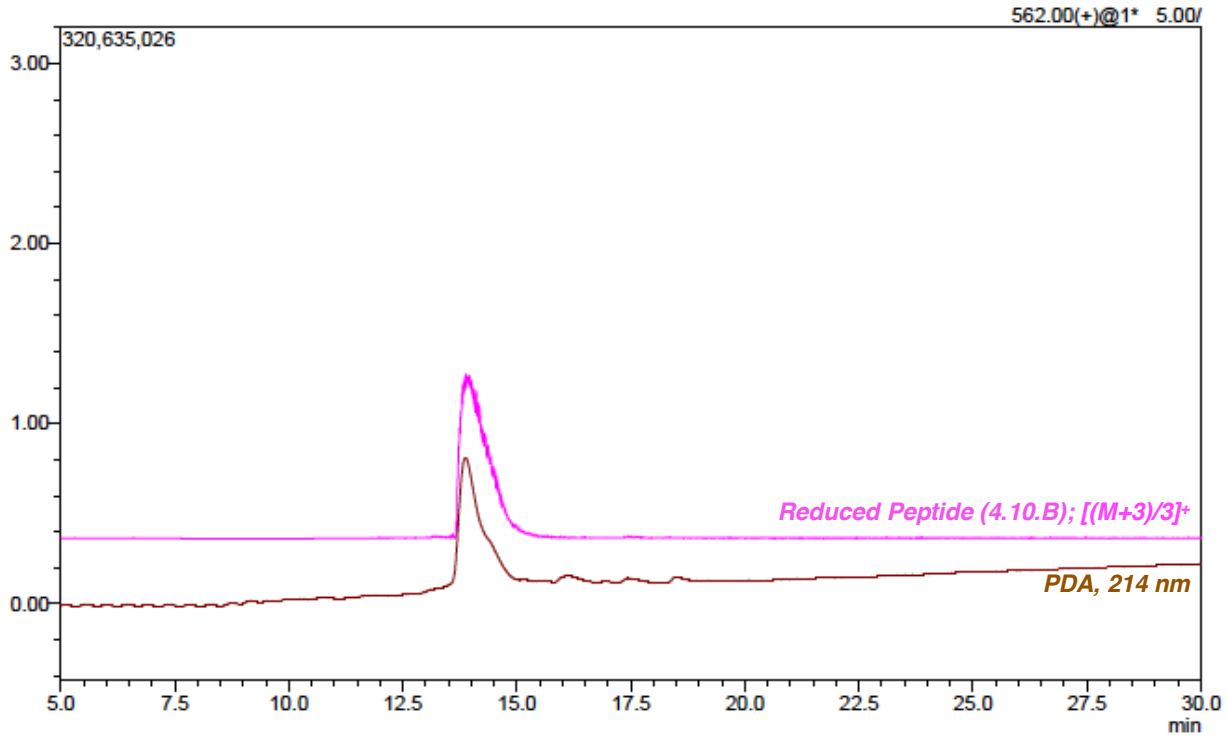
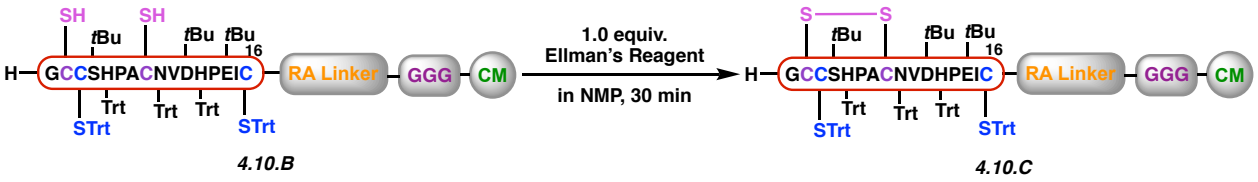


Figure 4.25 Analytical Column PDA for Scheme 4.11 – Step 1



Synthesis of Peptide 4.10.C. The peptide **4.10.B** (50 mg, 15 μmol) was first swelled in NMP (1 mL). Then it was subjected to a solution of Ellman's Reagent (6.0 mg, 15 μmol) in NMP for 30 min until the resin color changed from peach to yellow. Finally, the reaction solution was filtered and the resin was washed with NMP (6 x 1 mL) and DCM (6 x 1 mL). To analyze the reaction 10 mg of the resulting resin was subjected to 300 μL of the

cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 1 hour. The resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 4.10.C $[C_{66}H_{100}N_{22}O_{22}S_4 + 1]^+$: 1681.90; observed: 1682.60. m/z ES calc'd for $[(C_{66}H_{100}N_{22}O_{22}S_4 + 2)/2]^+$: 841.95; observed: 841.75. m/z ES calc'd for $[(C_{66}H_{100}N_{22}O_{22}S_4 + 3)/3]^+$: 561.63; observed: 561.50.

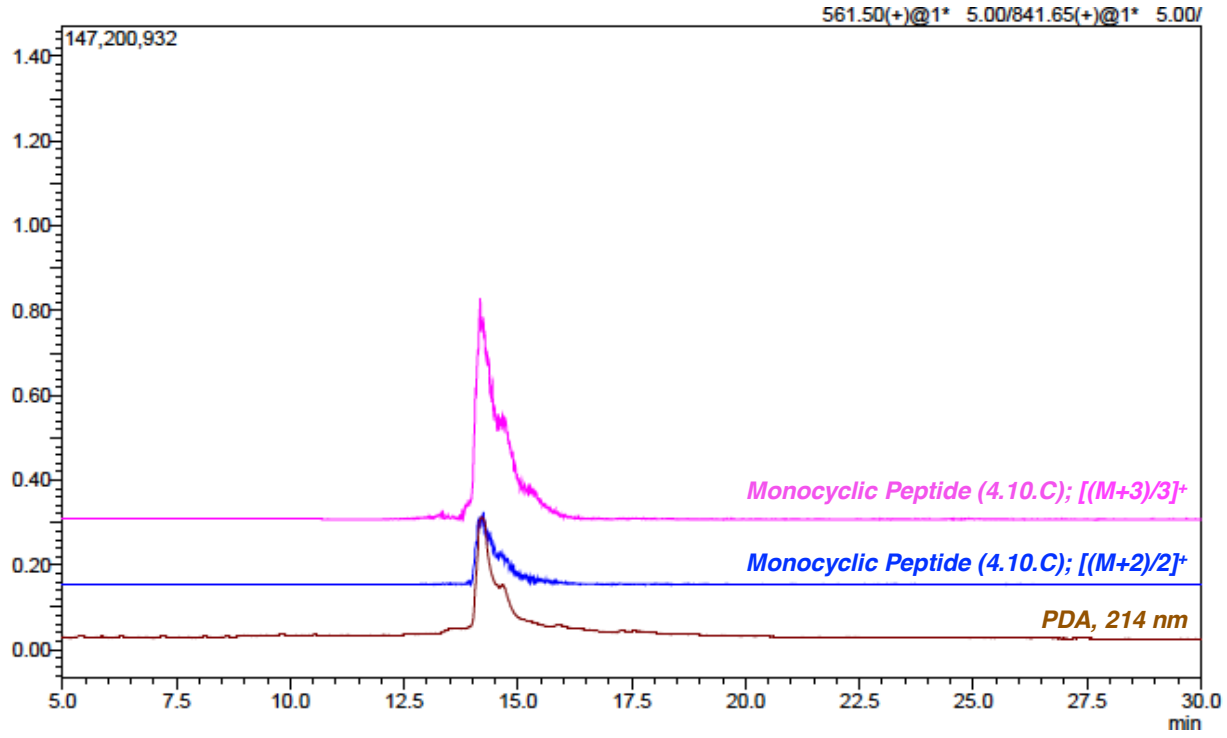
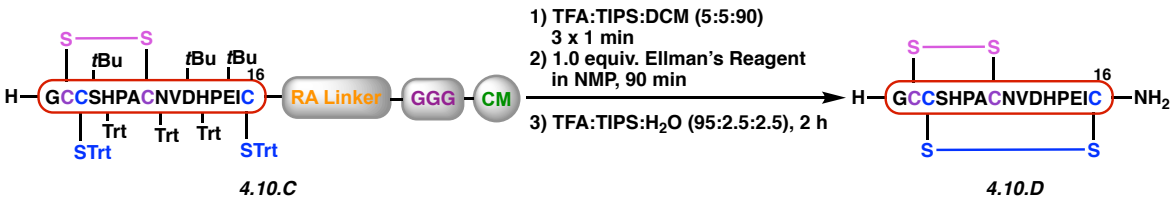


Figure 4.26 Analytical Column PDA for Scheme 4.11 – Step 2



Synthesis of α4/7-Conotoxin LvIA 4.10.D. The resin **4.10.C** (40 mg, 12 μmol) was first swelled in DCM (1 mL). Then, a freshly prepared solution of TFA:TIPS:DCM (5:5:90) was added and the reaction mixture was agitated at room temperature for 3 x 5 min. About 1 – 2 drops of more TIPS were added during the reaction until the orange-yellow color disappeared in the reaction mixture. The resulting TFA solution was filtered and the resin was washed with DCM (3 x 1 mL). After that, the resin was subjected to a solution of Ellman's Reagent (5.0 mg, 12 μmol) in DMF (1.5 mL) for 90 min until the resin color changed from peach to yellow. Finally, the reaction solution was filtered and the resin was washed with DMF (6 x 1 mL) and DCM (6 x 1 mL). In order to confirmed the total Trt group removal, the resulting peptide was retreated with a solution of TFA:TIPS:DCM (5:5:90) for 2 x 5 min and re-subjected to the stated amount of Ellman's Reagent in NMP. If the resin color changes to peach-orange color upon addition of Ellman's reagent, the reaction was agitated further until the resin color changed from peach to yellow. This procedure was repeated until no color change was observed. When no color change was observed, the solution of Ellman's reagent added was filtered and the resin was washed with DMF (6 x 1 mL) and DCM (6 x 1 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 2 hours. The resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was

dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP- HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 15 minutes. m/z ES calc'd for peptide 4.10.D $[C_{66}H_{98}N_{22}O_{22}S_4 + 1]^+$: 1679.88; observed: 1682.00. m/z ES calc'd for $[(C_{66}H_{98}N_{22}O_{22}S_4 + 2)/2]^+$: 840.94; observed: 840.75. m/z ES calc'd for $[(C_{66}H_{98}N_{22}O_{22}S_4 + 3)/3]^+$: 560.96; observed: 560.80.

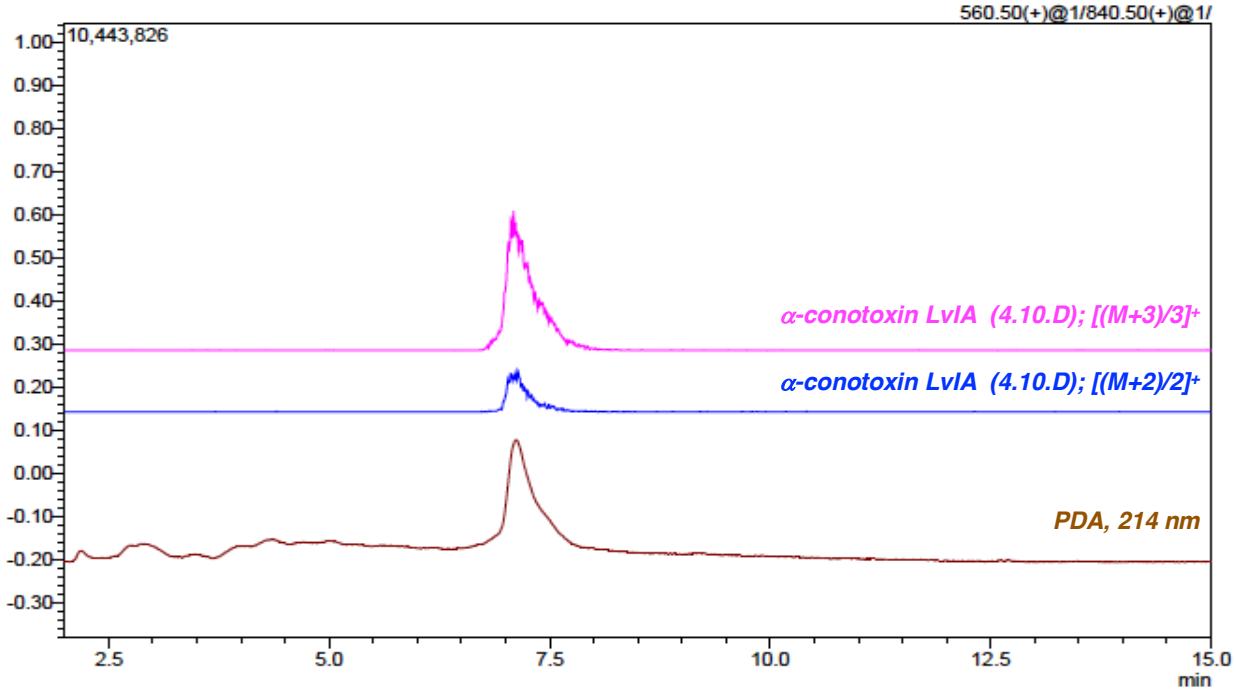


Figure 4.27 Analytical Column PDA of pure α-conotoxin LvIA **4.10.D**. Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 15 min. Retention time 7.18 min

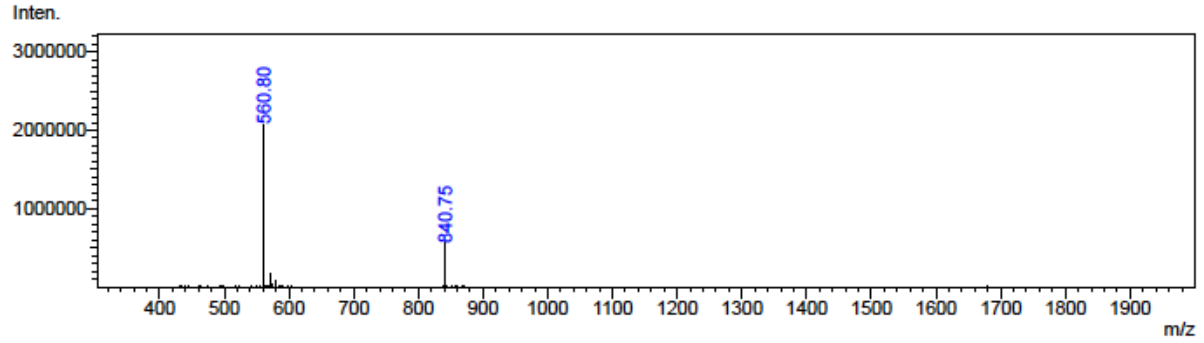


Figure 4.28 ES-MS spectrum for pure α-conotoxin LvIA **4.10.D** at retention time 7.18 min

CHAPTER 5: ON RESIN CONTROLLED FOLDING OF LINACLOTIDE

5.1 Introduction

A majority of cysteine-rich bioactive peptides contain three or more disulfide bonds. They have higher potential as a valuable tool in biomedical and pharmaceutical research. Duo to their small size, chemical synthesis has become the method of choice over recombinant expression. Although there are several on-resin folding methods have been developed to synthesize one or two disulfide bonds in peptides, successful regioselective formation of three or more disulfide bonds with resin-bound peptides has not been reported. This chapter details the progress towards on-resin regioselective disulfide bond formation to access linaclotide, which contains three disulfide linkages. The synthetic procedure demonstrates utility of Allocam group as a cysteine protecting group and applicability of new Allocam cleavage - oxidation method and Ellman's reagent - mediated on-resin disulfide bond formation method to access peptides with three disulfide bonds

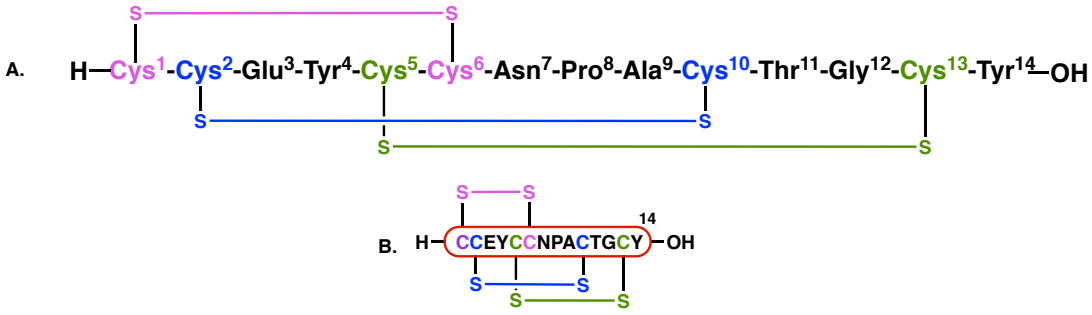
5.2. Linaclotide

5.2.1 Linaclotide: Structure and Bioactivity

Linaclotide is a cyclic peptide consists of 14-amino acid residues. It derived from heat-stable enterotoxins, produced by various *Escherichia coli* strains which are a frequent cause of diarrhea.[174] It is a small peptide with 43% cysteine residues in the sequence, and have three disulfide bonds in a constrained conformation. The disulfide bonds are lies between Cys^{1}-Cys^{6}, Cys^{2}-Cys^{10} and Cys^{5}-Cys^{13} (**Figure 5.1**). Linaclotide was approved by FDA in 2012 as a drug, marketed under the trade name Linzess in the US and Mexico, and as Constella in Canada. It can be used for the treatment of

gastrointestinal diseases such as chronic constipation (CC) and irritable bowel syndrome (IBS) in adults.[175] The drug provides well tolerated relief in patients and acts by binding to guanylate cyclase C on the surface of intestinal epithelial cells.[176,177,178] Activation of the cyclase triggers a signaling pathway that leads to chloride and fluid secretions into the lumen, increasing colonic transit. The location of this target in the intestine allows for linaclotide to be orally administered.[179]

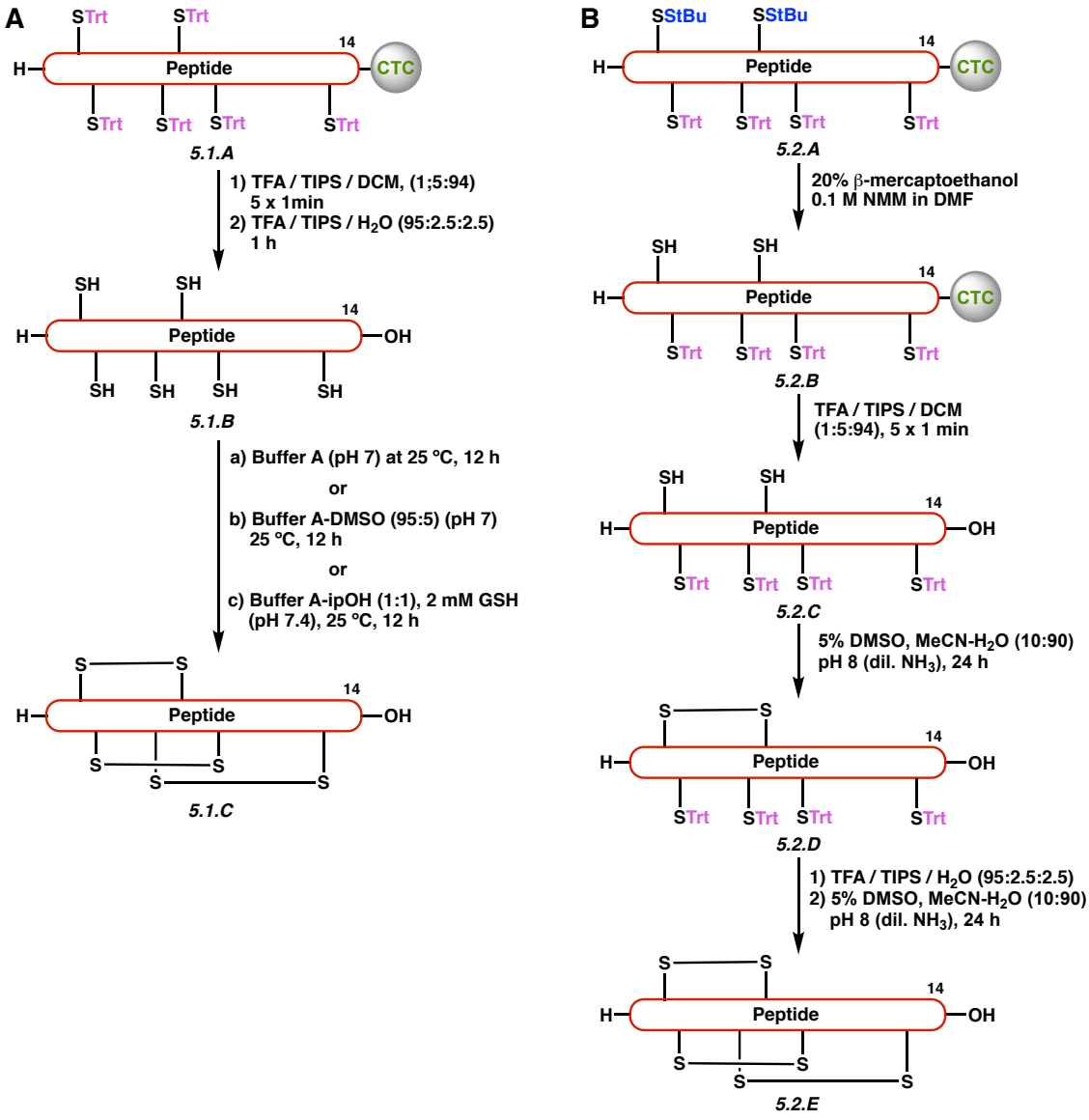
Figure 5.1 A. Structure of Linaclotide **B.** Graphical representation of Linaclotide structure



5.2.2 Reported Chemical Synthesis of Linaclotide

Albericio and co-workers have reported synthesis studies of linaclotide.[180] The study illustrated 14 available strategies and only 2 were successful to give correctly-linked product as the major product. The two succeeded strategies have Trt protection of all cysteines, or Trt protection of all cysteines except S*t*Bu protection of Cys[1] and Cys[6] in the sequence (**Scheme 5.1)**. The linear peptide **5.1.A** was synthesized on CTC resin and all six cysteine residues were incorporated with Trt protecting group for the random folding strategy (**A. Scheme 5.1**). First, the protected peptide was cleaved from the resin by 1% TFA in DCM in presence of TIPS and then, it was subjected to 95% TFA condition for total side group deprotection.

Scheme 5.1 (A) Random folding strategy for synthesis of Linaclotide Cys(Trt)
(B) Semi-directed folding strategy for synthesis of Linaclotide Cys(S*t*Bu) and Cys(Trt)



The resulting reduced peptide **5.1.B** was oxidized by three different in-solution folding conditions. An air oxidation was done by dissolving the peptide in buffer A (100 mM sodium phosphate, 2 M guanidine hydrochloride, pH 7.0) and stirred under open atmosphere at 25 °C for 12 hours. Using the same buffer conditions, DMSO-mediated oxidation was done with 5% DMSO and the reaction was stirred under open atmosphere at 25 °C for 12 hours. As the third method, the peptide was subjected to a mixture of

buffer A:2-propanol (1:1) and 2 mM glutathione and the solution stirred at 25 ºC for 12 hours under open atmosphere. In this strategy all the oxidation procedures gave completely oxidized peptide **5.1.C** with desired disulfide bonds. A semi-regioselective strategy was done by protecting Cys^1 and Cys^6 residues with S*t*Bu group (**B. Scheme 5.1**). After linear synthesis of the peptide **5.2.A** on CTC resin, it was first treated with 20% mercaptoethanol and 0.1 M *N*-methylmorpholine in DMF on resin. The resulting peptide **5.2.B** was then cleaved from the resin with 1% TFA in DCM. The cleavage mixture was poured over H_2O and the organic phase was evaporated under N_2 prevent an increase in concentration of TFA, which prevent premature cleavage of Trt groups. The peptide **5.2.C** is then lyophilized and subjected to DMSO oxidation form first disulfide bond. After purifying the mono cyclic peptide **5.2.D**, it was re-subjected to DMSO oxidation to form two remaining disulfide bonds randomly in solution. This strategy also gave fully oxidized product **5.2.E** with correct disulfide bond conformation.

5.3 Synthesis of Linaclotide using On-resin Regioselective Folding Method

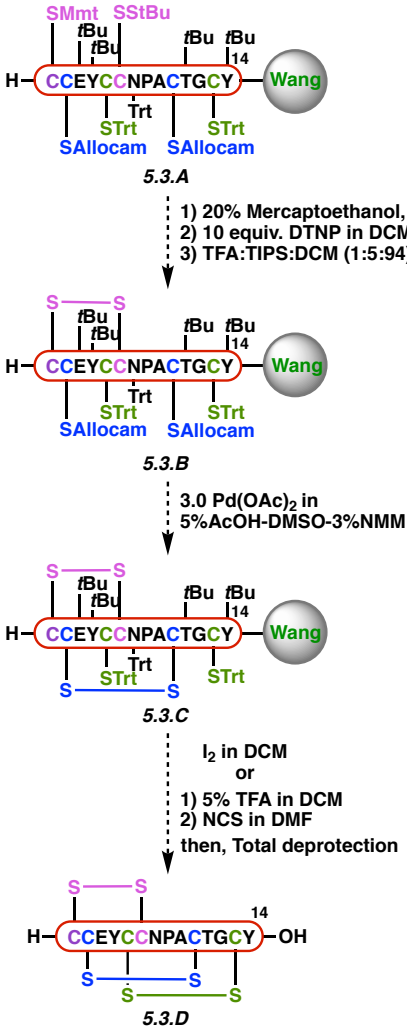
After analyzing both random and semi-regioselective strategies reported for linaclotide synthesis, we evaluated few on-resin regioselective folding methods to access correct disulfide bond pattern in for linaclotide. The findings form Albericio's work,[7] such as order of Cys residue pairing, best protecting group positioning for cysteine protection were applied to design our synthesis pathways.

5.3.1 Synthesis of Linaclotide using Cys(StBu), Cys(Mmt), Cys(Allocam) and Cys(Trt)

The synthesis of α-conotoxin LvIA by the combination of a displacement method and simultaneous Allocam removal and oxidation (see Chapter 02) inspired this design

of synthesis for linaclotide (**Scheme 5.2**). The linear synthesis of linaclotide **5.3.A** was done on Wang resin and S*t*Bu, Mmt, Allcam and Trt was used to protect cysteine residues.

Scheme 5.2. On-resin synthesis of Linaclotide using Cys(S*t*Bu), Cys(Mmt), Cys(Allocam) and Cys(Trt)



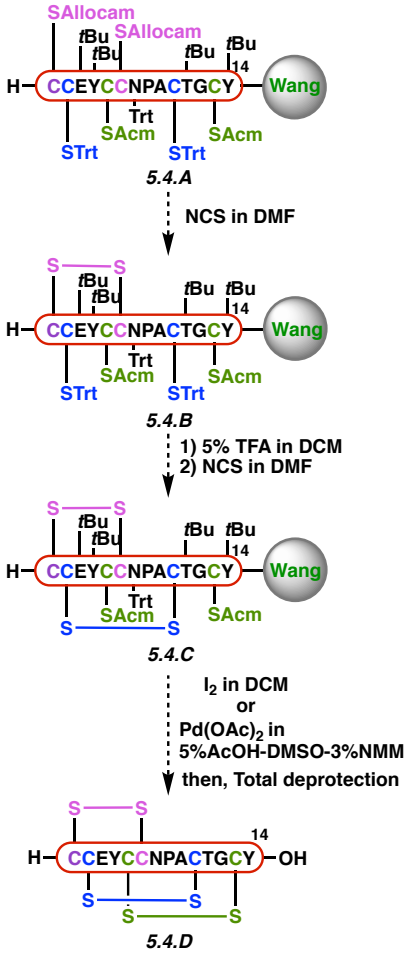
As described in Chaper 01 and Chapter 02, S*t*Bu and Mmt groups were used to form the first disulfide bond vis a displacement method. Deprotection of Cys(S*t*Bu) was achieved with 20% mercaptoethanol, 0.1 NMM in DMF at room temperature, after 18 hours. Microwave heating was also tried to remove S*t*Bu group with 30% mercaptoethanol, but significant peptide cleavage was observed. The free thiol group resulting from Cys(S*t*Bu)

deprotection was activated with 5-Npys group by treating the peptide with excess DTNP. To form the first disulfide bond, Mmt group needed to be removed in present of Trt group. This was a very challenging step since both the group have slightly different TFA tolerance. The general 1% TFA cleavage condition didn't work for removal of Mmt group in this substrate. When higher TFA conditions were used, Trt group was started to cleave together with Mmt group. Therefore, we couldn't continue the synthetic steps as planned. Failure to remove Mmt group in presence of Trt group made this proposed synthetic route a challenge.

5.3.2 Synthesis of Linaclotide using Cys(Allocam), Cys(Trt) and Cys(Acm)

A synthetic route using Cys(Allocam), Cys(Trt) and Cys(Acm) was designed to access correct disulfide bonds in linaclotide (**Scheme 5.3**). Allocam group was used to form the first disulfide bond by NCS oxidation. Neither Pd-DMSO based simultaneous Allocam removal and oxidation conditions nor iodine oxidation can be used in this step. Because from our previous studies in Chapter 02, we observed that Acm group is sensitive to Pd-DMSO based simultaneous Allocam removal and oxidation conditions and both Acm and Trt groups are not stable to iodine oxidation. Therefore, the choice of oxidation method was NCS-mediated oxidation. The linear peptide **5.4.A** was subjected to NCS in DMF and it caused cleavage of not only Allocam group, but also other two cysteine protecting group. This illustrated the sensitivity of both Trt and Acm groups toward NCS oxidation. There was no other method for Allocam cleavage and oxidation in presence of Trt and Acm groups, and therefore, this proposed synthetic design also failed to access desired selectivity in disulfide bond formation .

Scheme 5.3. On-resin synthesis of Linaclotide using Cys(Allocam), Cys(Trt) and Cys(Acm)

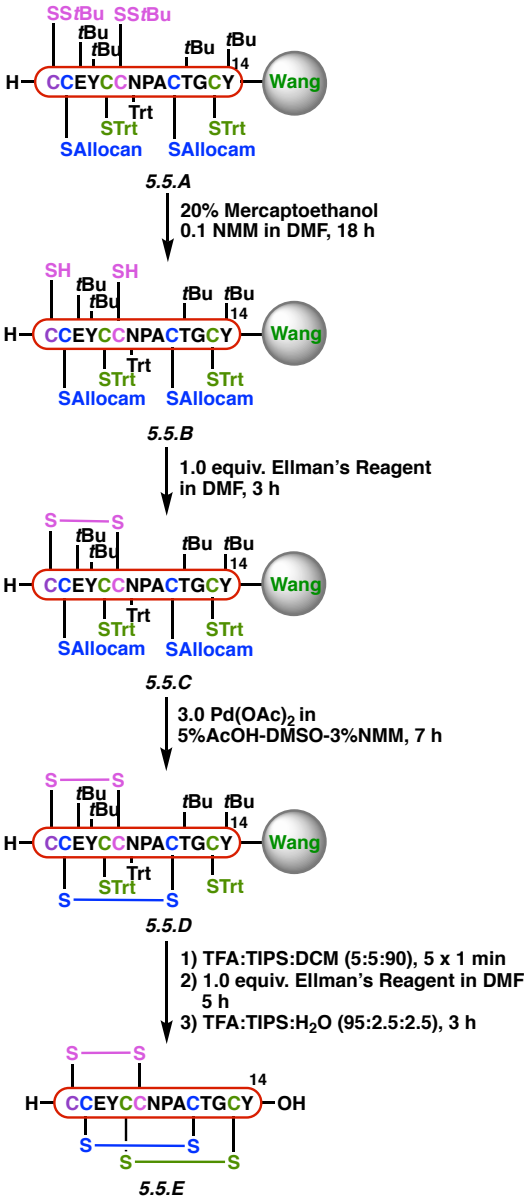


5.3.3 Synthesis of Linaclotide using Cys(StBu), Cys(Allocam) and Cys(Trt)

After the discovery of Ellman's reagent as a mild oxidant to form on-resin disulfide bonds in reduced peptides (see chapter 04), a new synthetic route was designed using combination of Cys(S*t*Bu), Cys(Allocam) and Cys(Trt) groups (**Scheme 5.4**). Linear synthesis of the peptide **5.5.A** was done on Wang resin using S*t*Bu, Allocam and Trt to protect cysteine residues. First, optimized Cys(S*t*Bu) deprotection condition reported in Albericio and co-workers' studies,[7] was applied to remove S*t*Bu groups in the peptide. The peptide was treated with 20% mercaptoethanol, 0.1 NMM in DMF for 18 hours and

the resulting free thiols in the peptide **5.5.B** were subjected to 1.0 equivalent of Ellman's reagent in DMF to form the first disulfide bond.

Scheme 5.4. On-resin regioselective synthesis of Linaclotide using Cys(S*t*Bu), Cys(Trt) and Cys(Allocam)



The reaction was monitors by color change of the resin, from dark peach-orange to yellow. After 3 hours, when the resin color changed to yellow, reagents were filtered off and the resin was washed thoroughly with DMF and DCM. With 1.0 equivalent of Ellman's reagent the mono-cyclic peptide **5.5.C** was obtained with zero by products. Next, the mono-cyclic

peptide **5.5.C** was subjected to Pd-DMSO based simultaneous Allocam removal and oxidation conditions and the second disulfide bond formation **5.5.D** was observed in 7 hours. Thereafter, the Cys(Trt) deprotection was done with 5% TFA in DCM using TIPS as the scavenger to get free cysteine residues. Finally, another Ellman's reagent-based on-resin oxidation was done to get the fully folded product **5.5.E**. In this last step, 1.0 equivalent of Ellman's reagent in DMF was used and the reaction was run for 5 hours until the resin color changes from dark peach-orange to yellow.

5.4 Summary and Future Directions

Different on-resin regioselective methods to access three disulfide bonds in peptides were evaluated using linaclotide as a target peptide. Out of the three proposed synthetic designs, orthogonal combination of S*t*Bu, Allocam and Trt groups allowed regioselective disulfide bond formation to access desired disulfide pattern. To our knowledge, this is the first on-resin regioselective synthesis of a peptide with more than two bonds.

Several modifications could be done to improve this on-resin regioselective synthesis of linaclotide. As iodine-mediated simultaneous Allocam removal and oxidation is more effective (see chapter 03) than Pd-DMSO mediated Allocam removal and oxidation, the Allocam-mediated disulfide bond formation step (Step 3) in linaclotide synthesis could be done using iodine oxidation. In order to perform an iodine oxidation on Allocam group, change in the positioning of cysteine protecting groups should be done in the synthetic design. As Allocam group is sensitive to iodine oxidation, Trt group removal with 5% TFA in DCM followed by Ellman's oxidation should be done to form the second

disulfide bond. Allocam protection should be done for Cys^5-Cys^{13} residues and iodine-mediated simultaneous Allocam removal and oxidation could be done to form the final disulfide bond. In addition, Ellman's oxidation could be done in NMP to increase the rate of disulfide formation (see chapter 4). The higher density of both cysteine residues and side-chain protecting groups in linaclotide made the on-resin synthesis a very challenging process. In order to have higher swelling power of the resin, which increases the reactivity of peptide (see chapter 2 and 3), the synthesis could be rerun on ChemMatric resin with Wang linker. Moreover, to increase the flexibility of peptide backbone and get effective folding of the peptide, a long linker could be introduce between the resin and the peptide, similar to the modification done in the synthesis of α4/7-conotoxin LvIA (see chapter 3).

5.5 Methods and Material

5.5.1 General Information

Unless otherwise specified, all commercially available reagents were purchased from Sigma-Aldrich and used without further purification. Anhydrous Et_2O, MeCN, DMF, DMSO, CH_2Cl_2 were purchased from Fisher. These were passed through a commercial solvent purification system (2 columns of alumina) and used without further drying. Hünig's base was distilled over CaH_2 immediately prior to use. All amino acids were purchased from Chem-Impex Int'l. Inc. unless otherwise noted. HATU were purchased from Chem-Impex Int'l. Inc. Unless otherwise noted, all reactions were performed in 2 mL, 5 mL and 10 mL Biotage reactor vials with PTFE frit (depend on reaction volume) at room temperature. All yields refer to chromatographically and spectroscopically pure products. All HPLC analyses and purifications were performed on a Custom Reverse Phase

Shimadzu Liquid Chromatograph Mass Spectrometer (LCMS-2020), which can toggle between analytical and semi-preparative columns. This instrument has a photodiode array (PDA) detector (D2 & W lamp), which collects a range of wavelengths, in place of a traditional single channel UV detector. RP-HPLC-MS mobile phases (MeCN and H_2O) contained 0.1% Formic Acid. Analytical HPLC was performed on a Phenomenex Kinetex C18 column (5 μm, 250 x 4.6 mm) and a Thermo Scientific Hypersil Gold C8 column (5 μm, 250 x 4.6 mm). Semi-Preparative HPLC was conducted using a Thermo Scientific Hypersil Gold C8 column (5 μm, 150 x 10 mm). All peptide yields are calculated based on the final loading.

SPPS General Information. Solid-phase peptide synthesis was executed on a Biotage Isolera+ semi-automated synthesizer with microwave heating.

- *Reactor Vials [Vial size (Volume range allowed)]:* 2 mL reactor vial (0.8-1.1 mL), 5 mL reactor vial (1.6-3.2 mL), and 10 mL reactor vial (3.2-6.4 mL)
- *Swelling + Heat:* DMF was added and vortexed at 1200 RPM for 20 m at 70 ºC. The solvent was then removed over 1 m followed by two DMF washes (DMF was added and the suspension was vortexed at 600 RPM for 45 s, followed by the removal of solvent (over 2 m)).
- *Coupling:* A solution of Fmoc-aa-OH (3 equiv), HATU (3 equiv), and DIPEA (6 equiv) in DMF was made immediately prior to addition to the reaction vial containing the resin. Once the solution was added, the suspension was heated to 75 ºC (except for Fmoc-Cys-OH which was heated to 50 ºC) for 5 minutes with a vortex rate of 1200 RPM. After the reaction, the solution was removed (over 2 m) and the resin was rinsed

with DMF 4 times (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).

- *Fmoc Removal (Deprotection):* The reactor vial was filled with 20% piperidine in DMF. The suspension was vortexed at 1200 RPM for 3 m at RT. The solvent is removed followed by addition of 20% piperidine in DMF. The suspension is vortexed again at 1200 RPM for 10 m at RT. The solvent was removed over 2 m, followed by 4 DMF washes (after addition of DMF, the suspension was agitated at a vortex rate of 1200 RPM for 1 m, solvent removal was at a rate of 2 m).
- *Wash*: DMF was added to the reaction vial and agitated at a vortex rate of 1200 RPM for 1 m. The solvent was removed over 1 m and repeated for a total of 4 times.
- *Final Wash:* Resin was rinsed with CH_2Cl_2 (3 x 1 mL) and MeOH (3 x 1 mL).
- *Drying for Storage/Weighing:* After the final wash, the resin was placed on the lyophilizer overnight for drying.

General Procedure for Capping - First, 25% acetic anhydride in DMF (3 mL) was added and agitated for 5 min. Then,1.5 eqiuv. of DIPEA was added to the reaction and agitated for 30 min. Finally, the reaction solution was filtered and the resin was washed with DMF (5 x 3 mL).

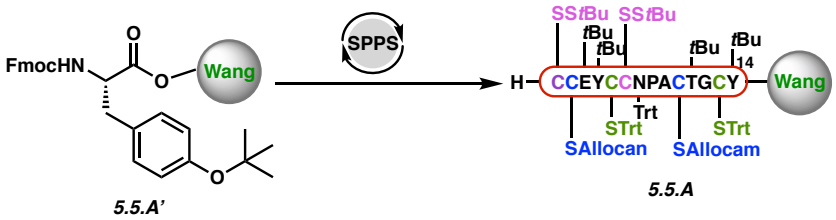
Procedure for Resin Loading Analysis. Initial and final loading of resin was analyzed based on amount of Fmoc group.

Fmoc Deprotection – 10 mg of the resin was weighed out and swelled in DMF in the peptide synthesizer. Then, 1.00 ml of 20% piperidine in DMF was added and it was allowed to vortex in the peptide synthesizer at room temperature for 3 min. After 3 min,

the filtrate was collected in to a separate tube, another 1.00 ml of 20% piperidine in DMF was added to it and allowed to vortex in the peptide synthesizer at room temperature for 10 min. The resulting filtrate was collected to the same tube and the deprotected resin was washed with DMF (4 x 1.75 ml).

Sample preparation for UV analysis – From the collected "deprotection solution" above, 100 μL was transferred in to an eppendorf tube and it was diluted with 900 μl of DMF. The resulting diluted sample was used to get the absorbance at 301 nm versus a DMF blank using a UV spectrometer with 1 cm cuvette.

5.5.2 Experimental Procedures and Spectroscopic Data



5.5.A' *5.5.A*

SPPS of Linear Peptide 5.5.A. The standard Fmoc-SPPS protocol described was used to synthesize peptide 5.5.A on pre-loaded Wang resin 5.5 A' (500 mg, 0.15 mmol). The peptide was capped before the last coupling step. To analyze the peptide, 10 mg of the resin was subjected 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 3 hours. The resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O

gradient for 30 minutes. Final loading of the resin: 0.10 mmol/g. m/z ES calc'd for peptide 5.5.A [($C_{77}H_{115}N_{17}O_{25}S_8$ + 1]$^+$: 1935.34; observed: 1936.50. m/z ES calc'd for [($C_{77}H_{115}N_{17}O_{25}S_8$ + 2)/2]$^+$: 968.67; observed: 968.45.

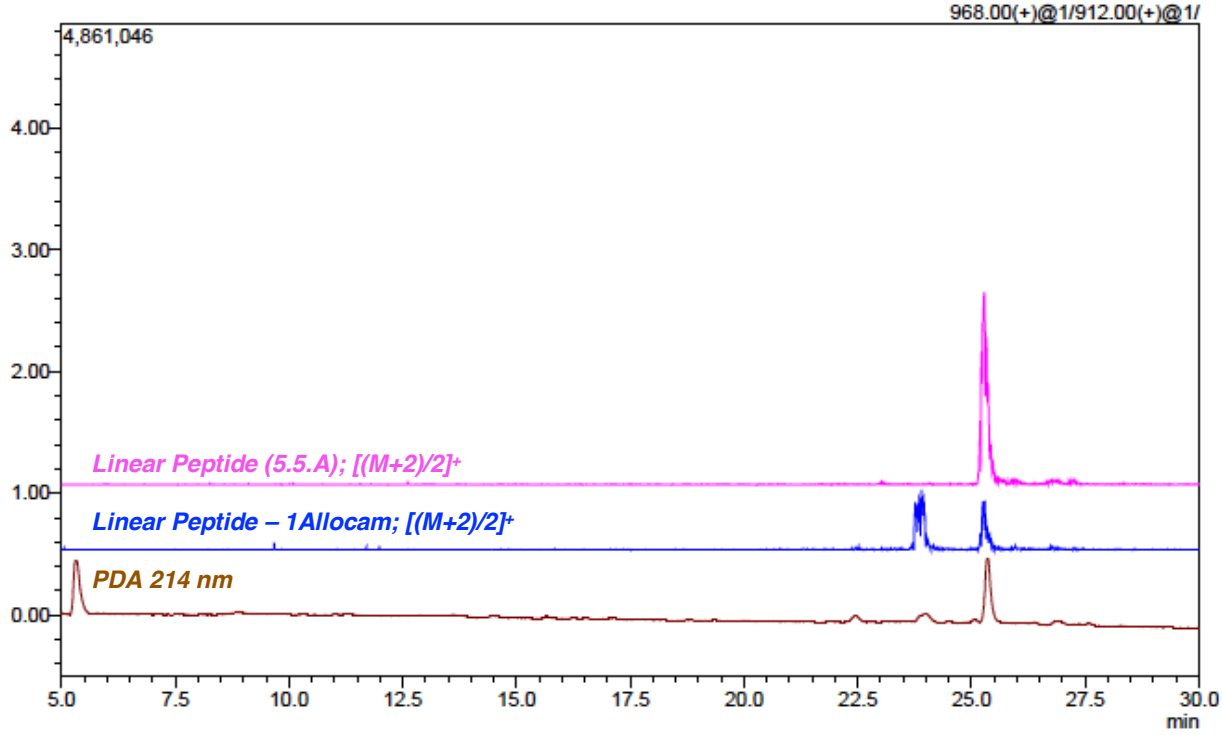


Figure 5.2. Analytical Column PDA for linear peptide **5.5.A**; Gradient: 10-50% MeCN/H_2O + 1% HCOOH over 30 min. Retention time 25.34 min

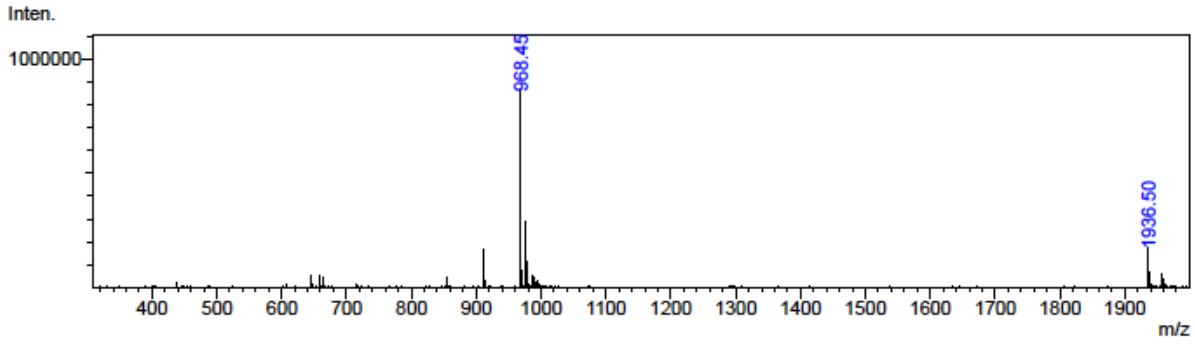
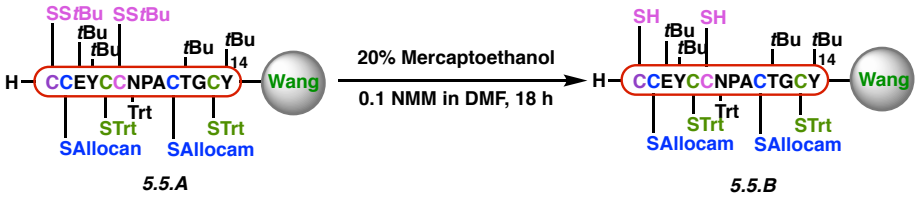


Figure 5.3. ES-MS spectrum for linear peptide **5.5.A** at retention time 25.34 min



5.5.A *5.5.B*

Synthesis of Reduced Peptide 5.5.B. The peptide **5.5.A** (200 mg, 60 μmol) was first swelled in DMF (3 mL). Then, a solution of 20% mercaptoethanol, 0.1 M NMM in DMF (5.0 mL) was added and agitated at room temperature for 18 hours. The reaction solution was filtered and the resin was washed with DMF (5 x 3 mL). To analyze the reaction 10 mg of the resulting resin was subjected to 300 μL of the cleavage mixture (95:2.5:2.5 TFA:TIPS:H_2O) for 1 hour. The resulting TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA and analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10–50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 5.5.B $[(C_{69}H_{99}N_{17}O_{25}S_6 + 1]^+$: 1759.01 observed: 1759.80. m/z ES calc'd for $[(C_{69}H_{99}N_{17}O_{25}S_6 + 2)/2]^+$: 880.50; observed: 880.20.

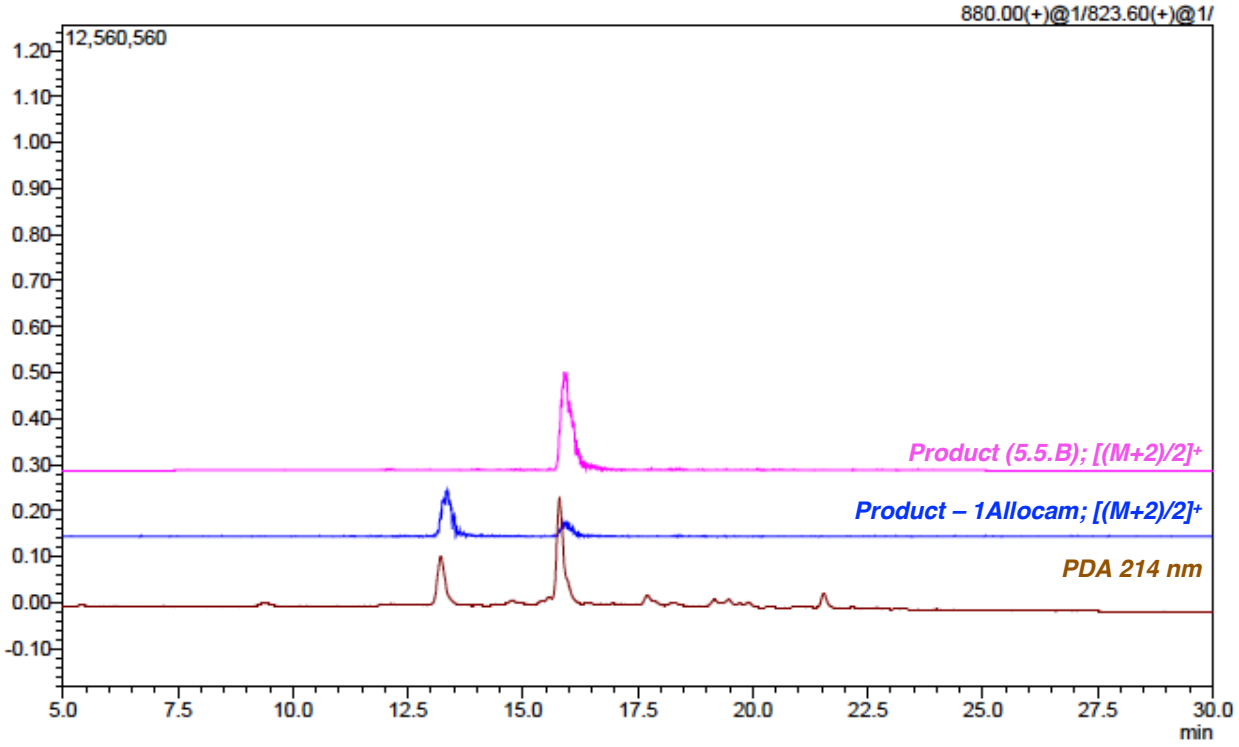
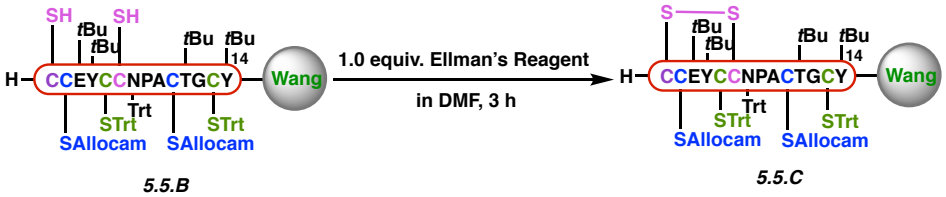


Figure 5.4. Analytical Column PDA for Scheme 5.4. Step 01



Synthesis of Mono Cyclic Peptide 5.5.C. The peptide **5.5.B** (200 mg, 60 μmol) was first swelled in DMF (3 mL). Then it was subjected to a solution of Ellman's Reagent (23.70 mg, 60 μmol) in DMF (3 mL) for 16 hours until the resin color changed from dark peach-orange to yellow. Finally, the reaction solution was filtered and the resin was washed with DMF (6 x 3 mL) and DCM (6 x 3 mL). To analyze the reaction 10 mg of the resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 3 hours. The resulting the TFA solutions were collected, concentrated under a constant stream of

air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-50% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 5.5.C $[(C_{69}H_{97}N_{17}O_{25}S_6 + 2)/2]^+$: 879.49; observed: 879.30.

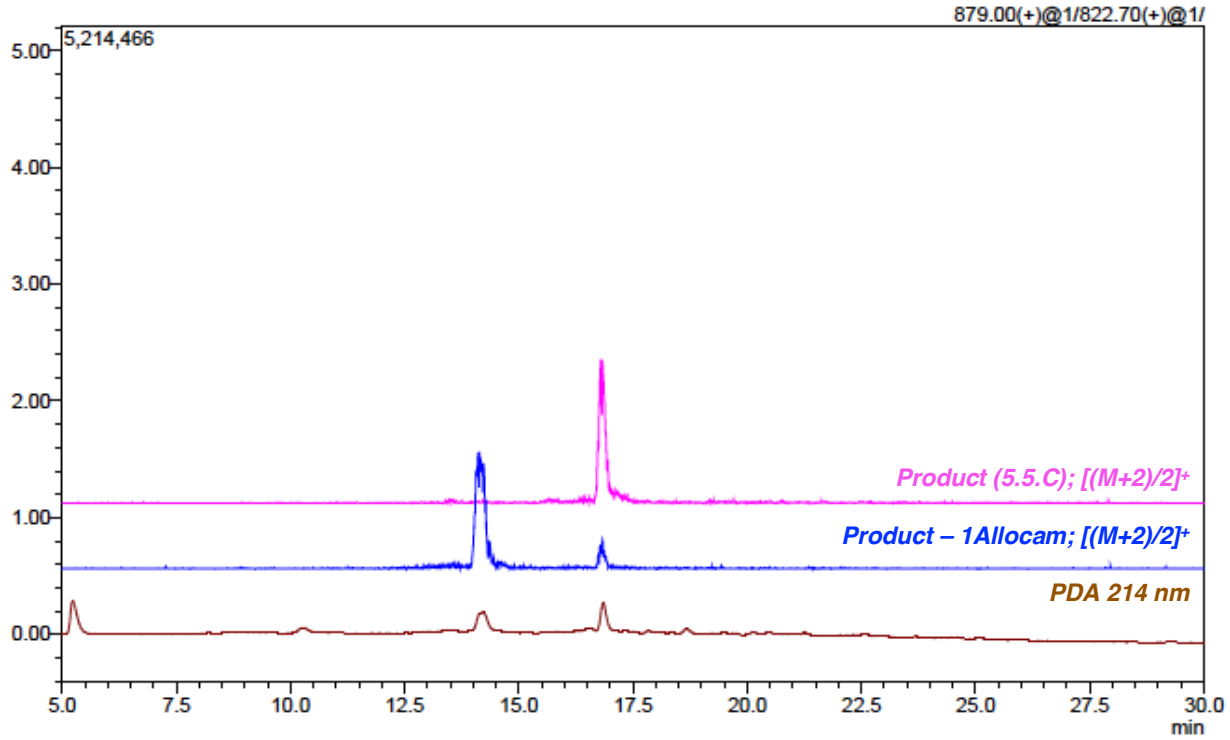
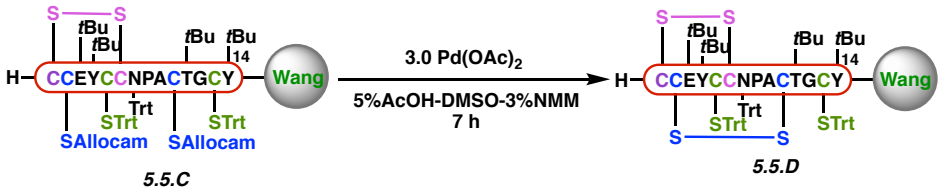


Figure 5.5. Analytical Column PDA for Scheme 5.4. Step 02



Synthesis of Bicyclic Peptide 5.5.D. First, the resin **5.5.C** (100 mg, 30 μmol) was swelled in DMF (3 mL). A solution of $Pd(OAc)_2$ (20.2 mg, 90 μmol) in 5% AcOH/DMSO (3.75 mL) solution was added, and then the NMM (113 μL) was added. The cartridge/vial was capped on the top, attached to a magnetic stir bar retriever using a rubber band, and agitated for 7 hours. The reaction was filtered, and the resin was washed successively with with DMF (3 x 2 min), CH_2Cl_2 (3 x 2 min), 0.2 M sodium diethyldithiocarbamate in DMF (3 x 30 min), MeOH (3 x 1 min), DMF (5 x 1 min) and CH_2Cl_2 (3 x 1 min). *Note: resin changes from dark orange/brownish to yellowish/tan. To analyze the reaction 10 mg of the resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 3 hours. The resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 10-40% MeCN/H_2O gradient for 30 minutes. m/z ES calc'd for peptide 5.5.D $[C_{59}H_{81}N_{15}O_{21}S_6 + 1]^+$: 1528.74; observed: 1528.45. m/z ES calc'd for $[(C_{59}H_{81}N_{15}O_{21}S_6 + 2)/2]^+$: 765.37; observed: 765.20.

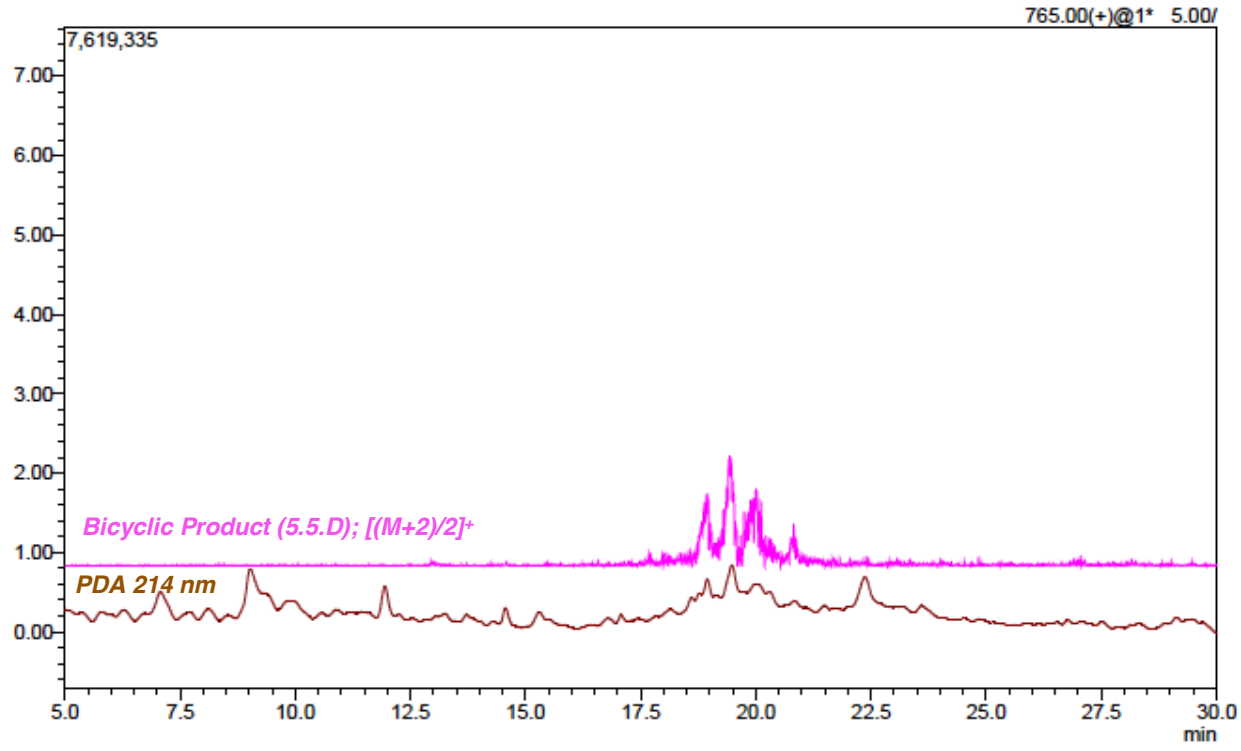
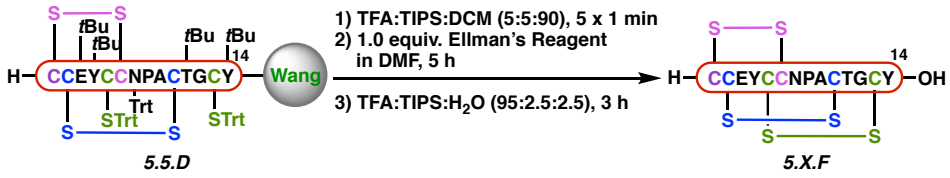


Figure 5.6 Analytical Column PDA for Scheme 5.4. Step 03



Synthesis of Linaclotide 5.5.F. The resin **5.5.E** (90 mg, 27 μmol) was first swelled in DMF (3 mL). Then, a freshly prepared solution (500 μL) of TFA:TIPS:DCM (5:5:90) was added and the reaction mixture was agitated at room temperature for 5 x 1 min. About 1 – 2 drops of more TIPS were added during the reaction until the orange-yellow color disappeared in the reaction mixture. The resulting TFA solution was filtered and the resin was washed with DCM (3 x 5 mL). After that, the resin was subjected to Ellman's Reagent (10.8 mg, 27 μmol) in DMF (2 mL) for 5 hours until the resin color changed from peach to yellow. Finally, the reaction solution was filtered and the resin was washed with DMF (6

x 3 mL) and DCM (6 x 3 mL). In order to confirmed the total Trt group removal, the resulting peptide was retreated with a solution (500 μL) of TFA:TIPS:DCM (5:5:90) for 5 x 1 min and re-subjected to the same amount of Ellman's Reagent in DMF. No color change was observed, the solution of Ellman's reagent added was filtered and the resin was washed with DMF (6 x 3 mL) and DCM (6 x 3 mL). The resulting resin was subjected to 300 μL of the cleavage mixture TFA:TIPS:H_2O (95:2.5.2.5) for 1 hour. The resulting the TFA solutions were collected, concentrated under a constant stream of air, crashed out with cold diethyl ether, centrifuged, and decanted to yield the crude peptide. The resulting pellet was dissolved in 10% MeCN:Water in presence of 0.05%TFA. This solution was analyzed via RP-HPLC-MS using an analytical Thermo Scientific Hypersil Gold C8 column at a flow rate of 1 mL/min with 15-45% MeCN/H_2O gradient for 20 minutes. m/z ES calc'd for peptide 5.5.E $[(C_{59}H_{79}N_{15}O_{21}S_6 + 2)/2]^+$: 764.37; observed: 764.20.

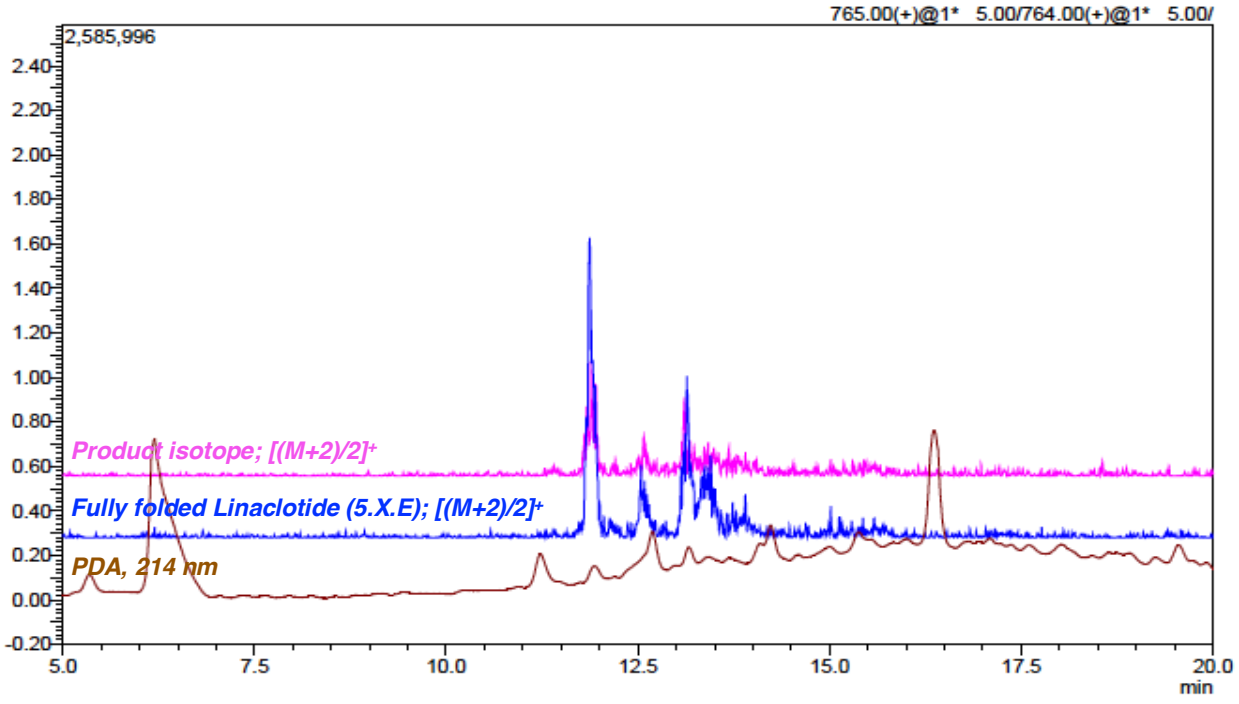


Figure 5.7 Analytical Column PDA for crude Linaclotide **5.5.E**; Gradient: 15-45% MeCN/H_2O + 1% HCOOH over 20 min. Retention time 11.89 min

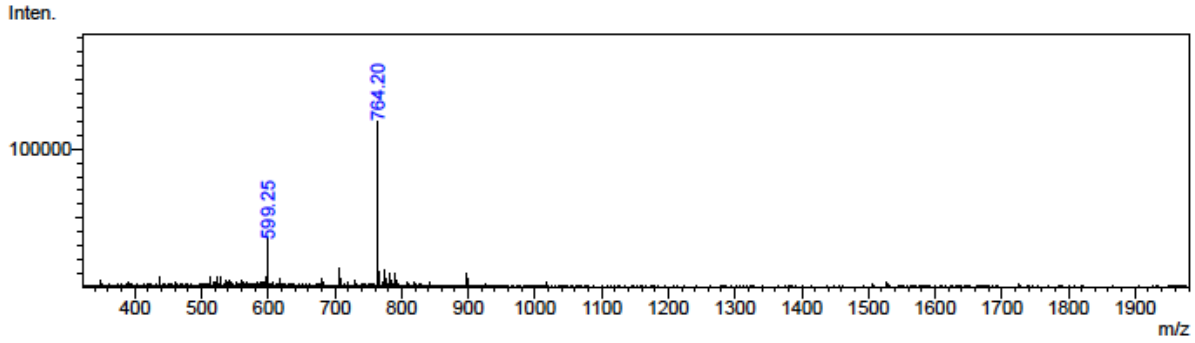


Figure 5.8 ES-MS spectrum for linear peptide **5.5.E** at retention time 11.89 min

CHAPTER 6: SUMMARY

Peptide – based pharmaceuticals are becoming increasingly important over traditional small molecule pharmaceuticals. This is mainly because of the greater specificity, potency and lower toxicity profile of the disulfide-rich mini proteins. Disulfide linkages play an important role to improve the thermal, chemical, and enzymatic stability of these polypeptides. The synthesis of disulfide – containing mini-peptides become a challenge in field of peptide chemistry, and hence, there is a demand for broadly applicable methods to construct correct disulfide connectivity in bioactive peptides. This dissertation detailed development of efficient deprotection methods for Allocam group and utility of Allocam as cysteine protecting group to form on-resin disulfide bonds in more complex peptides. In addition, oxidation potential of Ellman's reagent to access on-resin disulfide bonds was evaluated along with its interesting colorimetric behavior.

First, little-used Allocam group was studied for better π-allyl deprotection conditions to overcome limitations which prevent its general use in the field. A one-pot approach with Pd-mediated deprotection-oxidation was developed to form disulfide bonds on resin. As an on-resin method, it minimizes the number of time consuming purification steps and the reaction can be done with bench-stable reagents. The utility of the method was evaluated by solid phase synthesis and folding of the carboxy-oxytocin. However, detailed investigations into the mechanism of Pd-DMSO mediated simultaneous Allocam removal and oxidation needed to be done.

In addition to oxytocin, α4/7-conotoxin LvIA was synthesized to demonstrated the applicability of novel Pd-DMSO mediated simultaneous Allocam removal and oxidation

condition to access more complex disulfide – rich peptides. Two routes were introduced based on position of the Allocam protected cysteines and selection of compatible other cysteine protecting groups for each of the synthetic routes. In addition to Pd-DMSO based simultaneous on-resin Allocam removal and oxidation condition, both I_2 oxidation and NCS oxidation can be used in on-resin Allocam removal and oxidation. Selection of the best method is highly depend on design of synthetic protocol.

The potential of Ellman's reagent as a colorimetric and mild oxidizing agent to form on-resin disulfide bonds in peptides was evaluated. The reaction procedure is simple, no complex time consuming reaction set up is needed and the reaction can be monitored easily by observing the color change of the resin. The colorimetric behavior of Ellman's reagent can be seen both in DMF and NMP. Ellman's reagent – mediated disulfide bond formation in DMF is relatively slower while it shows faster reaction rates in NMP. The insight of colorimetric behavior of Ellman's reagent in disulfide bond formation process needed to be further investigated.

The new Allocam cleavage - oxidation method and Ellman's reagent - mediated on-resin disulfide bond formation method was carefully combined in a synthetic protocol to access linaclotide, which contains three disulfide bonds. For best of our knowledge, this is the first on-resin synthesis of three disulfide linkages.

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ABSTRACT

PROGRESS TOWARD CONTROLLED DISULFIDE FORMATION TO ACCESS NEUROACTIVE CONOTOXINS

by

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May 2018

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Conotoxins are mini proteins isolated from marine snails of the *Conus* family. They have shown activity in the selective inhibition of various ion channels. Therefore, they present an opportunity as possible therapy for a myriad of neurological ailments. The synthesis of conotoxins would enable both biological studies and evaluation of these structures as lead targets for therapeutics. However, establishing a reliable method to consistently folding disulfide rich proteins, including conotoxin, into their native structure has been challenging over few decades. In this research project, it is shown the progress towards a mild, efficient, and broadly applicable methods to generate the desired pattern of disulfide linkages in synthetic polypeptides.

π-allyl chemistry mediated deprotection conditions for Allocam group was optimized to overcome the limitations which prevent its general use in the field. A one-pot approach with Pd-mediated deprotection-oxidation was developed to form disulfide bonds on resin. The utility of the method was evaluated by solid phase synthesis and folding of the carboxy-oxytocin. In addition, α4/7-conotoxin LvIA was synthesized to demonstrate the

applicability of novel Pd-DMSO mediated simultaneous Allocam removal and oxidation condition to access more complex disulfide – rich peptides. Two routes were introduced based on position of the Allocam protected cysteines and selection of compatible other cysteine protecting groups for each of the synthetic routes.

The potential of Ellman's reagent as a colorimetric and mild oxidizing agent to form on-resin disulfide bonds in peptides was evaluated. The reaction procedure is simple, no complex time consuming reaction set up is needed and the reaction can be monitored easily by observing the color change of the resin. The colorimetric behavior of Ellman's reagent in different solvents were examined. Ellman's reagent – mediated disulfide bond formation in DMF is relatively slower while it shows faster reaction rates in NMP. This new colorimetric method was utilized in the synthesis of α4/7-conotoxin LvIA and α-conotoxin ImI and obtained excellent overall yields.

For the first time, a regioselective on-resin synthesis of three disulfide linkages was achieved by synthesizing linaclotide with careful orthogonal combination of the new Allocam cleavage - oxidation method and Ellman's reagent - mediated on-resin disulfide bond formation method.

AUTOBIOGRAPHICAL STATEMENT

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PUBLICATIONS

1. Arbour, C. A.; **Kondasinghe, T. D**.; Saraha, H. Y.; Vorlicek, T. L.; Stockdill, J. L.* Epimerization-Free Access to C-Terminal Cysteine Peptide Acids, Carboxamides, Secondary Amides, and Esters via Complimentary Strategies. *Chem. Sci.* **2018**, *9, 350-355*

2. **Kondasinghe, T. D.;‡** Saraha, H. Y.;‡ Odeesho S. B.; Stockdill, J. L. * Direct Palladium-Mediated On-Resin Disulfide Formation from Allocam Protected Peptides. *Org. Biomol. Chem.*, **2017**, 15, 2914 – 2918