A novel approach toward branched peptide synthesis

by

Chia Yun Yu

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Graduate Department of Chemistry
University of Toronto

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Abstract

Branched peptides are peptides featuring side chain-linked linear peptides which branch off from the main chain. Branched peptides have previously been employed in the development of small molecular carriers for drug delivery systems as well as in biodegradable materials and artificial enzymes. In our own efforts toward the synthesis of branched peptides, we have optimized reaction conditions adopted from Native Chemical Ligation (NCL) to prepare ligated peptides in a convergent synthesis. Herein we present the synthesis of *N*-terminal aziridine and *C*-terminal thioacid-functionalized peptides, followed by their ligation and subsequent transthioesterification with a C-terminal thioester to yield a tri-branched pentadecapeptide.

Acknowledgments

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1 Introduction

Presently, peptides are evaluated in clinical trials as treatments for various cancers and diseases, yet these linear peptide based drugs are limited as most of therapeutic agents due to their short half-life, fast proteolytic cleavage, and low oral bioavailability[3]. An additional limiting factor of linear peptides is that they tend to intramolecularly aggregate resulted in an unpredictable protein folding which impedes the design and synthesis of novel proteins[4].

Considering these limitations, branched peptides have made great strides in their application as small molecules, drug delivery carriers, and bioactive materials[5]. Stewart et al. were able to synthesize branched peptides that demonstrated enzymatic activity[6]. The four amphiphlic helical peptides were ligated at the branching point at the *C*-terminus and it was designed that the *N*-terminus contained the enzymatic chymotrypsin (Figure 1). Hartgerink et al. showed that branched peptide-amphiphile self-assembled to form a fibrous nanostructure , which can be widely applied in nanochemistry and material science[7].

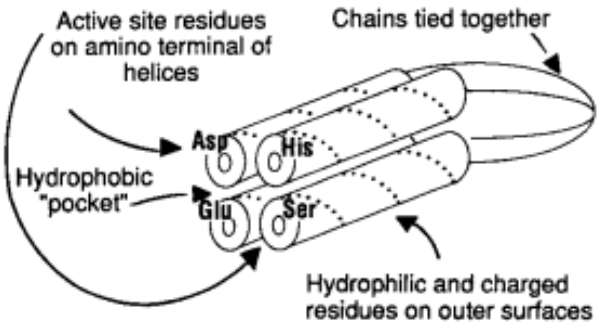


Figure 1 Chemical structure of peptide having chymotrypsin-like esterase activity[6]

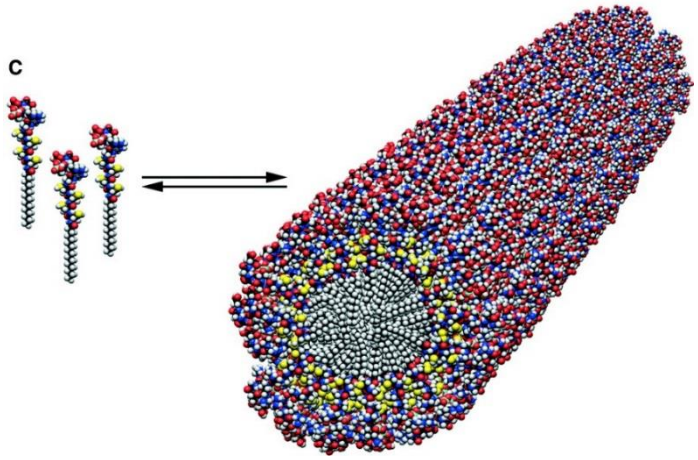
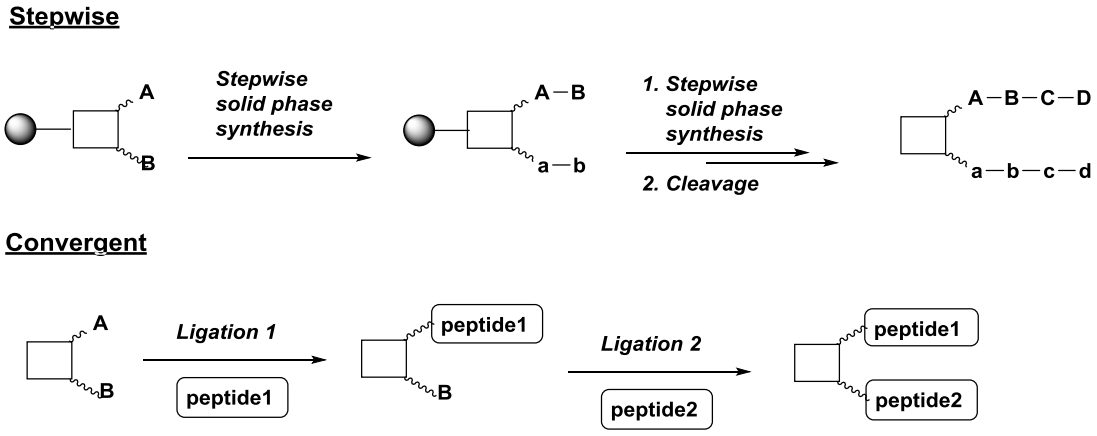


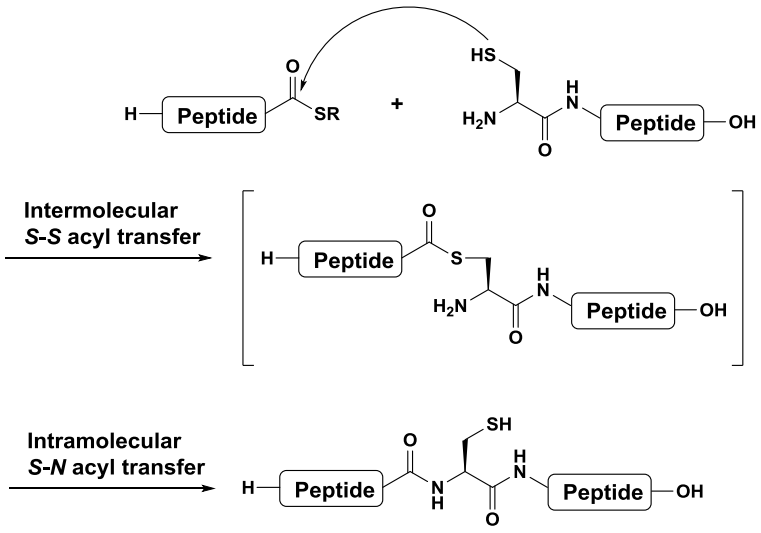
Figure 2 Molecular model of self-assembly peptide amphiphile molecules into a cylindrical micelle[7]

Currently, there are two widely accepted synthetic strategies to prepare branched peptides. The first route is through step-wise addition of amino acids onto the branching point of another peptide. This requires solid-phase synthesis and side chain group protection. Because the amino acids are added sequentially and the peptide is assembled one amino acid at a time, it is considered a time-consuming and inefficient process. The second method is through chemical ligation in a convergent synthesis. This can be done either by ligating peptide fragments onto the side chain of the parent peptides[8], or by a series of orthogonal ligations at the central core[9]. Although convergent syntheses increases the efficiency of synthesizing long peptides, the lack of selectivity and the difficulties associated with isolating desired products are still major problems that need to be overcome (Scheme 1).



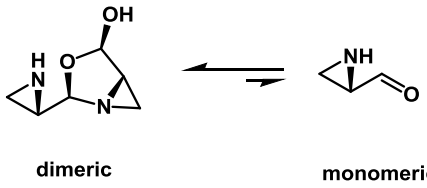
Scheme 1 Step-wise and convergent synthesis pathway toward branched peptide

Native Chemical Ligation (NCL), developed by Kent et al., has become the most practical method for chemically ligating unprotected peptide segments in aqueous solution[10]. A small protein containing up to 300 residues can be readily achieved by this novel chemical synthesis[10]. NCL requires an *N*-terminal cysteinyl peptide to chemoselectively attack a *C*-terminal thioester peptide to undergo transthioesterification, followed by *S*-to-*N* acyl transfer to yield the final ligated peptide product with a native amide bond at the ligation site[11] (Scheme 2).



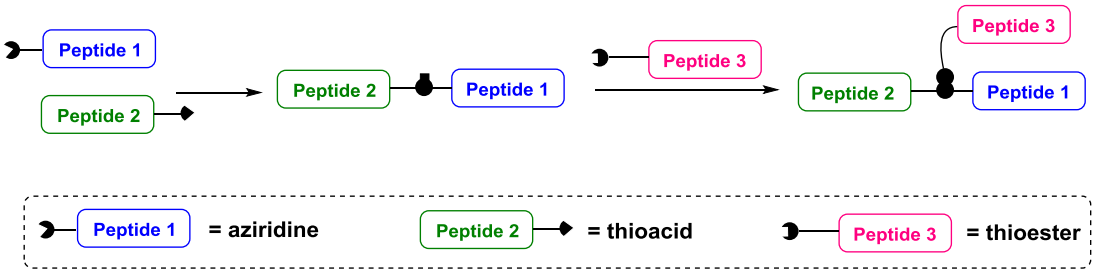
Scheme 2 Reaction mechanism of NCL[11]

In 2010, Assem et al.[12] reported a peptidomimetic ligation between a *C*-terminal thioacid and an *N*-terminal aziridine peptide, resulting in a reduced amide bond at the branching site. To prepare the *N*-terminal aziridine peptide, aziridine aldehydes were used. This class of molecule contains both unprotected aziridine and aldehyde functionalities in the same molecule, and was found to be kinetically stable in its dimeric form[13] (Scheme 3). The aziridine aldehydes were reductively aminated with the *N*-terminus of a linear peptide to generate the *N*-terminal aziridine peptide. We found that thioacids can attack only at the least sterically hindered *β*-position of the aziridine ring to yield a regioselective product[12]. In addition, the incorporated reduced amide bond is presented in a wide range of protease inhibitors, which displayed high affinity to their target but also resistance to protease degradation[14].



Scheme 3 Aziridine aldehydes in dimeric/monomeric equilibrium

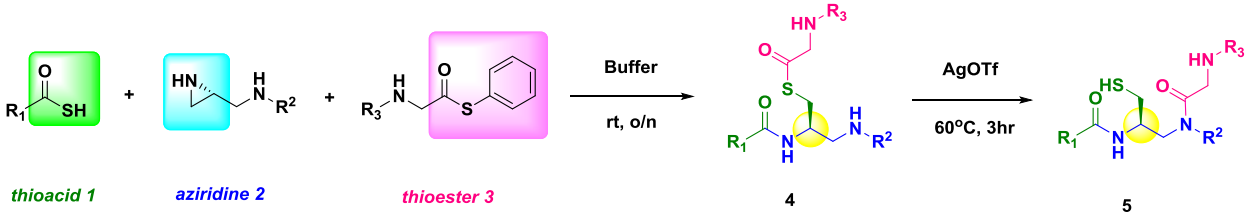
This discovery has led us, Dr. Naila Assem and myself to diversify branched peptide synthesis in a convergent fashion by taking advantage of a newly formed cysteine at the branching point that can serve as a handle for a second ligation to take place. In order to achieve this goal, a thioester peptide substrate was subjected to the ligation strategy to make our ultimate product – a tri-branched peptide (Scheme 4). No protecting groups are required for this synthesis as the second stage of the ligation will only take place after the thiol handle has formed upon the completion of the first ligation step. In addition, apart from ligating peptide fragments at the terminal ends of the peptides, we are able to provide a unique pathway to ligate peptides at the center of the peptide. To our gratification, this method has been successfully performed in a single amino acid ligation to yield a tri-branched peptide. *Therefore, my current goal is to extend the reactants to a pentapeptide system and further screen the substrate scope of the final macro-branched peptides in a biological assay.*



Scheme 4 Schematic pathway of tri-branched peptide synthesis

A pentapeptide system requires three components: thioacid-, aziridine-, and thioester-containing peptides (Scheme 5). The general strategy and goal was to conduct the reaction in phosphate buffer – 6M guanidine hydrochloride buffer containing 0.2M sodium phosphate and 0.05M tris 2-carboxyethyl phosphine hydrochloride (TCEP) under neutral conditions. The reason for phosphate buffer to be used in NCL is because it accelerates a ligation reaction. Guanidine hydrochloride prevents peptide aggregation, sodium phosphate maintains a neutral condition during the course

of the reaction, and TCEP is a reducing agent that prevents disulfide bond formation between the free thiols[15]. The peptidomimetic ligation can be monitored by HPLC/MS after 24 hours of stirring and followed by addition of the thioester in a one-pot two-step reaction. The tri-branched peptide can then be purified by preparative (prep) HPLC/MS. Compound **4,** dissolved in 0.2M NaOAc buffer and treated with silver triflate, can be added to accelerate the *S*-to-*N* acyl transfer step at 60°C under sonication. The final tri-branched peptide product **5** can then be isolated by preparative HPLC/MS. (Scheme 5)



Scheme 5 Schematic synthesis of tri-branched peptide **5**

Pentapeptides will be synthesized on solid phase using 2-chlorotrityl chloride resin as all the side chains need to be protected. These crude peptides will then be transformed to the corresponding reagent substrates.

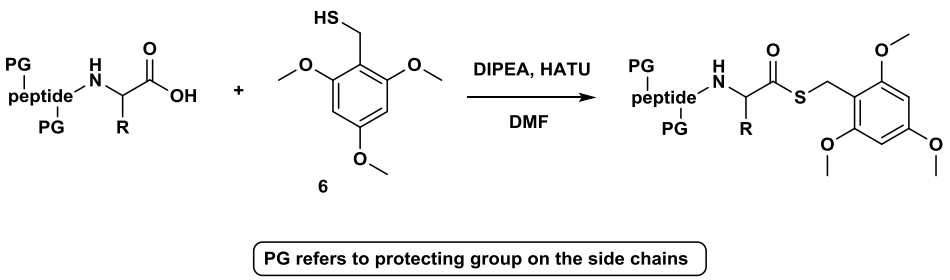
2 Results and Discussion

2.1 Ligation of long chain protected peptides in buffer

As compared to prior syntheses for preparing reactants in the single amino acid ligation[12], longer chain peptide substrates requires modification in the procedure of coupling functional groups onto the peptides. Additionally, all the protecting groups on the peptide reactants were removed as a requirement for conducting NCL. As a result, it can be treated as a different reaction system.

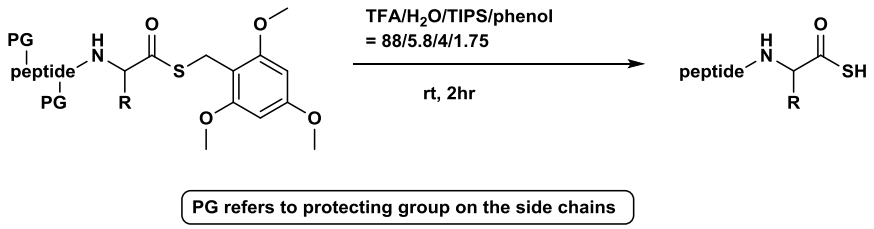
2.1.1 Long chain unprotected thioacid peptides

In order to make a thioacid-terminated peptide we needed to modify the standard preparation procedure. Instead of treating the peptide candidate with 1,1'-carbonyldiimidazole (CDI) and sodium sulfide (Na_2S) as in the standard procedure[12], a longer peptide (>2 residues) will be loaded with 2,4,6-trimethyoxyphenyl-methanethiol (TmobSH) **6**, prepared in two steps[16].



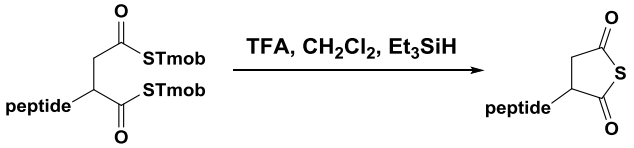
Scheme 6 Synthesis of Tmob protected thioester and its corresponding deprotection to yield the thioacid

TmobSH was coupled to peptide substrates using the coupling reagent HATU to afford the corresponding thioester. Two different pathways were conceived to remove the Tmob on the thioester to give the desired thioacid substrates. The first route was by purifying the resulting thioester before deprotecting with a TFA cocktail to give the desired thioacid substrate. However, purification by using both flash silica gel column chromatography (5% MeOH in DCM) and reverse-phase preparative HPLC/MS (50-90% MeCN in water) did not yield clean protected Tmob-thioester substrates. Therefore, we decided to reverse the process as we did in the first route, by charging the Tmob thioester with TFA cocktail before the purification step. We first searched for the best deprotection method to yield a desired deprotected thioacid peptide by subjecting the protected Tmob thioester peptide into a series of TFA based-cocktails (Table 1). The second cleavage cocktail solution worked ideally but the unbearable odor of ethanedithiol was undesirable[17]. The third method could only work for a linear dual end of terminal Tmob-thioester that cyclized right after the deprotection to form thiolactone (Scheme 7)[18]. Lastly, we tested the first method again by carefully degassing the TFA cocktail (30.0 mg of phenol, 0.1 ml of deionized water, 0.07 ml of triisopropylsilane, and 1.5 ml TFA) for two hours[19]. A thoroughly degassed cocktail solution was essential to avoid oxidation of the resulting thioacid. Once the deprotection reached completion, the reaction mixture was diluted with water/acetonitrile at -78°C to minimize exposure time to the atmosphere. The desired thioacid was obtained after lyophilization and was successfully detected by HPLC/MS. Taking advantage of the TmobSH coupling method, the long chain unprotected thioacid peptide was successfully prepared for use in the ligation reaction.



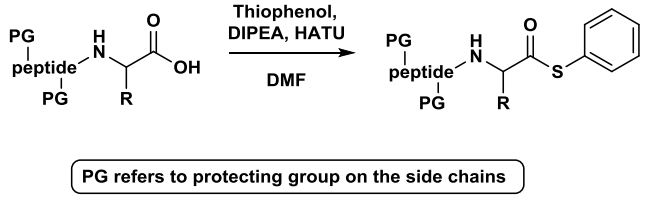
Method	Conditions	Reaction time (hr)
1	TFA/phenol/H_2O/TIPS = 88/1.7/5.8/4	2
2	TFA/1,2-Ethanedithiol/H_2O/TIPS = 95/2.5/2.5/2.5	2
3	TFA/CH_2Cl_2/Et_3SiH = 4/5/1	2

Table 1 Different approach toward deprotecting protected Tmob-thioester through various cocktail TFAs



Scheme 7 TFA deprotection of dual end Tmob protected thioester

2.1.2 Long chain protected thioester peptide

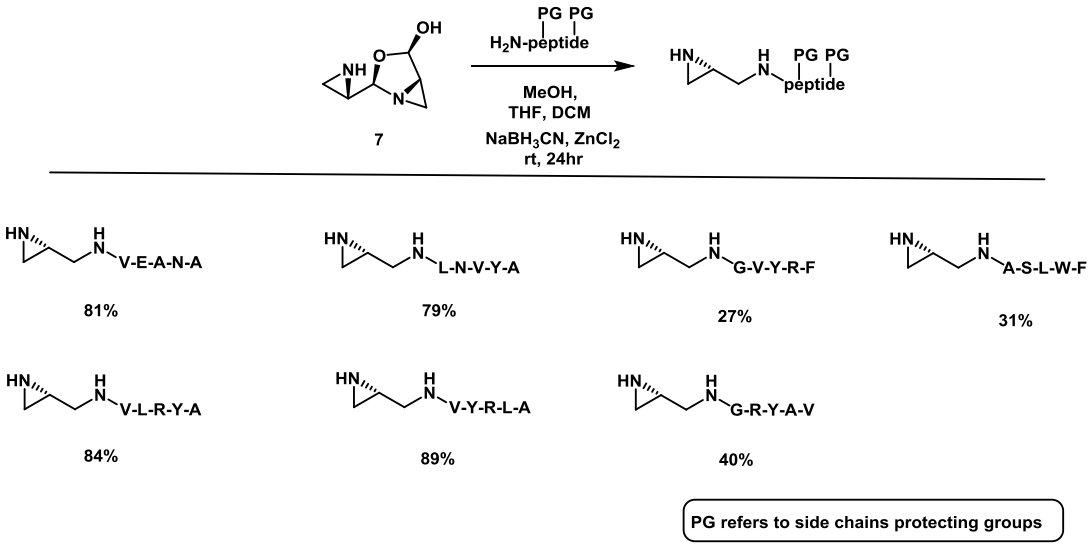


Scheme 8 Synthesis of phenyl thioester fully protected peptide and its corresponding deprotection to yield thioester substrate

According to past experimental results obtained from Dr. Naila Assem, an intermediate phenyl thioester is essential for transthioesterification process to occur at the second branching point, which is the thiol handle at cysteine[12]. Therefore, the thioester peptide substrate was prepared by adding thiophenol onto the *C*-terminus of the fully protected peptide (Scheme 8). In order to obtain

a clean protected thioester peptide substrate, we attempted two different pathways. Our first approach was to purify the protected phenyl thioester peptide by using silica gel chromatography that unfortunately resulted in inseparable impurities. As an alternative to chromatography, we triturated the protected phenyl thioester peptide with 1:1 water/brine. To our surprise, this water/brine reaction mixture gave two separable layers when we expected a miscible solution. After consulting with Dr. Jennifer Hickey, a small amount of isopropyl alcohol was added to yield a homogenous solution. The resulting solution was then subjected to prep HPLC/MS to obtain a clean protected phenyl thioester peptide.

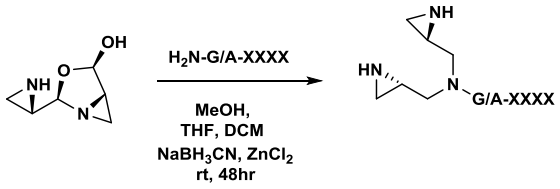
2.1.3 Long chain protected aziridine peptide



Scheme 9 Synthesis of *N*-terminal aziridine peptide and corresponding substrate scopes

The aziridine aldehyde precursor for *N*-terminal aziridine peptides was prepared by literature method[20]. This *N*-terminal aziridine peptide is one of the key components involved in the ligation reaction as it differs from the typical NCL. Our unique *N*-terminal aziridine peptide replaces a thioester susbtrate in the first stage of ligation. To prepare this peptide, an (2*R*,4*R*,5*S*)-2-((*S*)-aziridin-2-yl)-3-oxa-1-azabicyclo[3.1.0]hexan-4-ol (unsubstituted dimeric aziridine aldehyde) was reductively aminated with different peptide substrates to yield the desired aziridine peptide substrates (Scheme 9). Reductive amination using sodium cyanoborohydride was clean and required no purification. Zinc (II) chloride acts as the Lewis acid and is required for iminium ion formation. However, in contrast with preparing the single amino acid aziridine substrate (aziridine amino anilide), the same work-up procedure did not apply to the longer *N*-terminal aziridine

peptide as no product could be observed by HPLC/MS. Since reductive amination was relatively clean for the aziridine-peptide as compared to the thioester- and thioacid-peptide preparation, we used the crude product following HPLC/MS characterization and without purification for direct use in the ligation reaction. Another concern was that the reductive amination was substrate-dependent. This worry was confirmed after observing a wide range of product yields for different *N*-terminus amino acids on the peptide substrate following reductive amination with aziridine aldehyde. We first chose valine and leucine as our *N*-terminal amino acid in the peptide chain and after monitoring the reductive amination reaction by HPLC/MS, clean *N*-terminal aziridine peptide was exclusively observed. However, with the less sterically hindered amino acids at the *N*-terminus, such as alanine and glycine, we barely observed any consumption of the starting material over the course of the reaction. On the other hand, as we extended the reaction times to approximately 48 hours, an overalkylation was detected (Scheme 10). Therefore, we can conclude that the best *N*-terminated amino acid for our aziridine peptide substrate should contain a bulky side chain.



Scheme 10 Overalkylation of reductive amination

2.1.4 Long chain protected peptide ligation

Table 2 lists the functionalized pentapeptides that have been prepared using the previously mentioned methodologies for our ligation reactions. Phosphate buffer was used as the reaction medium for the long chain peptide systems as it worked smoothly in previous single amino acid ligations.

Thioacid 8	Aziridine 9
Ala-Ser-Leu-Trp-Ala-SH **8a**	Az-Val-Glu(tBu)-Ala-Asn(Trt)-Ala **9a** Az-Leu-Asn(Trt)-Val-Tyr(tBu)-Ala **9b**

Table 2 List of protected/unprotected pentapeptide substrates participated in the ligation reaction

We first started with ligating **8a** and **9a**. Unfortunately, there was no starting materials consumption nor product formation detected by HPLC/MS. Next, we tried peptidomimetic ligation between **8a** and **9b**, which afforded only 4-5% yield of product at an extended reaction time (48 hours). The majority of the reagents still remained unreacted. During the course of both reactions, we observed two layers in the reaction solution and significant amount of precipitation in the bottom layer. In a standard NCL[11, 21], reactants must be fully dissolved in the buffer solution in order for the ligation to proceed. These peptide reactants should be globally deprotected so that they are hydrophilic enough to be soluble in aqueous media. As a result, the hydrophobicity of the protected aziridine peptide was likely prohibiting the reaction. Thus, we decided to remove the side chain protecting groups on the aziridine and thioester peptides to increase the solubility of each in the ligation reaction.

2.2 Ligation of long chain unprotected peptide ligation in buffer

In order to remove all the protecting groups on the reactants, protected aziridine and thioester peptides were treated with the same TFA cocktail that was used in the global deprotection for thioacid peptides, but no degassing process was needed. The resulting unprotected aziridine and thioester peptides required further purification by prep HPLC/MS and to our delight, both peptides were detected, purified, and collected by HPLC/MS.

With all three reagents successfully isolated, we commenced the ligation reaction by first reacting the thioacid peptides with the aziridine peptides in phosphate buffer. Ligation reactions were

monitored by HPLC/MS. Unfortunately, no di-branched peptide product was observed. We tested several batches of reaction, each containing different unprotected peptide reagents (Table 3), yet none of these reactions yielded product.

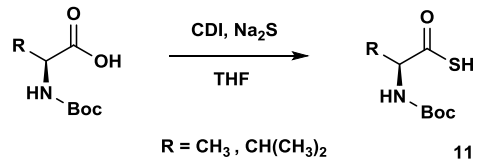
Thioacid 8	**Aziridine 9**	**Thioester 10**
Leu-Arg-Tyr-Gly-Ala-SH	Az-Gly-Arg-Tyr-Ala-Val	Val-Leu-Arg-Tyr-Gly-SPh
8b	**9c**	**10a**
Leu-Tyr-Arg-Gly-Ala-SH	Az-Ala-Ser-Leu-Trp-Phe	
8c	**9d**	

Table 3 List of unprotected pentapeptide substrates participated in the ligation reaction

2.3 Ligation of protected single amino acids in buffer

These unsuccessful outcomes led us to question whether phosphate buffer is the ideal medium for the long chain peptide system. In Assem et al., the peptidomimetic single amino acid ligation reactions were carried out in ethanol[12], an organic solvent. However, we wanted to pursue branched pentapeptide ligation in phosphate buffer, as used in NCL. Due to the lesser complexity of single amino acids involved in branched peptide synthesis, these simple peptides were chosen as model substrates in trial ligation reactions. We decided to run ligation reactions of single amino acids in phosphate buffer in order to confirm the reliability and feasibility of tri-branched peptide synthesis in the same media. The first three single amino acid reactants tested in the ligation reaction were boc-protected alanine thioacid **11**, aziridine-alanine anilide **13**, and boc-protected glycine thioester **16**.

2.3.1 Protected single amino thioacids



Scheme 11 Synthesis of single amino thioacids

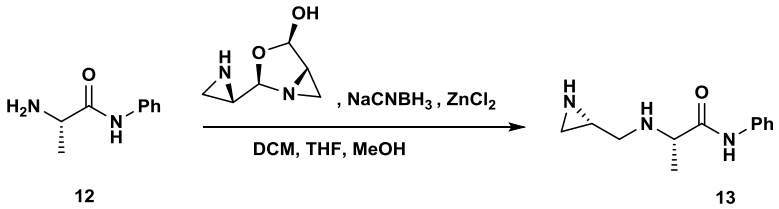
The desired Boc-protected thioacids were obtained by using 1,1'-carbonyldiimidazole (CDI) as a coupling reagent with sodium sulfide[12]. Once the reaction reached completion, an acidic work-up was performed under a nitrogen atmosphere at 0°C. 1M HCl was added dropwise until the solution reached pH 3. This careful addition of acid was crucial as a rapid change in pH resulted in undesired hydrolysis of the resulting thioacid product. After work-up, the Boc-thioacids required no further purification[12], and although the ^{1}H NMR spectrum of the Boc-Ala-SH showed traces of unidentified peaks, the impurities matched identically with the spectrum of Boc-Val-SH reported in literature[12].

2.3.2 Protected aziridine amino anilides

2.3.2.1 Aziridine analine anilide

The previously established syntheses of long chain aziridine peptides requires the reductive amination of aziridine aldehyde (1 eq.) and an amine-terminated peptide (1 eq.) using sodium cyanoborohydride (1.1 eq.) and zinc chloride (2.2 eq.). The only difference from the previous syntheses is that free amino acids or dipeptides require the *C*-terminus to be protected with anilide to prevent decomposition of the reductively aminated product[14]. Hence we used *C*-terminal anilide protected alanine (Ala-An) **12** as our protected amine in the reaction**,** which was prepared by a literature method[14]. Unfortunately, this single amino acid did not yield the desired result when subjected to reductive amination conditions. We then modified our reaction conditions as listed in Table 4. We first applied the standard reaction conditions, and after 24 hours, the majority of starting material remained unreacted according to HPLC/MS. Therefore, we added another equivalent of simple dimer, $NaCNBH_3$ and $ZnCl_2$ to the reaction mixture and stirred the mixture for another 24 hours. However, the original product mass had now disappeared by HPLC/MS, which indicated product decomposition (Table 4, #1). Alternatively, we first dissolved Ala-An and aziridine aldehyde in the solvent mixture of DCM, THF and MeOH (1/2.5/2.5) in the presence of $ZnCl_2$ and stirred for 10 minutes to pre-form the iminium intermediate, followed by the addition of the reducing agent. This mixture was allowed to stir for 24 hours, yet still no product was observed in the NMR spectrum (Table 4, #2). Additionally, based on the HPLC/MS results of the first and second reactions, we concluded that excess amounts of aziridine aldehyde led to overalkylation and gave double aziridine ring containing products.

Li et al. demonstrated that only 0.5 eq. of aziridine aldehyde was required in the reductive amination[14]. Therefore, we followed Li's procedure and increased the equivalency of the amine and reduced by half the amount of aziridine aldehyde to avoid overalkylation. Furthermore, since acidic conditions are ideal when using sodium cyanoborohydride in reductive amination, 20 eq. of acetic acid was added. In order to monitor the acidity during the course of the reaction, we used bromocresol green as an acid/base indicator. According to the HPLC/MS results, aziridine alanine analide was formed and the amount of overalkylation byproduct was dramatically reduced compared with our previous studies. However, the characteristic aziridine peaks – δ 1.23- 2.58 ppm – of our product still remained absent in the ^{1}H NMR spectra.



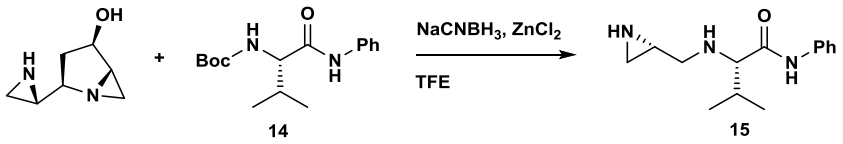
12 13

	Conditions (eq.)				Reaction times (hr)	Results
	Ala-An	Aziridine aldehyde	$NaCNBH_3$	$ZnCl_2$		
standard	1	1	1.1	2.2	24	No products
#1	1	1 + 0.5	1.1 + 0.5	2.2 + 1	72	No products
#2	1	1	1.1	2.2	24	No products
#3	1.2	0.5	1.5	2.2	2	No products
#4	1.5	0.5	1.5	2.2	2	No products

Table 4 Reaction conditions for synthesis of aziridine alanine anilide

Since no evidence of aziridine ring peaks were observed in ^{1}H NMR, we decided to synthesize aziridine valine anilide, the same compound that was reported by Li et al..

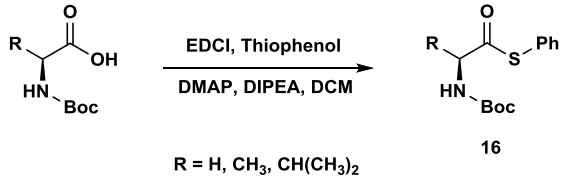
2.3.2.2 Aziridine valine anilide



Scheme 12 Synthesis of *C*-terminal anilide protected valine and corresponding valine aziridine anilide

We synthesized Az-Val-An by following the same procedure for Az-Ala-An, except that the reaction solvent mixture of DCM, THF and MeOH was replaced with TFE. Aziridine aldehyde is relatively more stable in TFE than MeOH[22], and the amino acid anilide dissolved adequately in TFE as well. A clean ^{1}H NMR spectrum was obtained for the product, Az-Val-An, and the characteristic aziridine ring peaks were correctly identified. This substrate was determined pure enough to undergo the ligation reaction. It was found that in both Li et al.[14] and Assem et al.[12] previous reports, that the aziridine anilide substrates with bulkier side chains on the amide bond gave improved yields of the final product. These observations corroborate with our own conclusions from the aziridine pentapeptide syntheses, where reductive amination does indeed work better with peptides containing a more hindered side chain at the *N*-terminal amino acid.

2.3.3 Protected single amino thioesters



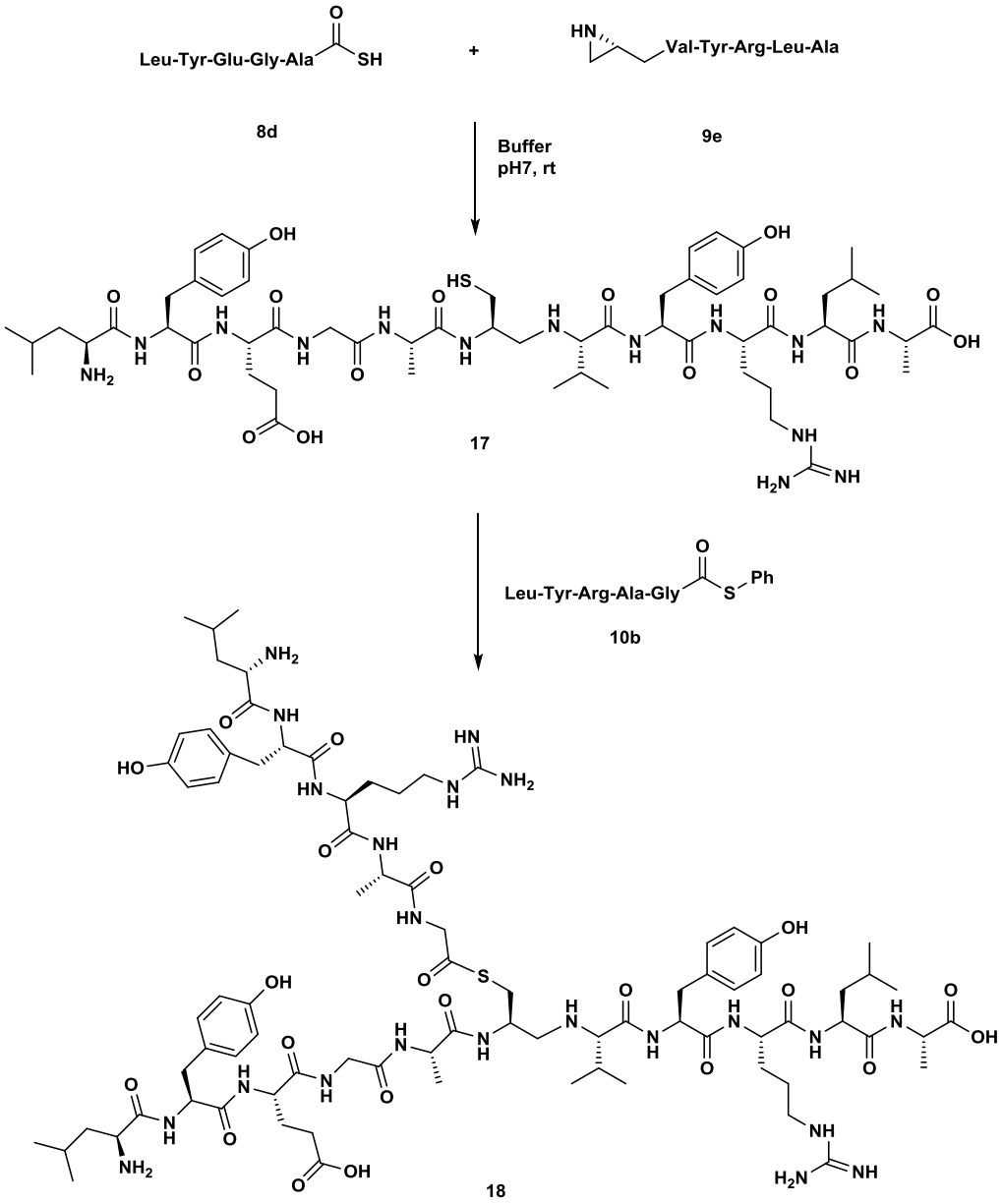
Scheme 13 Synthesis of protected single amino thioesters

The Boc-protected amino acid was first dissolved in DCM, cooled to 0°C, and EDCI was added to the solution followed by thiophenol, DMAP, and DIPEA. The reaction mixture was stirred at 0°C for three hours and slowly warmed up to room temperature and allowed to stir overnight. The addition of the coupling reagent (EDCI) to the reaction was done at 0°C to prevent the formation of unwanted side products. Once the reaction reached completion by HPLC/MS, the solvent was removed under reduced pressure and the residue was purified by flash silica gel column

chromatography (0-50% gradient of ethyl acetate in hexanes). Three different amino thioesters were made and determined pure by NMR.

2.4 Ligation of semi-protected long chain peptide in mixed solvents

Due to the time constraint of our project, we decided to focus on ligation of longer chain peptides. Shekhter et al.[23] have been using Leu-Tyr-Arg-Ala-Gly as their standard motif as this substrate works perfectly with all other substrates in their ligation studies. Knowing this, we have modified our peptide reagents adopting Shekhter's sequence to increase the accessibility of our own ligation reactions. In addition, side-chain unprotected peptides were still our reactants as their hydrophilicity remained an essential property for our ligation reaction[19, 24]. Therefore, all of our peptide reactants (**8d, 9e, 10b**) were deprotected and isolated by prep HPLC/MS. However, the product yield after prep HPLC/MS purification was low. This meant that when we tested different ligation conditions as listed in Table 5 using reactants **8d, 9e, 10b** (Scheme 14), it was on a relatively small scale and therefore difficult to properly asses.



Scheme 14 Modified peptide sequences in test ligation reactions

2.4.1 Ligation reaction conditions

Normally, a phosphate buffer in NCL is composed of 6M Guanidine hydrochloride, 0.2M sodium phosphate, 0.05M TCEP, and 0.02M 4-Mercaptophenylacetic acid (MPAA)[10]. Since the single amino acid ligation as performed previously was successful in phosphate buffer, we decided to continue with this medium. To eliminate any uncertainties that might hinder the reaction, we followed a buffer preparation method reported by Pollock et al.[15]. The entire preparation was done under an argon atmosphere. The phosphate buffer was kept at 4°C and used within three months of preparation. Pollock et al. suggested that buffer containing TCEP and MPAA should always be freshly made before use. Considering this, we prepared a stock solution of phosphate buffer (6M Guanidine hydrochloride, 0.2M sodium phosphate) and stored it in the fridge where TCEP and MPAA were only added to the buffer before conducting the ligation reaction. In addition, the pH should be kept at 7 for NCL. Acidic conditions disfavour ligation reactions hence, 0.2 M NaOH was added after the addition of peptide substrates to keep the pH at 7 if necessary.

	Ligation condition	**Results**
#1	3mM of each substrate + MPAA	No product
#2	3mM of each substrate + thiophenol added	No tri-branched peptide
#3	10.8 mM of each substrate	Observable di- and tri-branched peptides

Table 5 Ligation reaction conditions

MPAA is a thiol catalyst widely used in NCL that we decided to incorporate when we tried our ligation reactions with 3 mM each of thioacid and aziridine peptides in phosphate buffer (Table 5, #1). These reactions were monitored by HPLC/MS and stirred for 24 hours, yet no product was observed. Significant amounts of unreacted thioacid were observed and an unknown peak was also detected by HPLC/MS. At first, we thought this unidentified peak might be an MPAA-product adduct. However, the disulfide bond remained intact even after treating the reaction with an excess of the reducing agent TCEP. Therefore MPAA was determined to be unsuitable for our reaction.

We then tried using the same reaction conditions without any addition of MPAA (Table 5, #2). Only trace amounts of di-branched peptide formed after 24 hours. The reaction was monitored for

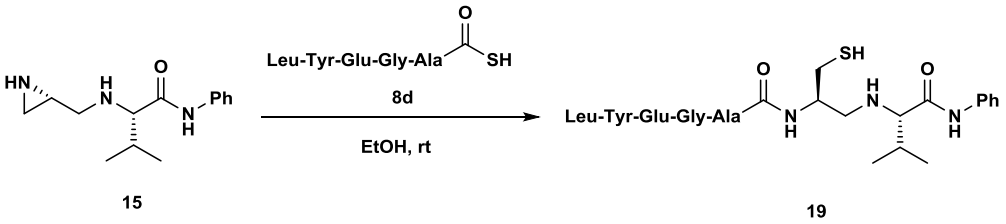
an additional 24 hours, yet unreacted thioacid continued to dominate the HPLC/MS spectrum. Alternatively, thiophenol was added to the reaction not only as a potential catalyst to transthioesterfication, but also as a reducing agent to prevent unwanted disulfide bond formation[25]. Unfortunately, this reaction turned into a cloudy suspension and no product was observed.

Lastly, instead of dissolving 3 mM of thioacid and the aziridine peptide in the ligation reaction as is the typical protocol for NCL, we tested 10.8 mM of reactants in phosphate buffer without adding MPAA or thiophenol (Table 5, #3). To our gratification, di-branched peptides were observed after 24 hours stirring and the tri-branched peptide was detected after the addition of a thioester peptide. Although we found that a concentration of 10.8 mM relative to peptide in the ligation reaction gave us promising results, this reaction was accompanied with significant amounts of unreacted starting material and low product yield. Due to the low yield of tri-branched peptide, it was impossible to conduct the acyl transfer in the next step. Since there was no NMR proof showing the existence of an aziridine ring and no mass of the aziridine peptide detected by HPLC/MS while running the ligation, we started to question the reliability of the aziridine peptide present in the ligation reaction altogether.

2.4.2 Ligation of mixed reactants

In order to run the ligation reaction, side chain protecting groups of each long peptide substrate were deprotected and purified by preparative HPLC/MS. However, the aziridine ring is very sensitive to the acidic TFA cocktail as well as the prep HPLC/MS conditions. Hydrolysis may easily occur resulting in the aziridine ring-opening.

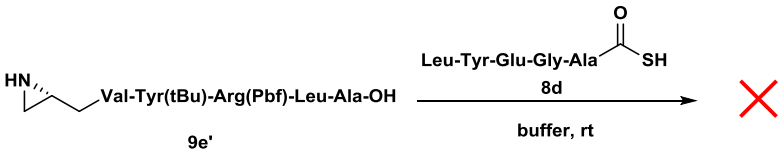
Since the previous ligation condition (Table5, #3) gave a low yield of product and large amounts of unreacted reactants, we decided to replace the long chain aziridine peptide with an aziridine amino anilide in a trial reaction. To reiterate, the single amino acid containing an aziridine ring has been successfully identified by ^{1}H NMR. Assem et al.[12] have reported that these single amino acids were ligated in ethanol, so we ran the reaction of long chain thioacid peptide **8d** with the Az-Val-An **15** in ethanol as well (Scheme 15). To our delight, we observed our product as the major peak by HPLC/MS.



Scheme 15 The ligation reaction between long chain thioacid peptide 8d and aziridine amino anilide **15**

The successful ligation reaction between the long chain thioacid peptide and the aziridine amino anilide has established that the thioacid SH nucleophile attacks the aziridine ring to form a thioacid di-branched peptide. Since the product yield of this reaction was high, the likely reason why the corresponding reaction on a long chain azirdine peptide was unsuccessful is likely because of a decomposed aziridine functionality. We therefore decided to use the protected crude aziridine pentapeptide directly from the reductive amination reaction in our branched peptide synthesis, as aziridine ring-opening could occur during purification.

This ligation reaction was done in the same phosphate buffer and reaction condition as listed in Table 5, #3. However, there was no product observed by HPLC/MS after reacting thioacid **8d** with the crude, protected aziridine peptide **9e'**, and the reaction resulted in a cloudy mixture (Scheme 16). Normally, the peptide reactants in NCL are free of side-chain protecting groups. Usually, the more polar the peptide, the better the solubility of the peptide is in phosphate buffer. The crude long chain aziridine peptide was completely side-chain protected, which increased the hydrophobicity of the reactant, preventing its dissolution into aqueous buffer.



Scheme 16 The ligation reaction between thioacid substrate and crude aziridine substrate

Dittmann et al.[26] reported that NCL of small hydrophobic peptides can be carried out in organic solvents with high yield. The best results were obtained by using DMF in the presence of triethylamine. Therefore, we tried DMF as our ligation solvent. The crude, hydrophobic long chain aziridine peptide dissolved in DMF, yet we only obtained a 30% yield. We tried other organic

solvents (EtOH, MeCN, DCM) and all resulted in a cloudy solution with no product formation observed. EtOH was attempted because ligation between **8d** and **15** was done successfully in EtOH.

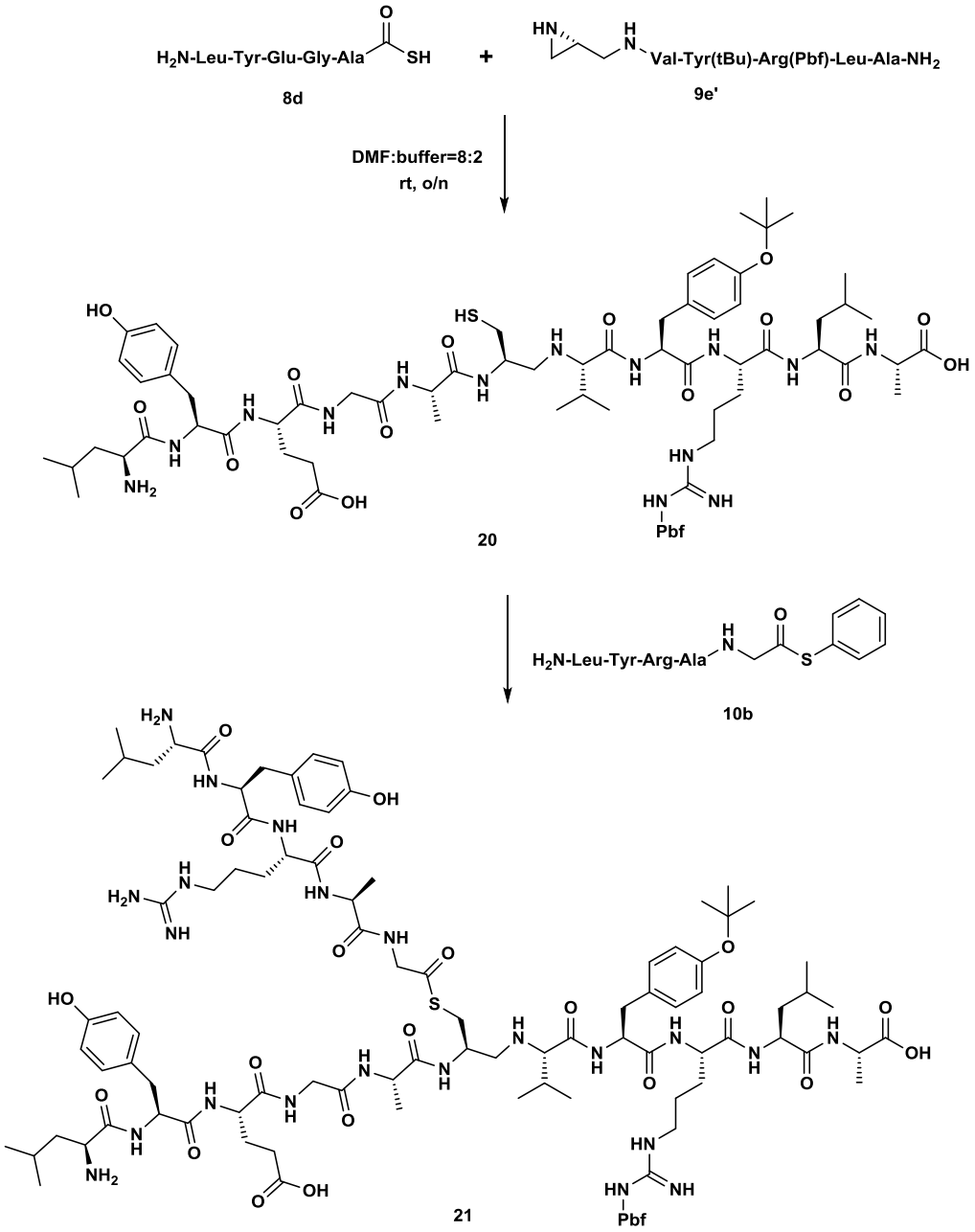
2.4.3 Ligation in mixed solvents

We tried mixing phosphate buffer with DMF and then monitored the ligation reaction of the deprotected pentapeptide reactants in four different ratios of solvents (Table 6) by HPLC/MS. In NCL, the reaction condition must remain at neutral pH (7.0). As reaction buffer became slightly acidic after dissolving peptide substrates into the solution, we added 2M NaOH to adjust the pH of the solution. Interestingly, it was found that at a slightly basic condition and a ratio of 2:8 buffer/DMF gave the best yield. As we increased the proportion of buffer in the mixed solvent, more hydrolysis of aziridine substrate was observed. The results are summarized in Table 6.

Solvent mixtures	pH	Results
Buffer*	7.0	poor solubility
DMF	7.0	30% yield
MeCN	7.0	0% yield
EtOH	7.0	0% yield
1:1 Buffer*/DMF	7.0	hydrolysis product
	8.0	hydrolysis product
3:7 Buffer*/DMF	7.0	40% yield
	8.0	50% yield
2:8 Buffer*/DMF	7.0	55% yield
	8.0	75% yield
1:9 Buffer*/DMF	7.0	40% yield
	8.0	40% yield

Table 6 Ligation reaction in different solvents and pH

This new optimized ligation condition has been applied to long chain thioacids, aziridines, and thioester peptides, and to our gratification, a significant amount of the desired tri-branched peptide has been observed by HPLC/MS. The thioacid substrate **8d** first reacted with the aziridine substrate **9e'** to form a di-branched thioester peptide **20**. Then in the same pot, thioester substrate **10b** was added to form the final tri-branched peptide **21** (Scheme 17). The desired product **21** was purified by reverse-phase preparative HPLC/MS (10-45% gradient of acetonitrile in water over 30 minutes, 10 mL/min) in a 2% yield.



Scheme 17 Reaction scheme of the formation of tri-branched peptide **21**

2.4.4 Purification approach to the ligation product

Even though the optimal ligation solvent mixture (2:8 buffer/DMF) gave us the highest product yield, the hydrolysis product was still observed over the course of the reaction. In addition, the longer the reaction was left to stir, the more hydrolysis occurred. We then tried microwave heating the ligation reaction in order to minimize the reaction time and reduce the possibility of hydrolysis. We first attempted microwave heating at 50°C for 10 minutes, but observed no product formation

or starting material consumption by HPLC/MS. Next, we tried increasing the temperature to 120°C and heated the reaction for 2 minutes, but unfortunately the reagents decomposed and no product was formed.

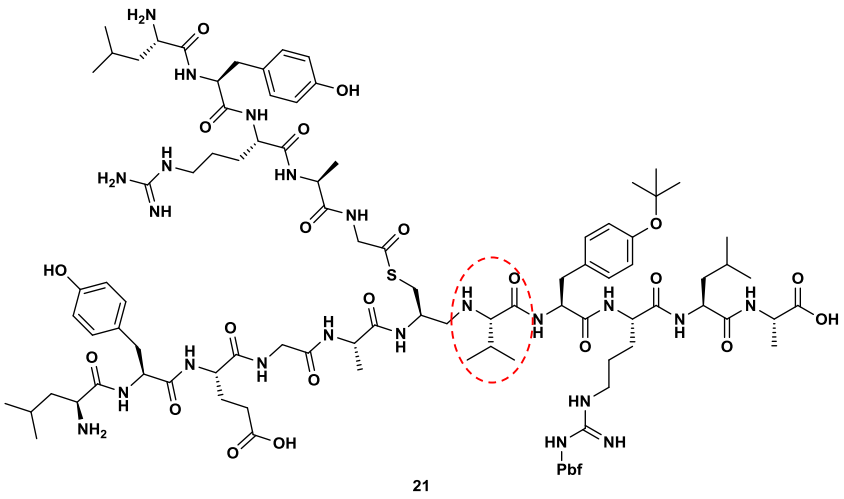
The purification step of compound **21** by prep HPLC/MS was inefficient giving a low yield (2%). We searched for an alternative purification method by taking advantage of the differences between the molecular weight of compound **21**, unreacted reactants and impurities. We chose a molecular weight cut-off filter (MWCO) at m/z 2000 as an alternative to preparative HPLC/MS purification method. Theoretically, as the reaction mixture gets flushed through the MWCO, the larger MW of compound **21** would remain behind and the smaller unwanted materials would be filtered through the membrane and collected in the bottom layer. However, there was an inherent problem with this method. This particular membrane, Hydrosart® is not compatible with organic solvents, including DMF – a necessary component of our reaction. Hence, a dilution by water was needed before filtering. Unfortunately, after checking the filtration results by HPLC/MS, no product was found in either the filtrate or the residue. We have attributed this failure to two possible reasons. Ideally, the membrane pore size should be at least 50% smaller than the size of the target compound needed to be separated, and the mass of our target compound is m/z 2112. In order to fullfill this standard requirement, a size of m/z 1000 would have been optimal. However, the smallest commercially available pore size is m/z 2000. Furthermore, the reaction, which was diluted with large amounts of water, might prevent its detection by HPLC/MS. As a result, prep HPLC/MS still remains the ideal purification method for separating the thioester tri-branched peptide despite its low product yield.

2.4.5 *S*-to-*N* acyl transfer

We assumed that after reacting compound **20** with **10b**, transthioesterification would take place followed by a spontaneous *S*-to-*N* acyl transfer to give us the tri-branched amide peptide. A macro-peptide whose mass is over m/z 2000 is difficult to characterize by NMR. Therefore, a more straight-forward and time-efficient identification method would be to react Aldrithiol-2 with **21**. Aldrithiol-1 is a reagent that will rapidly form a disulfide bond with any free thiol present in the reaction. If compound **21** is present as a tri-branched amide peptide, then the newly released cysteine thiol will react with Aldrithiol-2. Unfortunately, there is no disulfide adduct observed by

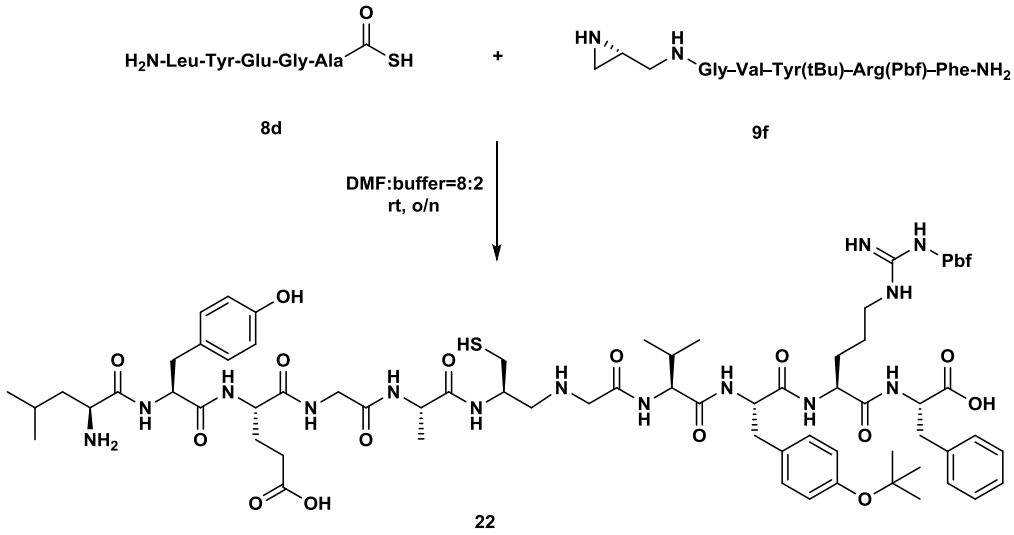
HPLC/MS. As a result, compound **21** is likely a thioester rather than an amide at the branching point.

Although it has been previously reported that there are no restrictions to the amino acid located at the branching point[27] (ie. the *N*-terminus of aziridine substrate) for spontaneous *S*-to-*N* acyl transfer to occur , those observations were done only on the ligation of a di-branched peptide and our tri-branched peptide ligation is more complicated and substrate-sensitive. We concluded that the bulky valine residue located by the reduced amide bond might block the spontaneous *S*-to-*N* acyl transfer. As a result, we selected a less bulky substrate – glycine as our *N*-terminus amino acids on the aziridine substrate. We hoped that this amino acid would negate any steric issues involved in the acyl transfer step.

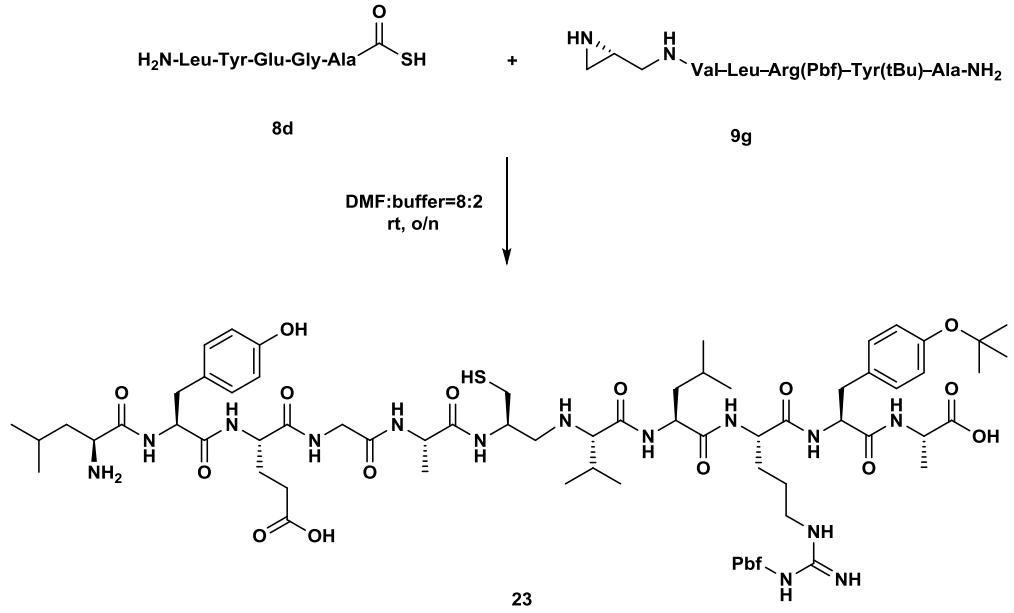


Scheme 18 The potential problematic hindered amino site on the tri-branched peptide **21**

However, with the *N*-terminal glycine pentapeptide **9f** as our aziridine substrate in branched peptide ligation (Scheme 19), the reaction also gave a low yield. The ligation reaction did not occur after 2 hours and a large amount of aziridine reactant remained. It was found that if we switched the *N*-terminus amino acid back to valine **9g** in the pentapeptide aziridine substrate (Scheme 20), we obtained a better yield. In addition, peptide ligation reaction must demonstrate high conversions in order to minimize purification efforts. As a result, valine situated at the *N*-terminus in the aziridine pentapeptide is essential for a high yielding ligation to occur despite the future limitation in the following acyl transfer step.



Scheme 19 Reaction scheme of ligation using *N*-terminal glycine pentapeptide **9f**



Scheme 20 Reaction scheme of ligation using *N*-terminal valine pentapeptide **9g**

Due to the unsuccessful modification of the amino acid at the branching site, we decided to subject compound **21** to forcing conditions to afford the product of acyl transfer. By taking advantage of the thiophilicity of silver[28], we added AgOTf to compound **21** in 0.2M NaOAc ligation buffer followed by sonicated the reaction at 60°C for three hours. Although we were initially encouraged by the success of these conditions in single amino acid ligations, neither the amide branched peptide nor thioester branched peptide **21** was observed at the end of the reaction. Since the

reaction was monitored by HPLC/MS, the reaction solution must first be filtered before being injected onto the HPLC column. Possible reasons for why we didn't observe any product or starting material might be due to the decomposition of the compounds at harsh conditions or the improper filtering of the reaction solution. We propose that by rinsing the reaction mixture with EDTA in buffer may help to elute the sample out from the filter membrane and aid in future analyses.

3 Conclusions

We have been able to optimize the reaction conditions for the synthesis of tri-branched peptides. Although we haven't observed spontaneous *N*-to-*S* acyl transfer, under forcing conditions we can form a peptoid-like branching point. A remaining major challenge is the aziridine substrate choice for the branched peptide ligation. Since the successful preparation of a tri-branched peptide requires a more bulky amino acid situated at the branching site, this amino acid inevitably becomes a hindrance for a spontaneous *S*-to-*N* acyl transfer to yield the final amide product. Nevertheless, the success of transthioesterification with the cysteine residue at the branching point of the long chain peptide substrates allow us to explore more potentially bioactive scaffolds. In addition, if the tri-branched thioester peptide remains intact even after reaction with AgOTf, it may act as a mimic of a stable amide bond that could act as an isostere in potential biomaterials.

4 Experiemental procedures

General information: Anhydrous methanol, toluene, dichloromethane, triethylamine and tetrahydrofuran were purified suing a Grubbs dry solvent system.

Chromatography: Flash column chromatography was carried out using Silicycle 230-400 mesh silica gel. Thin-layer chromatography (TLC) was performed on Macherey Nagel pre-coated glass backed TLC plates (SIL G/UV254, 0.25 mm). UV lamp and potassium permanganate ($KMnO_4$) stain were used to visualize each spot.

HPLC/MS:

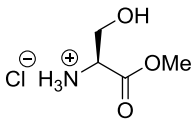
Analytical: HPLC equipped with a 254 nm and 214 nm detector was used. One of two reverse phase columns were used: 1) Poroshell 120 EC-C18 column (4.6 x 50 mm, 2.7 μm); or 2) Jupiter 4μ Proteo 90A column (150 x 4.60 mm, 4.0 μm). A linear gradient of 5-95% (v/v) solvent B in solvent A (flow rate 1mL/min) was run at various rates. Solvent A: HPLC grade acetonitrile (0.1% Formic acid v/v), Solvent B: HPLC grade water (0.1% Formic acid v/v).

Preparative: HPLC equipped with a 254 nm and 214 nm detector was used. A reverse phase column was used: Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). A linear gradient of 5-95% (v/v) solvent B in solvent A (flow rate 5mL/min) was run. Solvent A: HPLC grade acetonitrile (0.1% Formic acid v/v), Solvent B: HPLC grade water (0.1% Formic acid v/v).

Nuclear magnetic resonance spectra: ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Mercury 300, 400 MHz spectrometers or on the Agilent VNMRS 500 MHz spectrometer. ^{1}H NMR spectra were referenced to TMS (0ppm) in $CDCl_3$ or to solvent that the NMR was taken in. ^{13}C NMR spectra were referenced to solvent peaks the NMR was taken in. Peak multiplicities are designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; ds, doublet of singlets; dd, doublet of doublets; ddd, doublet of doublet of doublets.

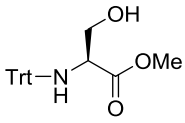
Mass spectroscopy: High-resolution mass spectra were obtained on a ABI/Sciex Qstar mass spectrometer with ESI source, MS/MS and accurate mass capabilities.

4.1 Synthesis of aziridine aldehyde dimers



(*S*)-methyl serinate hydrochloride

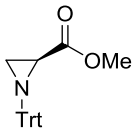
This compound was prepared using an unpublished method[20]. A 1000-mL, one-necked, round-bottomed flask was equipped with a magnetic stirring bar and charged with methanol (170 mL). The flask was fitted with a 100-mL pressure-equalizing addition funnel, then cooled to 0°C in an ice-brine bath with stirring. The addition funnel was then charged with thionyl chloride (44 mL, 600 mmol) over 30 minutes. The solution was then stirred for an additional 30 minutes at 0°C. L-serine (21.0 g, 200 mmol) was added as a solid at the same temperature. The reaction solution was then heated to 60°C and stirred at this temperature for 8 hours. The resulting clear solution was then cooled to room temperature and concentrated under reduced pressure. The resulting crystalline solid was triturated with 400 mL of diethyl ether and dried overnight under vacuum pressure to yield 31.7 g of (*S*)-methyl serinate hydrochloride (99% yield). ^{1}H NMR (400 MHz, Methanol-d_4) δ 4.16 (ddt, *J* = 4.4, 3.2, 1.7 Hz, 1H), 4.04 (d, *J* = 4.5 Hz, 0H), 4.01 (d, *J* = 4.5 Hz, 1H), 3.96 (dd, *J* = 3.5, 1.9 Hz, 1H), 3.93 (dd, *J* = 3.5, 1.9 Hz, 0H), 3.88 (s, 3H), 3.33 (p, *J* = 1.7 Hz, 1H). ^{13}C NMR (101 MHz, MeOD) δ 169.38, 60.69, 56.12, 53.70.



***N*-trityl-L-serine methyl ester**

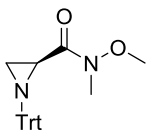
This compound was prepared using an unpublished method[20]. A 1000-mL, 2-necked, round-bottomed flask was equipped with a magnetic stirring bar and charged with (*S*)-methyl serinate hydrochloride (25.0 g, 160 mmol) and dichloromethane (100 mL). The resulting white suspension was stirred and cooled to 0 °C in an ice-brine bath, followed by the addition of triethylamine (44.6 mL, 320 mmol) over 30 minutes. Then trityl chloride (44.6 g, 160 mmol) pre-dissolved in dichloromethane (100 mL) was added to the reaction flask, drop-wise over 1 hour. The flask was then stirred at 4 °C for 20 hours. The flask is then warmed to room temperature and the resulting white suspension was vacuum filtered, and was washed with an addition dichloromethane (150 mL). The combined filtrate was concentrated under reduced pressure and then re-suspended in ethyl acetate (500 mL). This suspension was washed sequentially with an ice-cold aqueous solution of 0.5 M citric acid (2 x 200 mL), water (2 x 200 mL) and brine (1 x 200 mL). The organic

layer is then collected and dried with anhydrous sodium sulfate. The solution was then filtered and concentrated under reduced pressure to give 51.3 g of *N*-trityl-L-serine methyl ester (89 % yield) that was used without further purification.



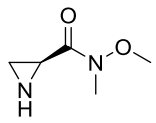
(*S*)-methyl 1-tritylaziridine-2-carboxylate

This compound was prepared using an unpublished method[20]. To a 1000-mL, one-necked, round-bottomed flask charged with *N*-trityl-L-serine methyl ester (56.0 g, 155 mmol) was added a magnetic stirring bar and dissolved in dichloromethane (320 mL). The solution was cooled to 0°C in an ice-brine bath. Methanesulfonyl chloride (15 mL, 194 mmol) was then added to the reaction followed by the drop-wise addition of triethylamine (33 mL, 237 mmol) over 30 minutes. The solution was then stirred for an addition 30 minutes at 0°C and then diluted with dichloromethane (200 mL). The solution was washed sequentially with an ice-cold, aqueous solution of 0.5 M citric acid (200 mL) and water (200 mL). The combined aqueous layers were backwashed with dichloromethane (50 mL) and the combined organic fractions were collected dried with anhydrous magnesium sulfate. The solution was filtered then concentrated under reduced pressure and the resulting mesylate was dissolved in THF (400 mL). Triethylamine (44 mL, 316 mmol) was then added to the solution, and was heated to 70 ^{o}C for 90 hours. After the reaction was done, the solution was concentrated under reduced pressure and suspended in ethyl acetate (500 mL). The solution was sequentially washed with an ice-cold aqueous solution of 0.5 M citric acid (2 x 200 mL), water (1 x 200 mL) and brine (1 x 200 mL). The organic layer was collected and dried with anhydrous magnesium sulfate. The solution was then filtered and concentrated under reduced pressure to yield the crude product as an orange solid. The product was then recrystallized by dissolving the crude solid in 300 mL of hot absolute ethanol. After cooling down the solution, the resulting crystals were collected by vacuum filtration and washed with an additional 150 mL of cold (-20 ^{o}C) absolute ethanol. The crystals were then dried to provide 46.8 g of (*S*)-methyl 1-tritylaziridine-2-carboxylate (85% yield).



(*S*)-*N*-methoxy-*N*-methyl-1-tritylaziridine-2-carboxamide

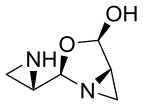
This compound was prepared using an unpublished method[20]. A 1000-mL, one-necked, round-bottomed flask was equipped with a magnetic stirring bar and charged with (*S*)-methyl 1-tritylaziridine-2-carboxylate (34.3 g. 100 mmol), *N*, *O*-dimethylhydroxylamine hydrochloride (14.6 g, 150 mmol) and THF (300 mL). The resulting suspension was vigorously stirred and cooled to 0°C in an ice-brine bath, followed by the addition of a 2.0 M solution of isopropylmagnesium chloride in THF (157 mL, 300 mmol) over 1 hour. The solution was then stirred for an additional 45 minutes at 0°C, at which point TLC analysis indicated complete consumption of starting material. A saturated aqueous solution of ammonium chloride was added drop-wise over 30 minutes at 0°C to quench the reaction. The solution was then warmed to room temperature and diluted with deionized water. The product was extracted with ethyl acetate (3 x 150 mL), and the combined organic fractions were then collected and dried with anhydrous sodium sulfate. The solution was then filtered by vacuum filtration and concentrated under reduced pressure to provide the crude product (*S*)-*N*-methoxy-*N*-methyl-1-tritylaziridine-2-carboxamide that was used directly in the next step without further purification.



(*S*)-*N*-methoxy-*N*-methylaziridine-2-carboxamide

This compound was prepared using an unpublished method[20]. A 1000-mL, one-necked, round bottomed flask containing the crude product (*S*)-*N*-methoxy-*N*-methyl-1-tritylaziridine-2-carboxamide was equipped with a magnetic stirring bar and charged with dichlormethane (500 mL) and triethylsilane (24 mL, 150 mmol). The resulting suspension was vigorously stirred and cooled to 0°C in an ice-brine bath, followed by the addition of trifluoroacetic acid (23 mL, 300 mmol) over 30 minutes. The solution was stirred at this temperature for 30 minutes, at which point TLC analysis indicated complete consumption of starting material. The round-bottomed flask was then removed from the ice-brine bath and concentrated under reduced pressure. The crude residue was then dissolved in 500 mL of diethyl ether and extracted into deionized water (2 x 100 mL, then 2 x 50 mL). The combined aqueous extracts were backwashed with diethyl ether (1 x 50 mL). The aqueous solution was then basified to pH 9 with solid sodium bicarbonate. The product was then extracted into chloroform through the use of a continuous liquid-liquid extractor. The collected $CHCl_3$ was dried with anhydrous magnesium sulfate. The solution was filtered and then concentrated under reduced pressure to provide the crude product as a yellow oil. The product was then purified by vacuum distillation using a bulb-to-bulb distillation apparatus at Bp = 92 – 102

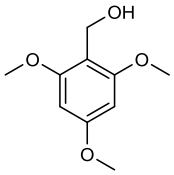
°C (0.7 mm Hg) to provide 5.80 g of (*S*)-*N*-methoxy-*N*-methylaziridine-2-carboxamide(45 % yield over two steps). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.78 (s, 3H), 3.25 (s, 3H), 2.97 (d, *J* = 5.9 Hz, 1H), 1.89 (d, *J* = 4.7 Hz, 1H), 1.83 (d, *J* = 5.4 Hz, 1H), 1.25 (s, 1H).



(2*R*,4*R*,5*S*)-2-((*S*)-aziridin-2-yl)-3-oxa-1-azabicyclo[3.1.0]hexan-4-ol (7)

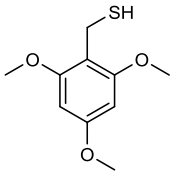
This compound was prepared using an unpublished method[20]. A one-necked, 250 mL Schlenk flask, equipped with a magnetic stirring bar was charged with $LiAlH_4$ (0.42 g, 11 mmol). THF (40 mL) was then added to the flask and the resulting suspension was stirred and cooled to -78°C in a dry ice-acetone bath. (*S*)-*N*-methoxy-*N*-methylaziridine-2-carboxamide (1.31 g, 10 mmol) in THF (10 mL) was added drop-wise to the reaction suspension along the wall of the Schlenk flask over 20 minutes. The Schlenk flask was then warmed to 0°C and was stirred at this temperature for 20 minutes, until TLC analysis indicated complete consumption of starting material. The flask was then cooled back down to -78°C and diluted with THF (40 mL) drop-wise, along the wall of the Schlenk flask over 20 minutes. The reaction was then quenched by adding a saturated aqueous solution of Na_2SO_4 (1 mL) drop-wise. Reaction flask was then warmed to room temperature. The suspension was stirred vigorously for an addition 25 minutes and then filtered by vacuum filtration. The precipitate was washed with additional THF (100 mL) and methanol : dichloromethane solution (20 : 80 mL). The filtrate was concentrated under reduced pressure to yield a crude, off-white residue, which was immediately purified by column chromatography to afford 0.49 g of (2*R*,4*R*,5*S*)-2-((*S*)-aziridin-2-yl)-3-oxa-1-azabicyclo[3.1.0]hexan-4-ol (68 % yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.28 (s, 1H), 4.94 – 4.91 (m, 1H), 2.63 (dd, *J* = 5.3, 3.4 Hz, 1H), 2.45 (ddd, *J* = 6.6, 4.0, 1.2 Hz, 1H), 1.86 (d, *J* = 6.6 Hz, 1H), 1.77 (d, *J* = 5.3 Hz, 2H), 1.70 (d, *J* = 4.0 Hz, 2H), 1.29 – 1.21 (m, 4H). ^{13}C NMR (126 MHz, $cdcl_3$) δ 96.4, 94.5, 43.8, 31.3, 27.7, 21.1.

4.2 Synthesis of Tmob thiol



(2,4,6-trimethoxyphenyl)methanol

This compound was prepared using a literature method. A 500-mL, one-necked, round bottomed flask containing 2,4,6-trimethoxybenzaldehyde (10g, 51 mmol) was equipped with a magnetic stirring bar and charged with methanol (300 mL), followed by the addition of sodium borohydride (1.93 g, 51 mmol). The resulting suspension was vigorously stirred at room temperature for 2 hours. The solution was removed under reduced pressure and the residue was redissovled in ethyl acetate (200 mL). The crude product was washed with a saturated aqueous solution of sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was then dried with sodium sulfate. The solvent was removed under reduced pressure and the resulting crude (2,4,6-trimethoxyphenyl)methanol was used directly in the next step without further purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.13 (d, *J* = 1.6 Hz, 2H), 5.15 (s, 1H), 4.71 (d, *J* = 6.6 Hz, 1H), 3.87 – 3.76 (m, 8H), 2.05 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.1, 159.2, 100.0, 90.5, 55.7, 55.4, 54.4.



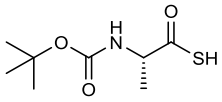
(2,4,6-trimethoxyphenyl)methanethiol (6)

This compound was prepared using a literature method[16]. A 500-mL, one-necked, round bottomed flask containing (2,4,6-trimethoxyphenyl)methanol (6.35 g, 32 mmol) and thiourea (4.88 g, 64 mmol) was equipped with a magnetic stir bar and charged with water/acetone (1:1 v/v) (65 mL). 5N HCl (9.6 mL) was added dropwise and the acidic solution was stirred at room temperature for 24 hours until TLC analysis indicated complete consumption of starting material. Then the solution was extracted with EtO_2 (2 x 25 ml) and the aqueous phase was then brought to alkaline pH by carefully addition of 5 N NaOH under nitrogen atmosphere and heated to reflux for 3 hours. The reaction was followed by TLC (hexane: ethyl acetate 8:2). The thiol was isolated after acidification with HCl by extraction with EtOAc (4 x 25 ml). The combined organic phases were dried, and removed under reduced pressure. After flash silica gel column chromatography (100 % hexane to 50 % ethyl acetate in hexane) purification, the title compound was isolated as a white solid in 41 % yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.12 (s, 2H), 3.82 (d, *J* = 13.8 Hz, 9H), 3.73 (d, *J* = 7.9 Hz, 2H), 1.95 (t, *J* = 7.9 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.3, 158.2, 90.6, 55.8, 16.7.

4.3 Synthesis of single amino acid substrates

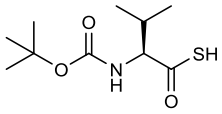
4.3.1 Synthesis of single amino thioacids

To a 0.67M solution of a Boc-protected amino acid in MeCN, carbonyldiimidazole (CDI) (1.1 eq.) was added under a nitrogen atmosphere. The reaction was left to stir for 30 minutes. Sodium sulfide (3 eq.) was subsequently added to the reaction mixture. The reaction was left to stir at room temperature for two hours. The solvent was then removed under reduced pressure and the residue was dissolved in ethyl acetate. The newly formed solution was then cooled to 0°C. At 0°C, prechilled 1N HCl was added dropwise to the solution until the aqueous layer was acidified to a pH 3. The organic layer was washed with brine (3 x) and dried under sodium sulfate. Once filtered, the solvent was removed under reduced pressure to yield clear sticky oil. The resulting residue was then purified by reverse phase preparative HPLC/MS.



(*S*)-2-((*tert*-butoxycarbonyl)amino)propanethioic *S*-acid (11a)

^{1}H NMR (400 MHz, Chloroform-d) δ 4.33 (s, 1H), 1.52 – 1.42 (m, 13H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.31, 154.84, 76.70, 28.26, 18.02.



(*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanethioic *S*-acid (11b)

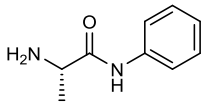
^{1}H NMR (500 MHz, Chloroform-d) δ 4.23 (dd, J = 9.2, 4.6 Hz, 1H), 2.39 – 2.23 (m, 1H), 1.47 (d, J = 3.0 Hz, 9H), 0.96 (dd, J = 51.4, 7.0 Hz, 7H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.14, 66.27, 60.39, 30.54, 28.31, 21.04, 19.44, 14.20.

4.3.2 Synthesis of aziridine amino anilides

4.3.2.1 Preparation of amino anilide

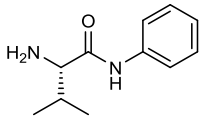
To a 0.25M solution of the desired Boc-protected amino acid in DCM, aniline (1 eq.), HBTU (1 eq.), and diisopropylethylamine (3 eq.) were added. The reaction was left to stir for 2 hours until TLC indicated that reaction had gone to completion. The solvent was removed under reduced pressure and the resulting residue was dissolved in ethyl acetate. The ethyl acetate solution was washed with 10% (w/v) aqueous solution of citric acid (2 x), saturated sodium bicarbonate (2 x),

and brine (2 x). The organic layer was then dried under sodium sulfate. TFA/DCM 1:1(v/v) was added to the target compound and stirred for 30 minutes. The resulting solution was then removed under reduced pressure, followed by redissovling in a minimal amount of ethyl acetate and the TFA salt was precipitated with diethyl ether. The filtered salts were then free-based with 30% aqueous solution of ammonium hydroxide. The aqueous layer was extracted with DCM (4 x). The combined organic layers were then washed with brine and dried with sodium sulfate. The solution was filtered and the solvent removed under reduced pressure. The crude product was used directly in the reductive amination without further purification.



(*S*)-2-amino-*N*-phenylpropanamide (12)

^{1}H NMR (400 MHz, Chloroform-d) δ 7.65 – 7.55 (m, 2H), 7.39 – 7.24 (m, 2H), 7.14 – 7.05 (m, 1H), 3.62 (q, *J* = 7.0 Hz, 1H), 2.80 (s, 1H), 1.43 (d, *J* = 7.0 Hz, 3H)

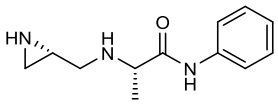


(S)-2-amino-3-methyl-N-phenylbutanamide (14)

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.50 (s, 1H), 7.64 – 7.57 (m, 2H), 7.39 – 7.28 (m, 2H), 7.14 – 7.04 (m, 1H), 3.37 (d, *J* = 3.7 Hz, 1H), 2.45 (td, *J* = 6.9, 3.6 Hz, 1H), 1.04 (d, *J* = 7.0 Hz, 3H), 0.88 (d, *J* = 6.9 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 172.58, 137.81, 128.96, 123.98, 119.42, 60.45, 38.62, 30.77, 19.83, 15.96.

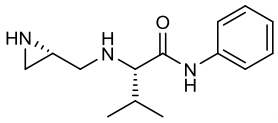
4.3.2.2 Reductive amination

A round bottom flask equipped with a magnetic stirring bar a 0.05M solution of the peptide anilide (1.5 eq.) in a 1:2.5:22.5 DCM:MeOH:THF solvent mixture was stirred under nitrogen at room temperature. The aziridine aldehyde (0.5 eq.) was then added to the reaction mixture followed by the addition of $ZnCl_2$ (2.2 eq.), $NaCNBH_3$ (1.5 eq.), and acetic acid (20 eq.). The reaction was stirred at room temperature for 2 hours at which point HPLC/MS indicated that the reaction had reached to completion. The solvent was then removed under reduced pressure and the resulting residue was redissolved in ethyl acetate. The organic solution was then washed with a saturated solution of sodium bicarbonate (3 x), water (2 x), and brine (2 x). The organic layer was then dried with sodium sulfate, and removed under reduced pressure.



(*S*)-2-((((*S*)-aziridin-2-yl)methyl)amino)-*N*-phenylpropanamide (13)

^{1}H NMR (500 MHz, Chloroform-d) δ 7.59 (ddd, J = 8.5, 2.2, 1.1 Hz, 2H), 7.32 (ddd, J = 8.5, 7.4, 2.1 Hz, 2H), 7.13 – 7.06 (m, 1H), 2.76 (dd, J = 11.7, 7.1 Hz, 1H), 2.67 (dd, J = 11.7, 7.2 Hz, 1H), 1.44 (d, J = 7.0 Hz, 3H), 1.40 (d, J = 7.0 Hz, 3H), 1.33 – 1.21 (m, 16H), 1.16 (t, J = 7.1 Hz, 3H)

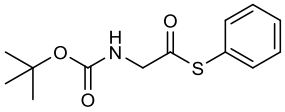


(*S*)-2-((((*S*)-aziridin-2-yl)methyl)amino)-3-methyl-*N*-phenylbutanamide (15)

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.68 – 7.56 (m, 2H), 7.39 – 7.29 (m, 2H), 7.13 – 7.06 (m, 1H), 3.03 (d, *J* = 4.5 Hz, 1H), 2.85 (dd, *J* = 12.7, 3.9 Hz, 1H), 2.49 (dd, *J* = 12.7, 7.4 Hz, 1H), 2.22 (tt, *J* = 7.0, 3.6 Hz, 2H), 1.87 (d, *J* = 6.1 Hz, 1H), 1.45 (d, *J* = 3.5 Hz, 1H), 1.25 (s, 1H), 1.05 (d, *J* = 7.0 Hz, 3H), 0.96 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 172.11, 137.86, 128.95, 128.20, 123.93, 119.42, 69.00, 53.41, 38.59, 31.60, 23.41, 19.61, 17.80.

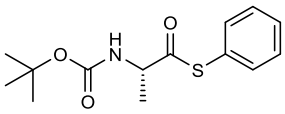
4.3.3 Synthesis of single amino thioesters

A round bottom flask equipped with a magnetic stir bar, a 0.77M solution of Boc-amino acid was dissolved in DCM and cooled to 0°C. EDCI (1.25 eq.), thiophenol (1.25 eq.), DMAP (0.1 eq.), and DiPEA (2.5 eq.) were then added to the reaction at 0°C and stirred for three hours. The reaction was then warmed up to room temperature and stirred overnight under nitrogen atmosphere. Once the reaction has reached to completion, the solvent was removed under reduced pressure and the resulting residue was purified by flash silica gel column chromatography (0-50% gradient of ethyl acetate in hexanes). The title compound was presented as solid.



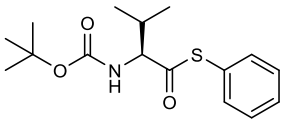
***S*-phenyl 2-((tert-butoxycarbonyl)amino)ethanethioate (16a)**

^{1}H NMR (400 MHz, Chloroform-d) δ 7.42 (s, 5H), 5.11 (s, 1H), 4.15 (d, *J* = 6.1 Hz, 2H), 1.48 (s, 10H)



***S*-phenyl (*S*)-2-((tert-butoxycarbonyl)amino)propanethioate (16b)**

^{1}H NMR (400 MHz, Chloroform-d) δ 7.41 (s, 5H), 4.99 (d, *J* = 8.4 Hz, 1H), 4.63 – 4.43 (m, 1H), 1.49 (s, 9H), 1.46 – 1.43 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 134.67, 129.43, 28.37, 18.78.



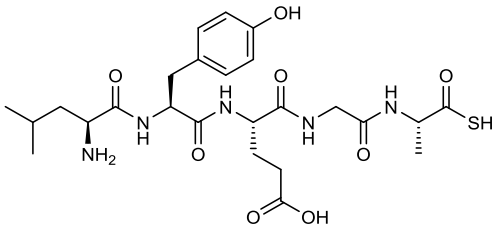
***S*-phenyl (*S*)-2-((tert-butoxycarbonyl)amino)-3-methylbutanethioate (16c)**

^{1}H NMR (400 MHz, Chloroform-d) δ 7.40 (d, *J* = 1.8 Hz, 5H), 5.02 (d, *J* = 9.6 Hz, 1H), 4.41 (dd, *J* = 9.5, 4.6 Hz, 1H), 2.40 – 2.27 (m, 1H), 1.49 (s, 10H), 1.04 (d, *J* = 6.8 Hz, 4H), 0.93 (d, *J* = 6.9 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.76, 148.99, 134.63, 129.44, 127.45, 99.99, 65.28, 31.10, 28.37, 19.45, 16.93.

4.4 Synthesis of pentapeptide substrates

4.4.1 Synthesis of pentapeptide thioacid

A 20-mL scintillation vial equipped with a magnetic stir bar, a 0.05 M solution of the Boc-protected pentapeptide charged with DMF was stirred under nitrogen at room temperature. To the reaction HATU (3 eq.), trimethoxylbenzenethiol (4 eq.) and DiPEA (3 eq.) were added. The reaction was left to stir overnight and was monitored by HPLC/MS. Once the reaction had gone to completion, the solvent was removed under a stream of nitrogen. The resulting residue was then deprotected by degassed cocktail TFA. The resulting residue was purified by reverse phase preparative HPLC/MS.

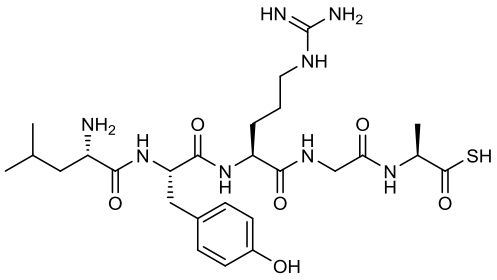


Leu-Tyr-Glu-Gly-Ala-SH (8d)

Product was purified by reverse phase preparative HPLC/MS (5-35% gradient of acetonitrile in water over 25 minutes (5 mL/min) with stop time of 30 minutes using Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). Yield 21%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 5 minutes with stop time of 6.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 μm).

Retention time: 2.89 minutes

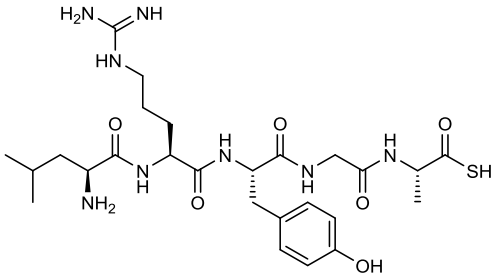


Leu-Tyr-Arg-Gly-Ala-SH (8c)

Product was purified by reverse phase preparative HPLC/MS (5-25% gradient of acetonitrile in water over 20 minutes (5 mL/min) with stop time of 25 minutes using Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). Yield 35%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 μm).

Retention time: 3.41 minutes

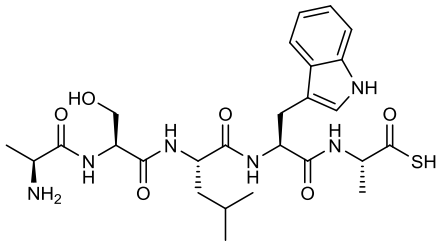


Leu-Arg-Tyr-Gly-Ala-SH (8b)

Product was purified by reverse phase preparative HPLC/MS (10-45% gradient of acetonitrile in water over 40 minutes (5 mL/min) with stop time of 50 minutes using Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). Yield 23%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 5 minutes with stop time of 6.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

Retention time: 2.80 minutes



Ala-Ser-Leu-Trp-Ala-SH (8a)

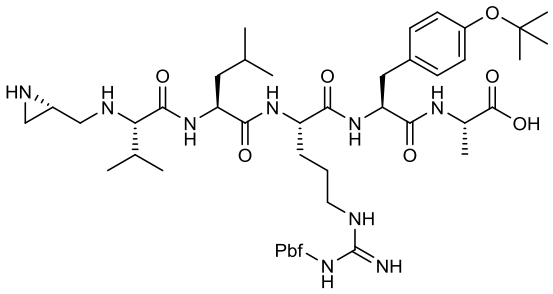
Product was purified by reverse phase preparative HPLC/MS (20-60% gradient of acetonitrile in water over 40 minutes (5 mL/min) with stop time of 50 minutes using Jupiter 4µ Proteo 90A (250 x 10.00 mm, 4.0 µm). Yield 15%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

Retention time: 3.56 minutes

4.4.2 Synthesis of aziridine pentapeptide

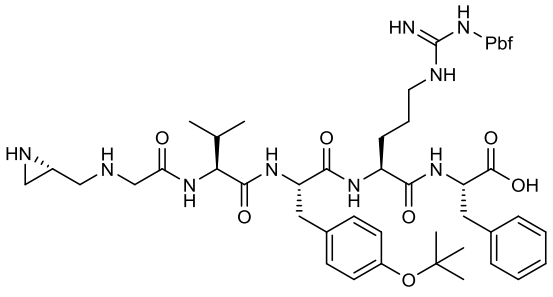
A round bottom flask equipped with a magnetic stir bar, a 0.05 M solution of pentapeptde in a 1:2.5:2.5 DCM:MeOH:THF solvent mixture was stirred under nitrogen at room temperature. The aziridine aldehyde (1 eq.) was then added to the reaction mixture followed by the addition of $ZnCl_2$ (3.3 eq.), and $NaCNBH_3$ (2.2 eq.). The reaction was allowed to stir at room temperature overnigh at which point HPLC/MS showed the completion of the reaction. The solvents were then removed under reduced pressure. The crude protected product was used directly in the next step without further purification. On the hand, the deprotected product was obtained by deprotecting with cocktail TFA, and purified by reverse phase preparative HPLC/MS.



Az-Val-Leu-Arg(Pbf)-Tyr(tBu)-Ala (9g)

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

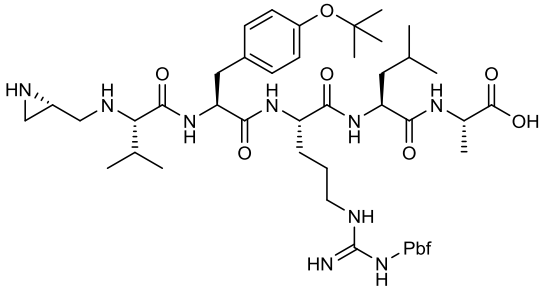
Retention time: 7.96 minutes



Az-Gly-Val-Tyr(tBu)-Arg(Pbf)-Phe (9f)

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

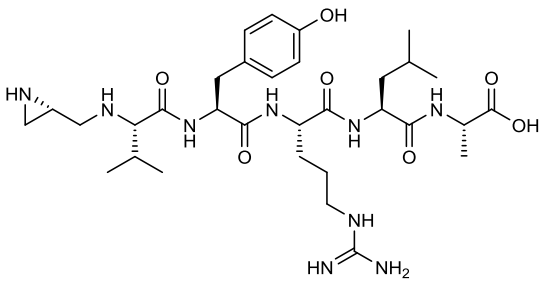
Retention time: 8.01 minutes



Az-Val-Tyr(tBu)-Arg(Pbf)-Leu-Ala (9e')

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

Retention time: 7.36 minutes

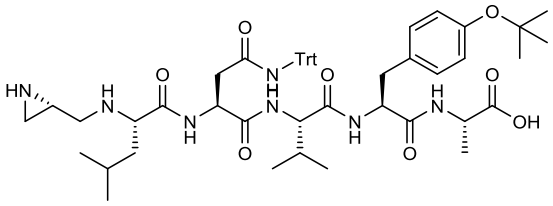


Az-Val-Tyr-Arg-Leu-Ala (9e)

Product was purified by reverse phase preparative HPLC/MS (20-80% gradient of acetonitrile in water over 40 minutes (5 mL/min) with stop time of 50 minutes using Jupiter 4µ Proteo 90A (250 x 10.00 mm, 4.0 µm). Yield 15%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

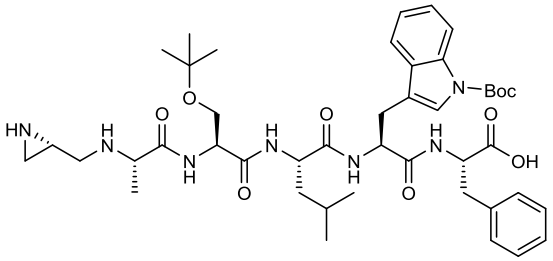
Retention time: 4.10 minutes



Az-Leu-Asn(Trt)-Val-Tyr(tBu)-Ala (9b)

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

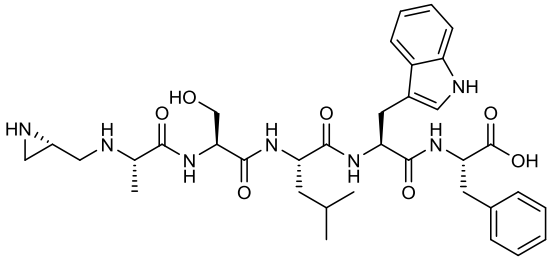
Retention time: 8.44 minutes



Az-Ala-Ser(tBu)-Leu-Trp(Boc)-Phe (9d')

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 5 minutes with stop time of 6.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

Retention time: 4.43 minutes

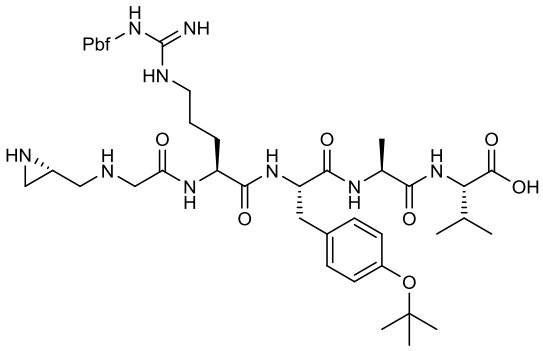


Az-Ala-Ser-Leu-Trp-Phe (9d)

Product was purified by reverse phase preparative HPLC/MS (20-60% gradient of acetonitrile in water over 40 minutes (5 mL/min) with stop time of 50 minutes using Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). Yield 15%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 14.5 minutes with stop time of 16 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 μm).

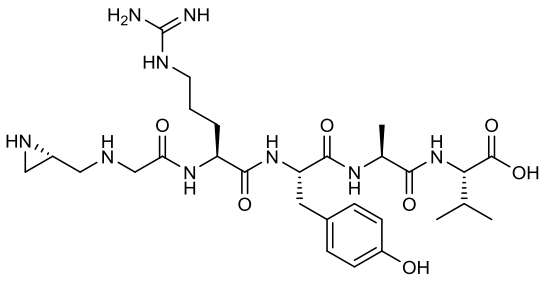
Retention time: 5.25 minutes



Az-Gly-Arg(Pbf)-Tyr(tBu)-Ala-Val (9c')

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 14.5 minutes with stop time of 16 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 μm).

Retention time: 7.49 minutes

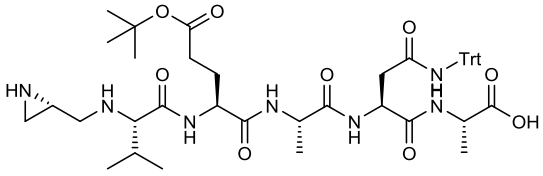


Az-Gly-Arg-Tyr-Ala-Val (9c)

Product was purified by reverse phase preparative HPLC/MS (15-70% gradient of acetonitrile in water over 50 minutes (5 mL/min) with stop time of 60 minutes using Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). Yield 15%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 9 minutes with stop time of 12.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 μm).

Retention time: 2.71 minutes



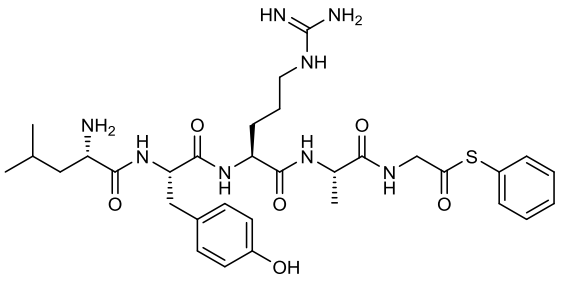
Az-Val-Glu(tBu)-Ala-Asn(Trt)-Ala (9a)

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 14.5 minutes with stop time of 16 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 μm).

Retention time: 8.82 minutes

4.4.3 Synthesis of pentapeptide thioester

A scintillation vial equipped with a magnetic stir bar, a 0.1 M solution of the Boc-protected pentapeptide in DMF was stirred under nitrogen at room temperature. To the reaction HATU (3 eq.), thiophenol (10 eq.) and DiPEA (3 eq.) were added. The reaction was left to stir at room temperature under nitrogen atmosphere overnight. The reaction was monitored by HPLC/MS. After the reaction had gone to completion, the DMF was evaporated off with a stream of nitrogen. The title compound was triturated by cold 1:1 water/brine mixture, and then treated with cocktail TFA. The resulting residue was purified by reverse phase preparative HPLC/MS.

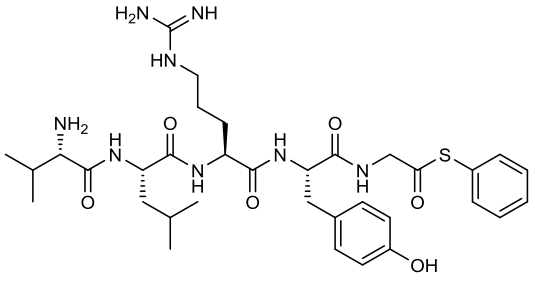


Leu-Tyr-Arg-Ala-Gly-SPH (10b)

Product was purified by reverse phase preparative HPLC/MS (25-50% gradient of acetonitrile in water over 30 minutes (5 mL/min) with stop time of 40 minutes using Jupiter 4µ Proteo 90A (250 x 10.00 mm, 4.0 µm). Yield 43%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 5 minutes with stop time of 6.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

Retention time: 3.33 minutes



Val-Leu-Arg-Tyr-Gly-SPh (10a)

Product was purified by reverse phase preparative HPLC/MS (25-50% gradient of acetonitrile in water over 50 minutes (5 mL/min) with stop time of 60 minutes using Jupiter 4µ Proteo 90A (250 x 10.00 mm, 4.0 µm). Yield 33%

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) over 5 minutes with stop time of 6.5 minutes using Poroshell 120 EC-C18(4.6 x 50 mm, 2.7 µm).

Retention time: 3.47 minutes

4.4.4 Global deprotection

A flame dried 25-mL Schlenk tube equipped with a magnetic stir bar, trifluoroacetic acid (92 %), deionized water (2.5 %), phenol (2.5 %), and triisopropyl silane (2.5 %) were added. The cleavage cocktail was then degassed by freeze-pump-thaw cycling. Once the cocktail was degassed, the

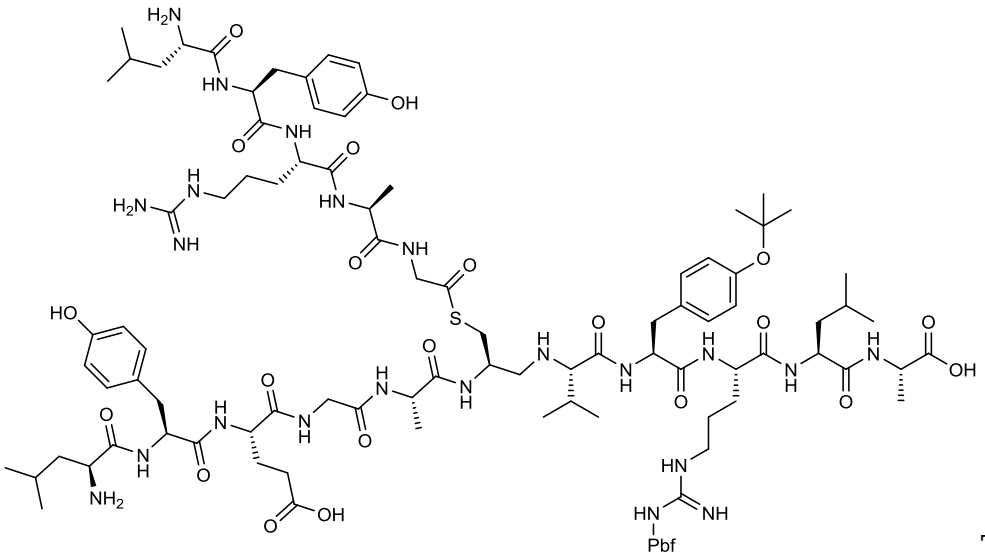
solution was then carefully added to the protected peptide. The cleavage was left to stir under nitrogen atmosphere for 2 hours. Then the resulting reaction mixture was transferred to a lyophilization flask that was prechilled at -78°C. Water/acetonitrile (1:1 v/v) was added to the lyophilization flask, ensuring a quantitative transfer. followed by lyophilizing overnight to afford the desired deprotected peptide.

4.5 Synthesis of branched peptide

4.5.1 Phosphate buffer preparation[15]

A 20-mL scintillation vial containing sodium phosphate dibasic (0.28 mg, 1.47 mmol) and guanidine hydrochloride (5.73 g, 60 mmol) was charged with water (6 mL). Once the solid had completely disoolved, the solution was poured into a 10 mL volumetric flask, more water was added if necessary up to 10 mL. This stock solution was stored at 4°C for three months. Prior to use, a 1-dram vial containing 0.2 micron filtered stock buffer was degassed with argon, followed by the addition of TCEP (7.00 mg, 24 mmol). The phosphate buffer was used directly in the ligation reaction.

4.5.2 Ligation reaction



Tri-branched thioester peptide (21)

A 2-dram vial containing the thioacid **8d** (30 mg, 0.053 mmol) and aziridine **9e'** (52 mg, 0.053 mmol) was equipped with a magnetic stirring bar and charged with DMF:buffer (8:2 v/v) (4.53

mL), followed by the addition of 2 M NaOH to adjust to pH 7.6. The resulting solution was stirred at room termerature for 18 hours, at which point HPLC/MS analysis indicated the formation of di-branched peptide product. Then thioester **10b** was added to the reaction vial and stirred for additional 18 hours. Once the reaction had gone to completion, the solvent was removed under reduced pressure and the branched thioester was then purified by reverse phase reparative HPLC/MS (20-60% gradient of acetonitrile in water over 50 minutes (5mL/min) with stop time of 60 minutes using Jupiter 4μ Proteo 90A (250 x 10.00 mm, 4.0 μm). Yield 2%.

Analytical reverse phase HPLC/MS (5-95% gradient of acetonitrile in water (1% formic acid v/v) gradient over 14.5 minutes with stop time of 16 minutes using Jupiter 4μ Proteo 90A (150 x 4.6 mm, 4 μm).

Retention time: 5.98 minutes

HRMS (ESI+) [M+H]+ calcd. For $C_{100}H_{154}N_{22}O_{24}S_2$ 2112.1, found 2112.1

5 References

1. Tam, J. P.; Yu, Q.; Yang, J.-L., Tandem Ligation of Unprotected Peptides through Thiaprolyl and Cysteinyl Bonds in Water†. *Journal of the American Chemical Society* **2001,** *123* (11), 2487-2494.

2. (a) Otvos, L., Jr., Peptide-Based Drug Design: Here and Now. In *Peptide-Based Drug Design*, Otvos, L., Ed. Humana Press: 2008; Vol. 494, pp 1-8; (b) Cirillo, D.; Pentimalli, F.; Giordano, A., Peptides or small molecules? Different approaches to develop more effective CDK inhibitors. *Current medicinal chemistry* **2011,** *18* (19), 2854-2866.

3. Bellmann-Sickert, K.; Beck-Sickinger, A. G., Peptide drugs to target G protein-coupled receptors. *Trends in pharmacological sciences* **2010,** *31* (9), 434-441.

4. (a) Creighton, T. E., Toward a better understanding of protein folding pathways. *Proceedings of the National Academy of Sciences* **1988,** *85* (14), 5082-5086; (b) Montelione, G. T.; Scheraga, H. A., Formation of local structures in protein folding. *Accounts of Chemical Research* **1989,** *22* (2), 70-76.

5. Dolphin, G. T., A Designed Branched Three-Helix Bundle Protein Dimer. *Journal of the American Chemical Society* **2006,** *128* (22), 7287-7290.

6. Hahn, K. W.; Klis, W. A.; Stewart, J. M., Design and synthesis of a peptide having chymotrypsin-like esterase activity. *Science* **1990,** *248*, 1544+.

7. Hartgerink, J. D.; Beniash, E.; Stupp, S. I., Self-Assembly and Mineralization of Peptide-Amphiphile Nanofibers. *Science* **2001,** *294* (5547), 1684-1688.

8. Kumar, K. S. A.; Bavikar, S. N.; Spasser, L.; Moyal, T.; Ohayon, S.; Brik, A., Total Chemical Synthesis of a 304 Amino Acid K48-Linked Tetraubiquitin Protein. *Angewandte Chemie International Edition* **2011,** *50* (27), 6137-6141.

9. Sadler, K.; Tam, J. P., Peptide dendrimers: applications and synthesis. *Reviews in Molecular Biotechnology* **2002,** *90* (3–4), 195-229.

10. Dawson, P.; Muir, T.; Clark-Lewis, I.; Kent, S., Synthesis of proteins by native chemical ligation. *Science* **1994,** *266* (5186), 776-779.

11. Otaka, A.; Sato, K.; Ding, H.; Shigenaga, A., One-Pot/Sequential Native Chemical Ligation Using N-Sulfanylethylanilide Peptide. *The Chemical Record* **2012,** *12* (5), 479-490.

12. Assem, N.; Natarajan, A.; Yudin, A. K., Chemoselective Peptidomimetic Ligation Using Thioacid Peptides and Aziridine Templates. *Journal of the American Chemical Society* **2010,** *132* (32), 10986-10987.

13. Hili, R.; Yudin, A. K., Amphoteric Amino Aldehydes Reroute the Aza-Michael Reaction. *Journal of the American Chemical Society* **2009,** *131* (45), 16404-16406.

14. Li, X.; Yudin, A. K., Epimerization- and Protecting-Group-Free Synthesis of Peptidomimetic Conjugates from Amphoteric Amino Aldehydes. *Journal of the American Chemical Society* **2007,** *129* (46), 14152-14153.

15. Pollock, S. B.; Kent, S. B. H., An investigation into the origin of the dramatically reduced reactivity of peptide-prolyl-thioesters in native chemical ligation. *Chemical Communications* **2011,** *47* (8), 2342-2344.

16. Vetter, S., Direct Synthesis of DI-and Trimethoxybenzyl Thiols from the Corresponding Alcohol. *Synthetic Communications* **1998,** *28* (17), 3219-3223.

17. Bryson, D. I.; Zhang, W.; Ray, W. K.; Santos, W. L., Screening of a branched peptide library with HIV-1 TAR RNA. *Molecular BioSystems* **2009,** *5* (9), 1070-1073.

18. Aubry, S.; Sasaki, K.; Eloy, L.; Aubert, G.; Retailleau, P.; Cresteil, T.; Crich, D., Exploring the potential of the [small beta]-thiolactones in bioorganic chemistry. *Organic & Biomolecular Chemistry* **2011,** *9* (20), 7134-7143.

19. Wang, P.; Danishefsky, S. J., Promising General Solution to the Problem of Ligating Peptides and Glycopeptides. *Journal of the American Chemical Society* **2010,** *132* (47), 17045-17051.

20. Rotstein, B. H.; Rai, V.; Hili, R.; Yudin, A. K., Synthesis of peptide macrocycles using unprotected amino aldehydes. *Nat. Protocols* **2010,** *5* (11), 1813-1822.

21. (a) Johnson, E. C. B.; Kent, S. B. H., Insights into the Mechanism and Catalysis of the Native Chemical Ligation Reaction. *Journal of the American Chemical Society* **2006,** *128* (20), 6640-6646; (b) Camarero, J. A.; Muir, T. W., Native Chemical Ligation of Polypeptides. In *Current Protocols in Protein Science*, John Wiley & Sons, Inc.: 2001.

22. Hili, R.; Yudin, A. K., Amphoteric Amino Aldehydes Enable Rapid Assembly of Unprotected Amino Alcohols. *Angewandte Chemie International Edition* **2008,** *47* (22), 4188-4191.

23. Shekhter, T.; Metanis, N.; Dawson, P. E.; Keinan, E., A residue outside the active site CXXC motif regulates the catalytic efficiency of Glutaredoxin 3. *Molecular BioSystems* **2010,** *6* (1), 241-248.

24. Shang, S.; Tan, Z.; Danishefsky, S. J., Application of the logic of cysteine-free native chemical ligation to the synthesis of Human Parathyroid Hormone (hPTH). *Proceedings of the National Academy of Sciences* **2011**.

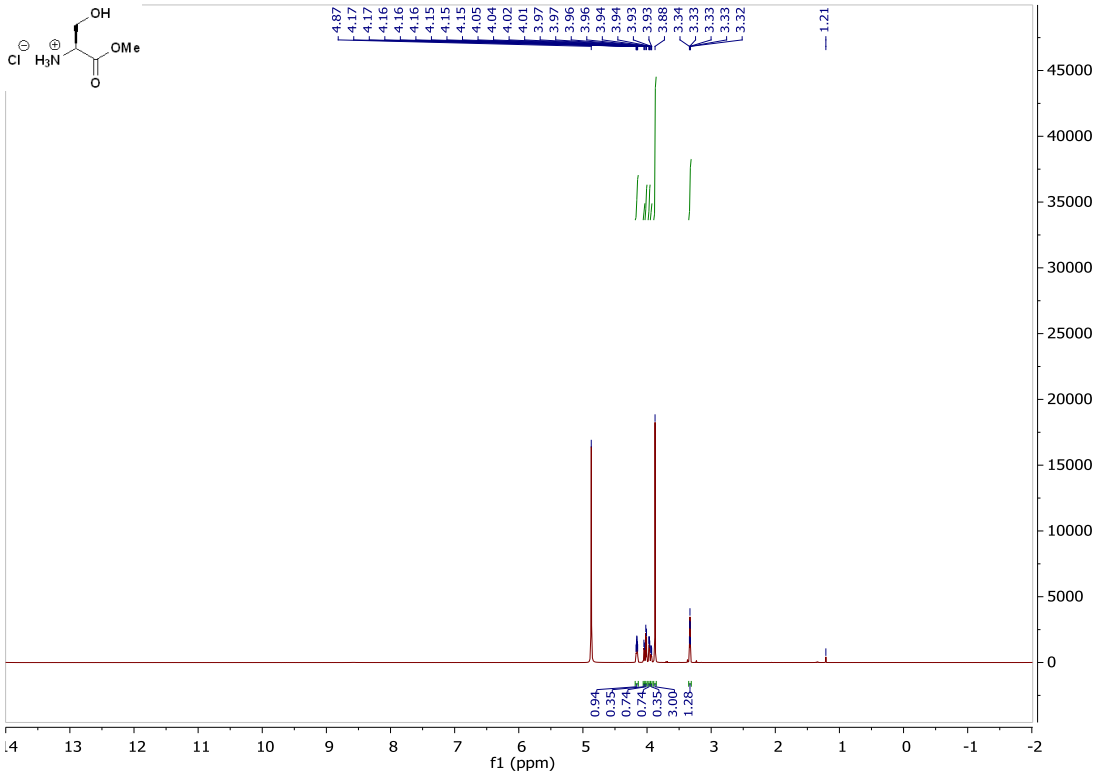
25. Hackeng, T. M.; Griffin, J. H.; Dawson, P. E., Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. *Proceedings of the National Academy of Sciences* **1999,** *96* (18), 10068-10073.

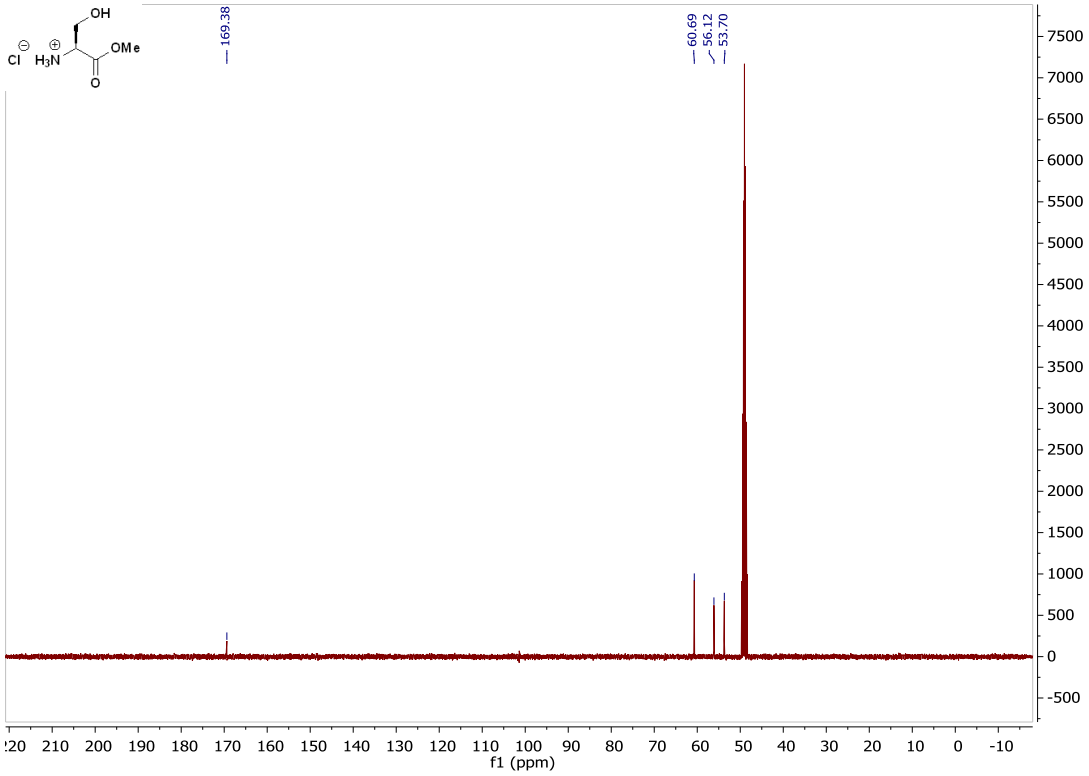
26. Dittmann, M.; Sauermann, J.; Seidel, R.; Zimmermann, W.; Engelhard, M., Native chemical ligation of hydrophobic peptides in organic solvents. *Journal of Peptide Science* **2010,** *16* (10), 558-562.

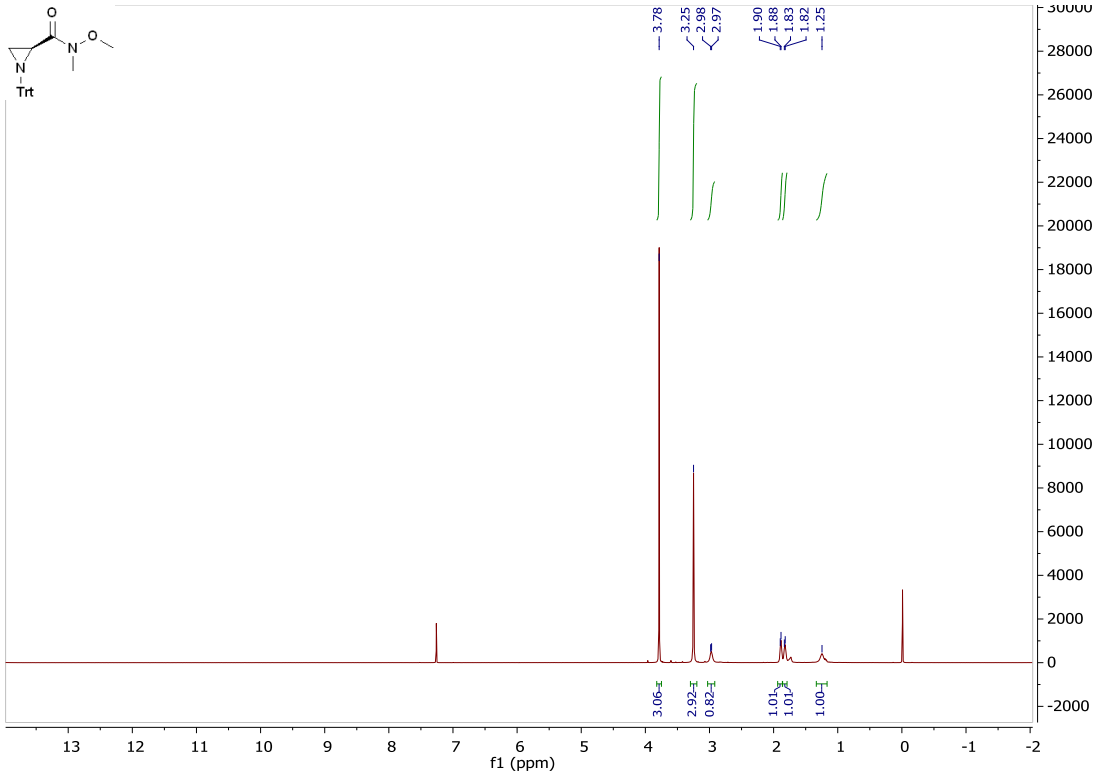
27. Tam, J. P.; Yu, Q., Methionine ligation strategy in the biomimetic synthesis of parathyroid hormones. *Biopolymers* **1998,** *46* (5), 319-327.

28. Zhang, L.; Tam, J. P., Lactone and Lactam Library Synthesis by Silver Ion-Assisted Orthogonal Cyclization of Unprotected Peptides†. *Journal of the American Chemical Society* **1999,** *121* (14), 3311-3320.

Appendix – 1H NMR, 13C NMR, ESI-MS and HPLC Spectra



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N
O
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Trt
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3.25
2.98
2.97
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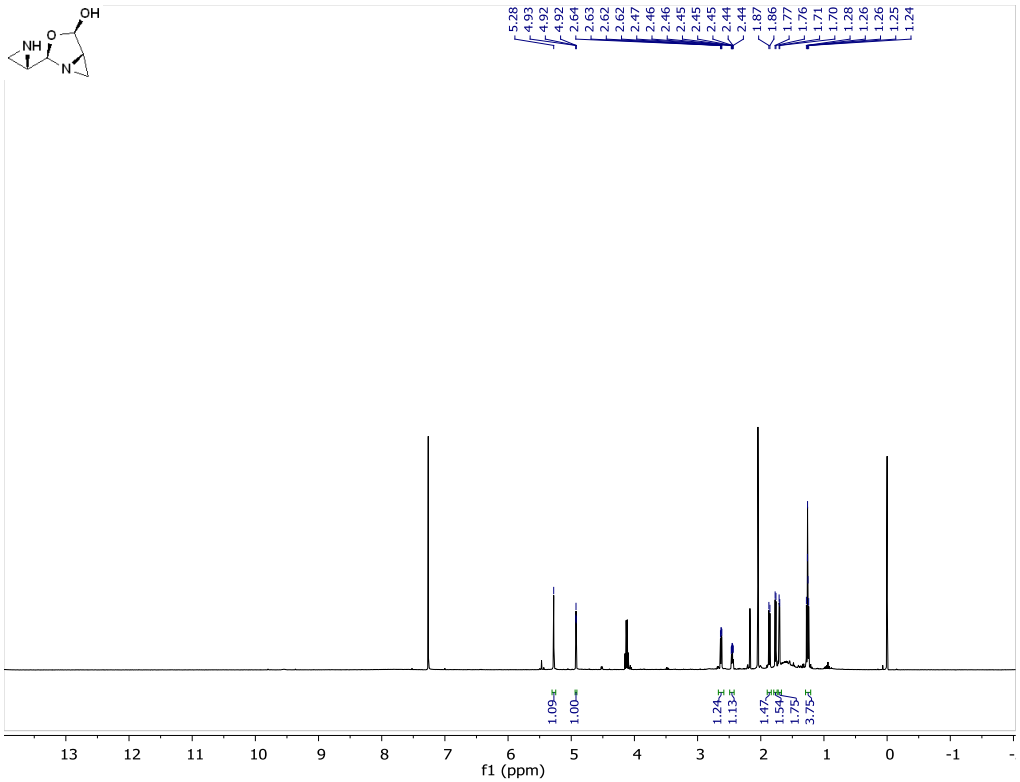
1.82
1.25
3.06
2.92
0.82
1.01
1.01
1.00
f1 (ppm)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O
NH
N
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4.93
4.92
4.92
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2.63
2.62
2.62
2.47
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2.45
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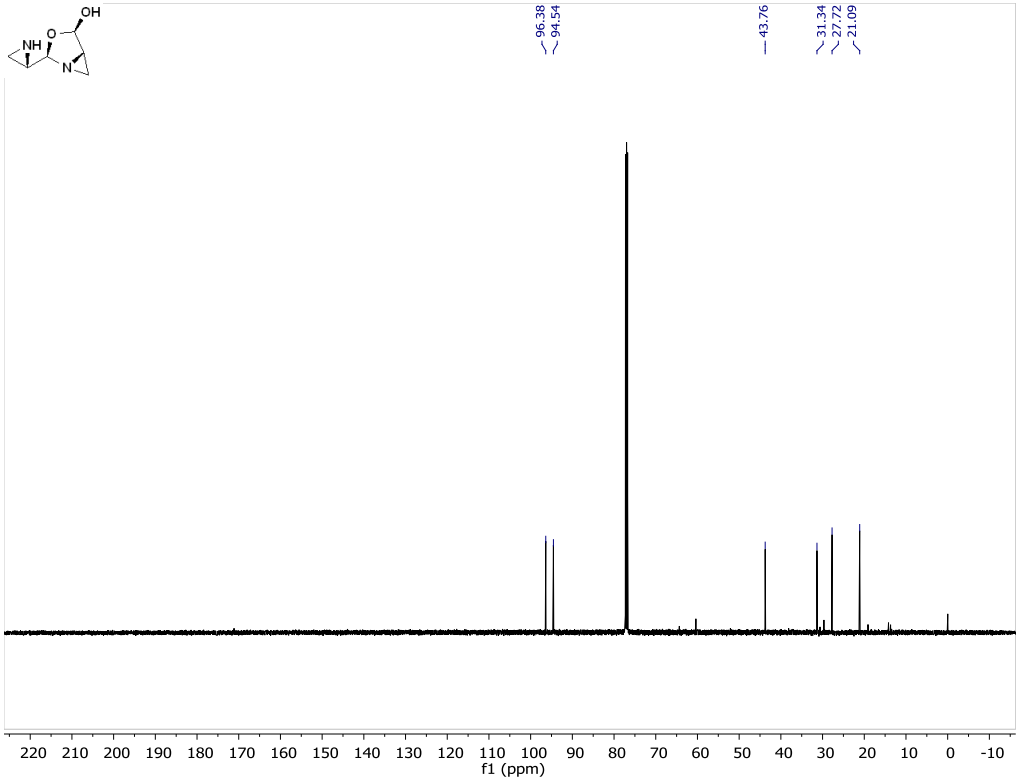
2.45
2.44
2.44
1.87
1.86
1.77
1.76
1.71
1.70
1.28
1.26
1.26
1.25
1.24
1.09
1.00
1.24
1.13
1.47
1.54
1.75
3.75
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f1 (ppm)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N
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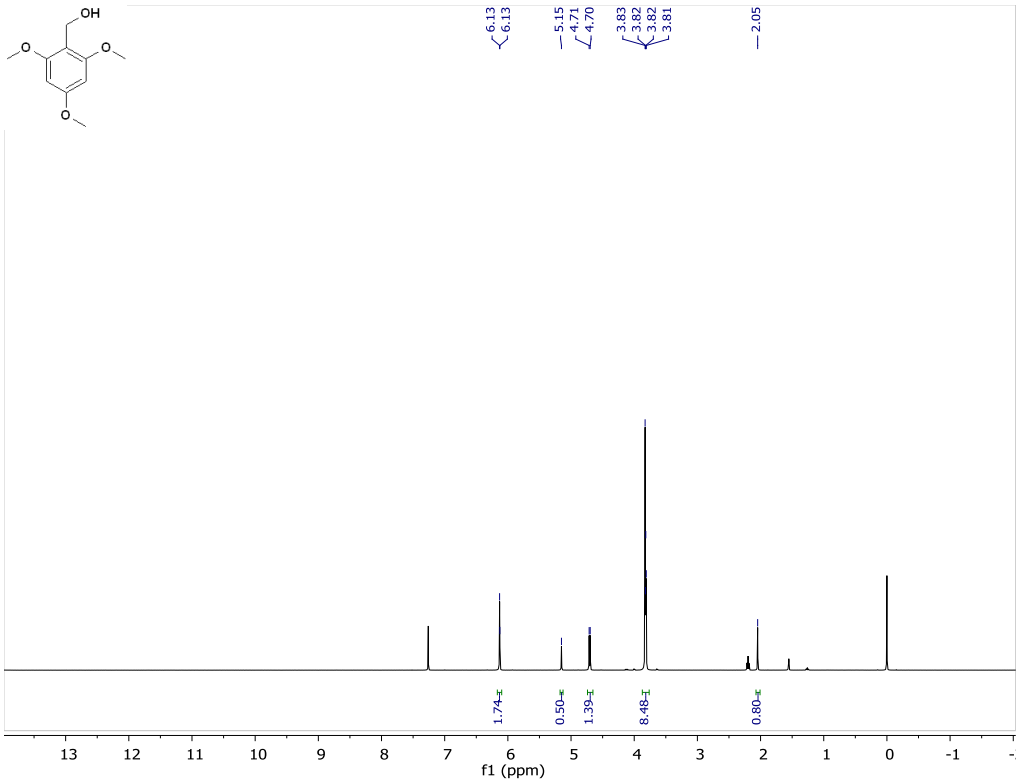
21.09
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)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O
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3.82
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13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
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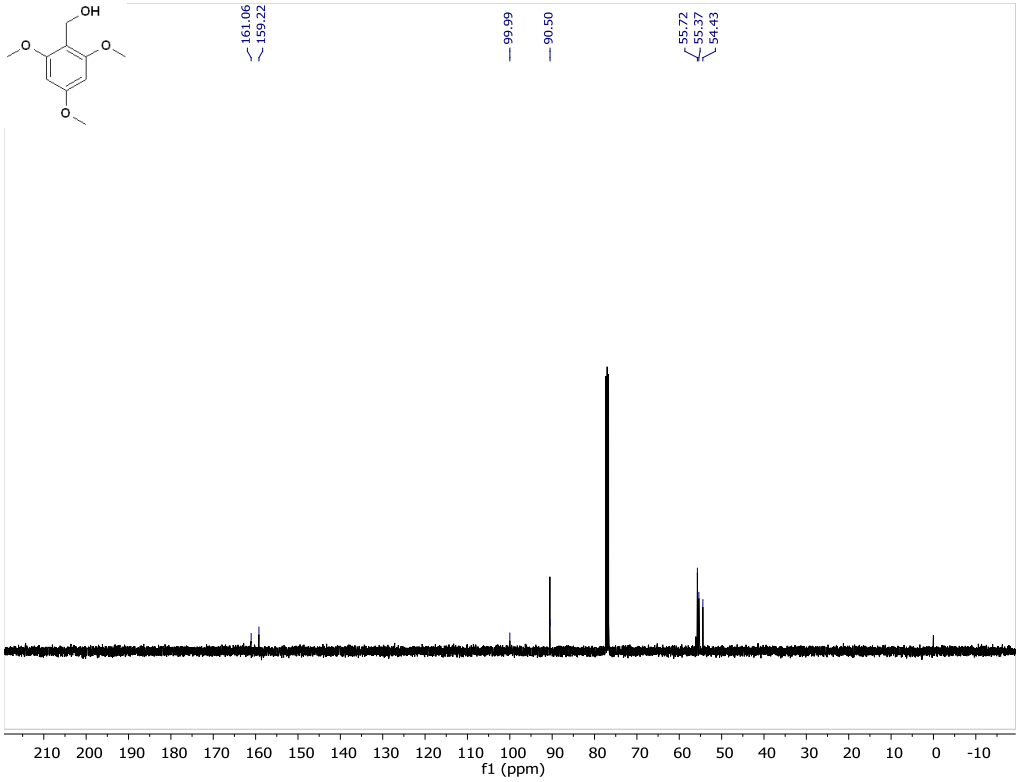
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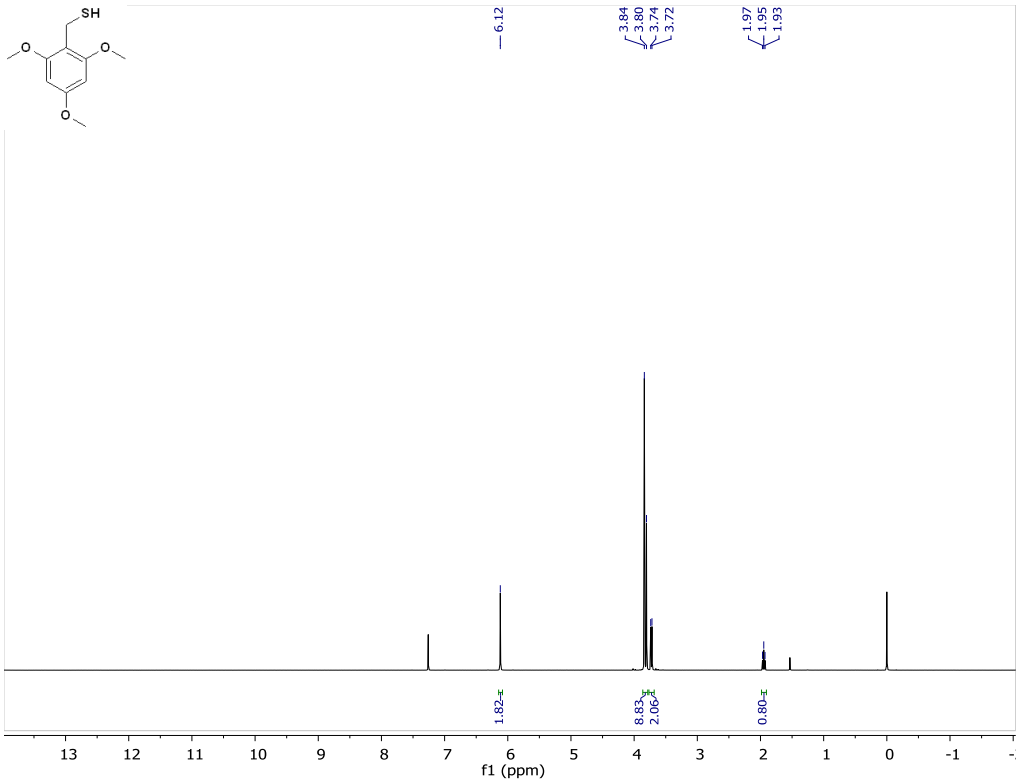
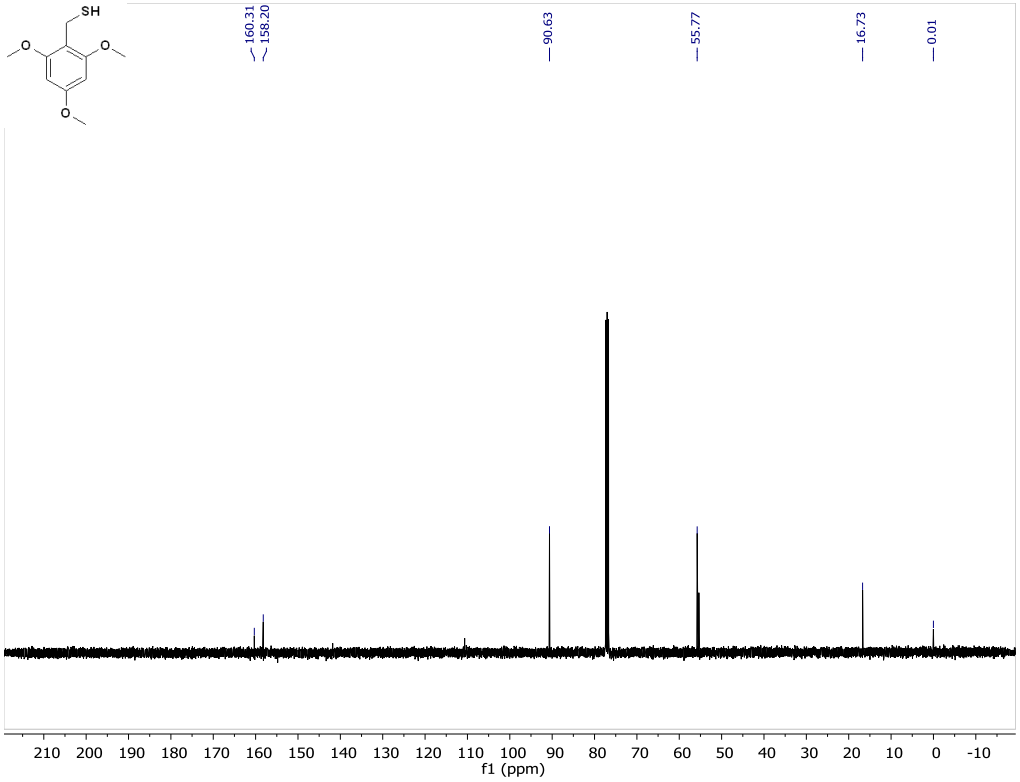
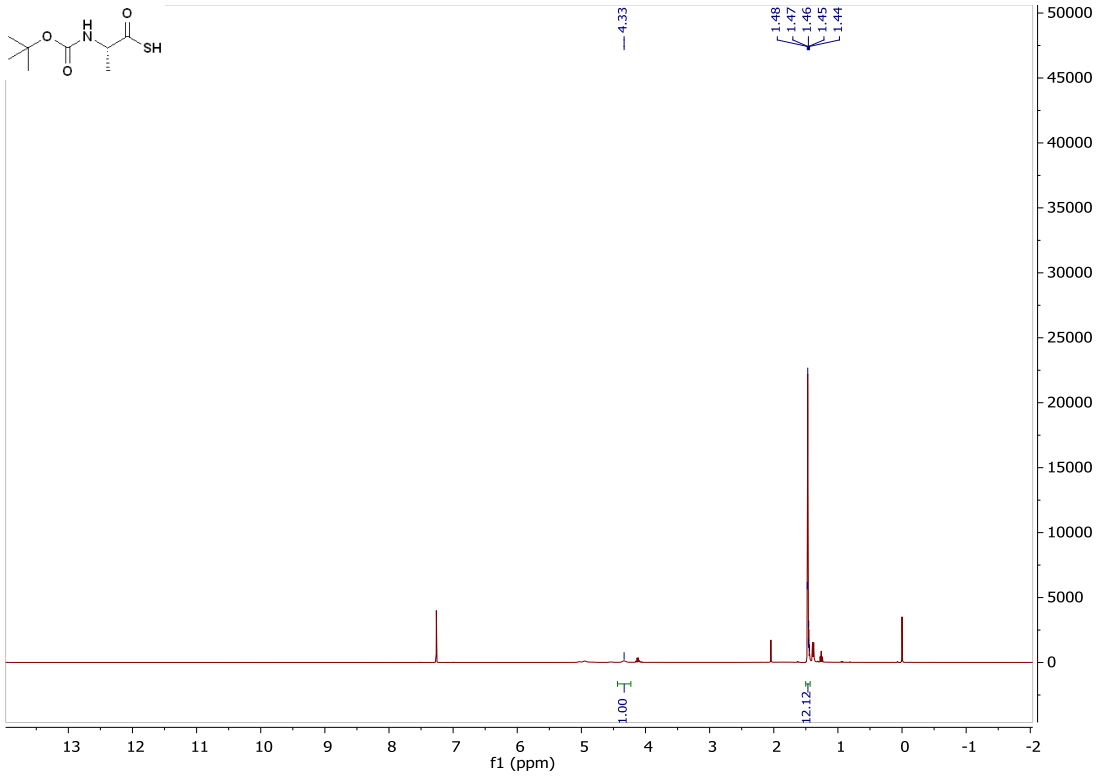
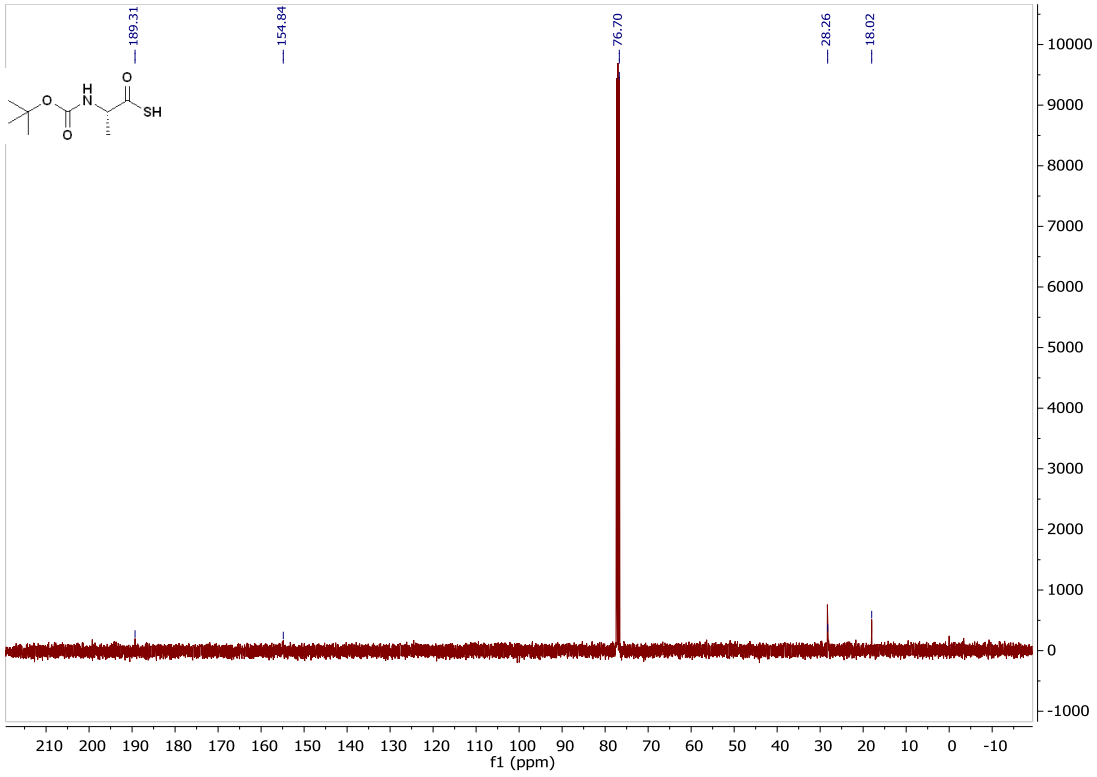
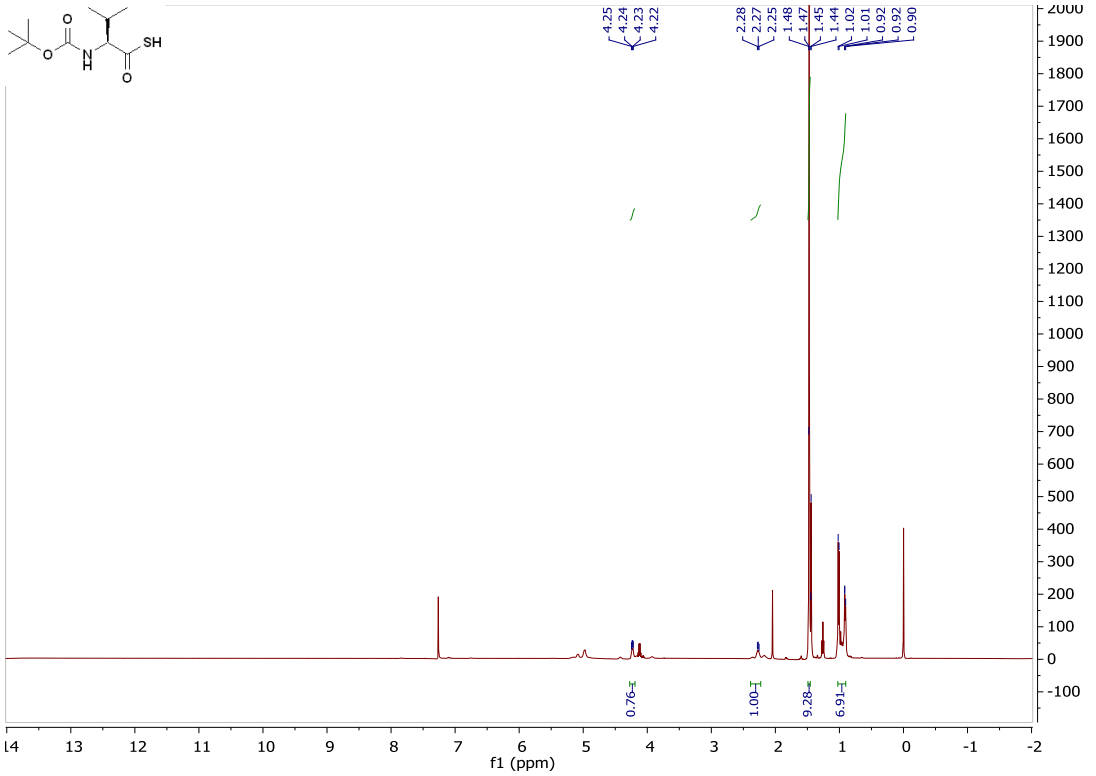
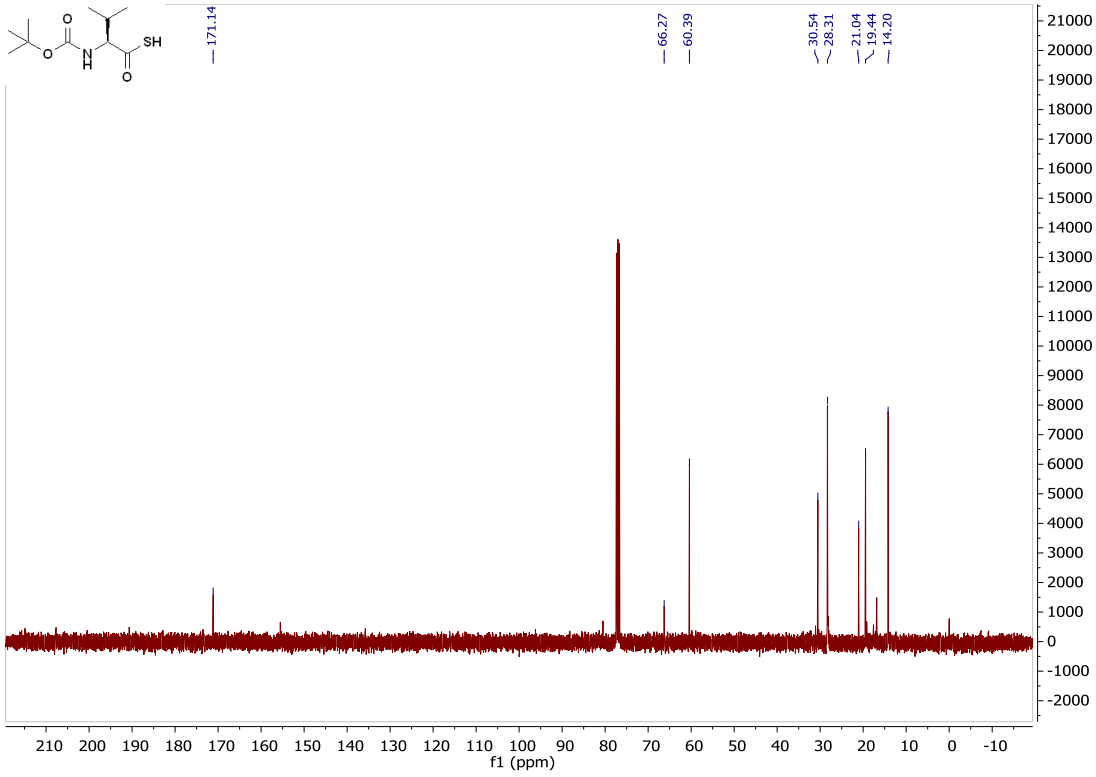

SH
O
O
O
6.12
3.84
3.80
3.74
3.72
1.97
1.95
1.93
1.82
8.83
2.06
0.80
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f1 (ppm)

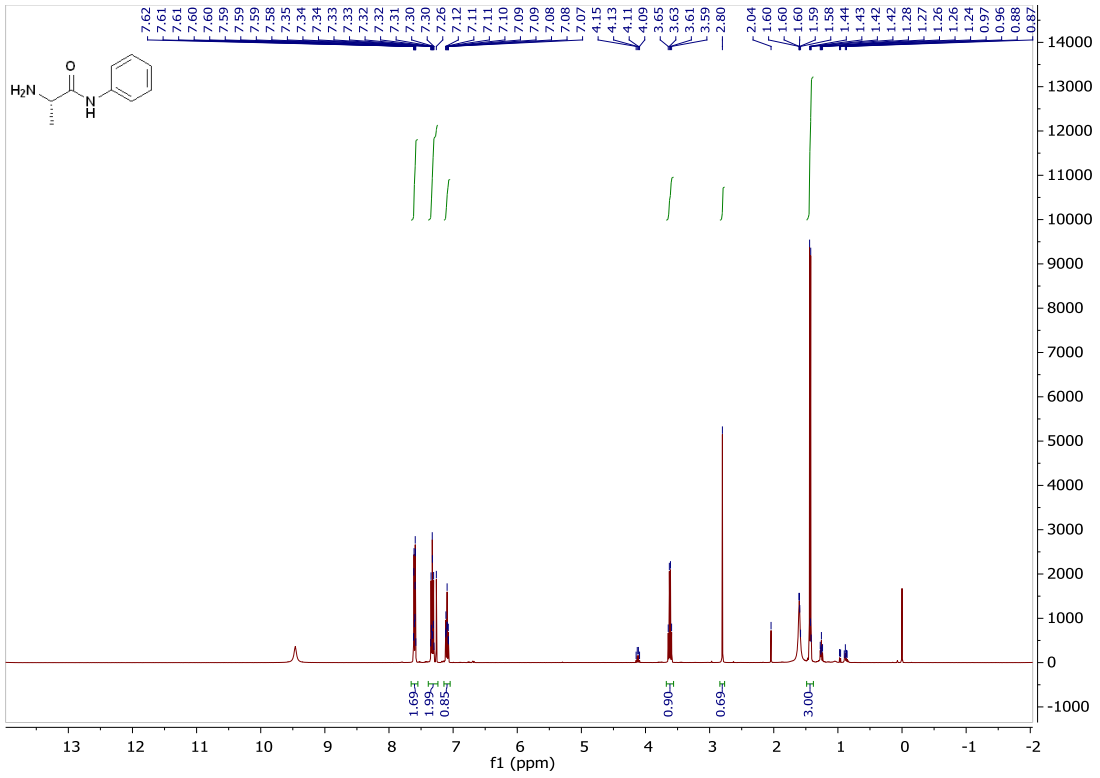

SH
O
O
O
160.31
158.20
90.63
55.77
16.73
0.01
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

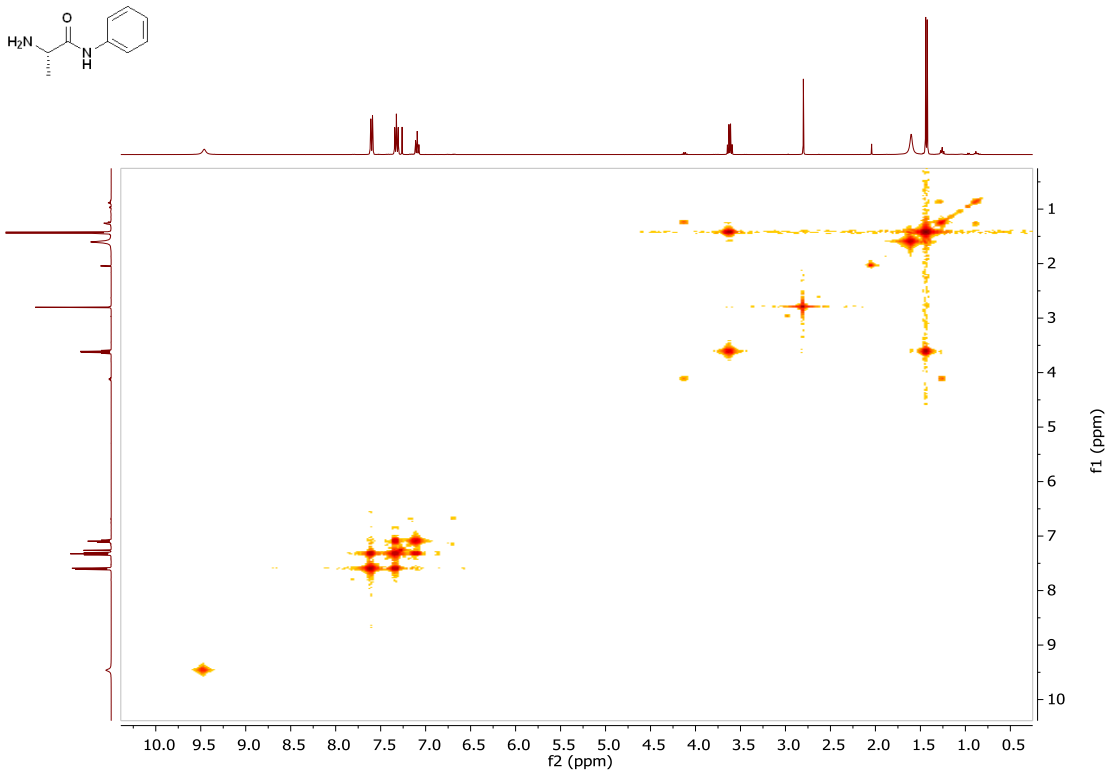

4.33
1.48
1.47
1.46
1.45
1.44
1.00
12.12
f1 (ppm)

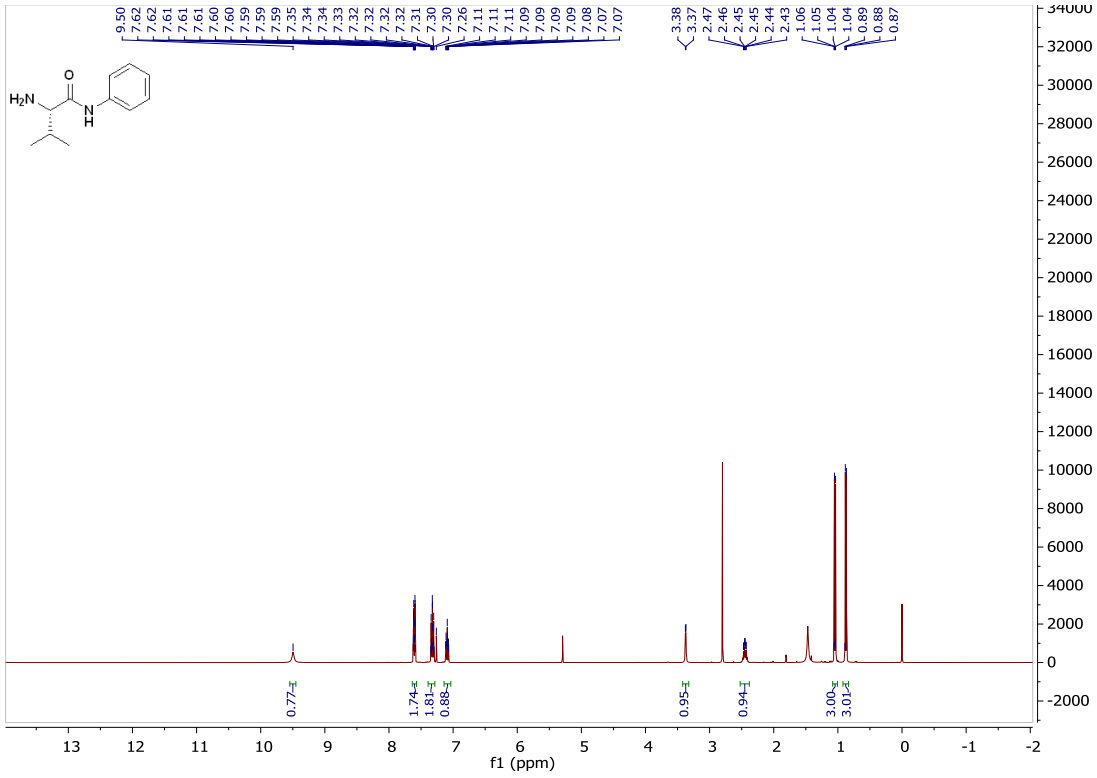

189.31
154.84
76.70
28.26
18.02
f1 (ppm)

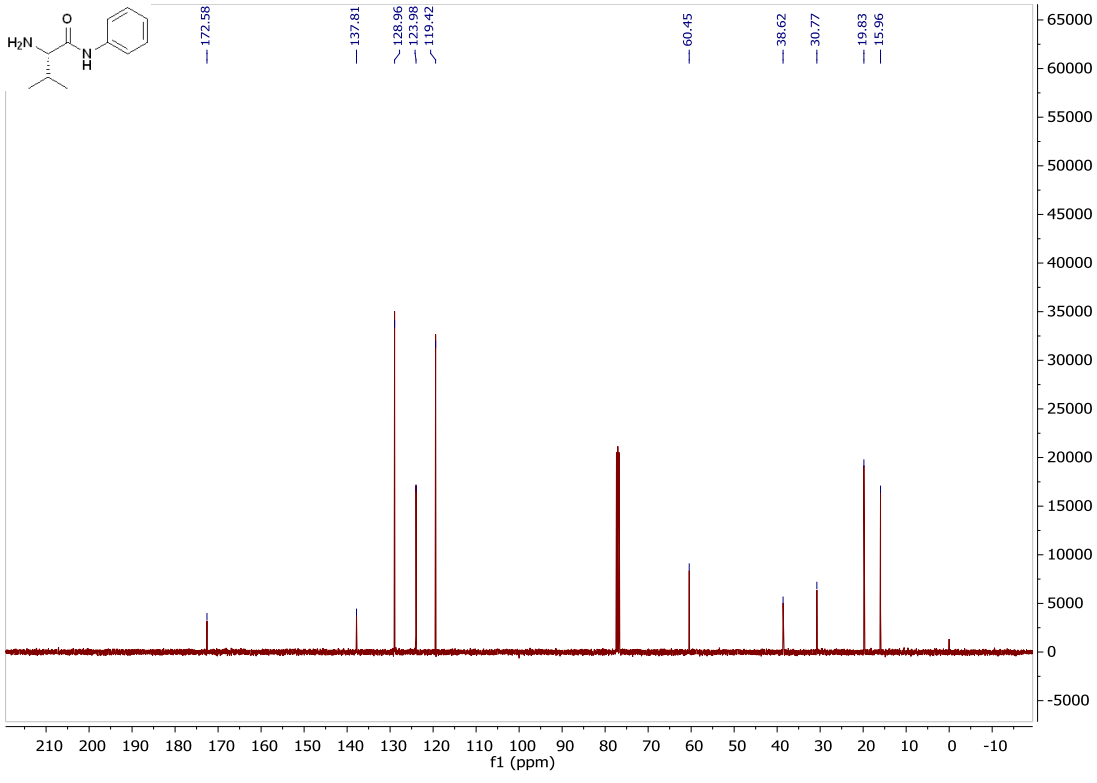

4.25
4.24
4.23
4.22
2.28
2.27
2.25
1.48
1.47
1.45
1.44
1.02
1.01
0.92
0.92
0.90
0.76
1.00
9.28
6.91
f1 (ppm)

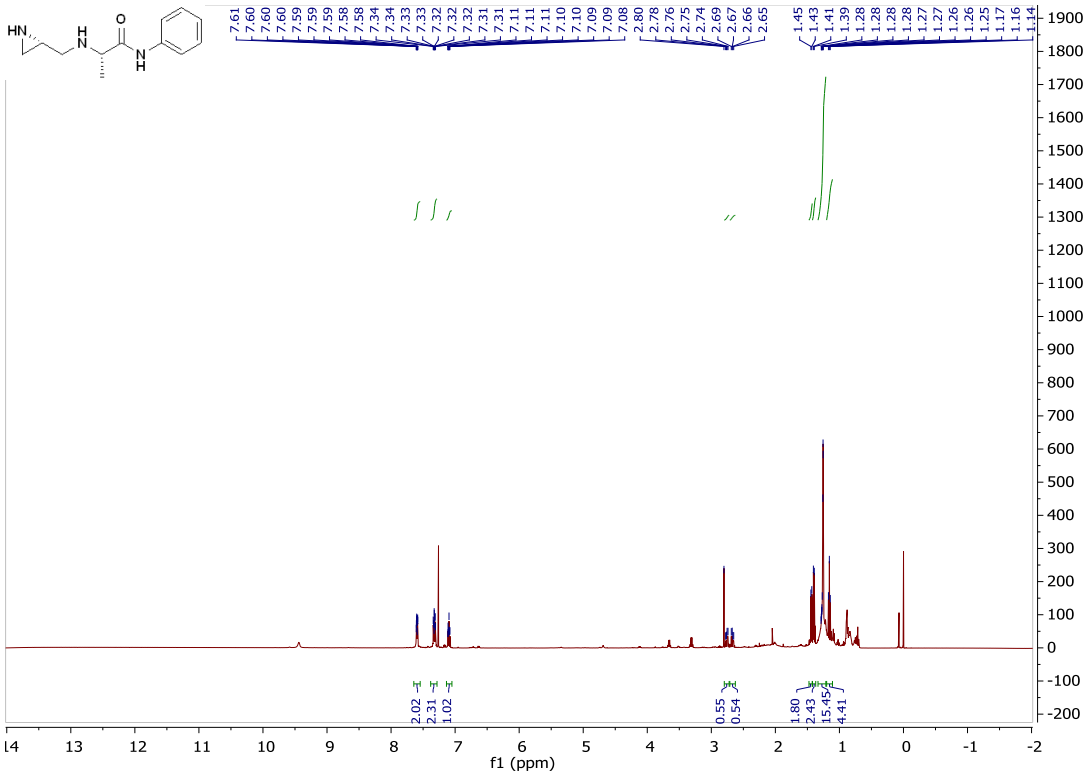

171.14
66.27
60.39
30.54
28.31
21.04
19.44
14.20
f1 (ppm)

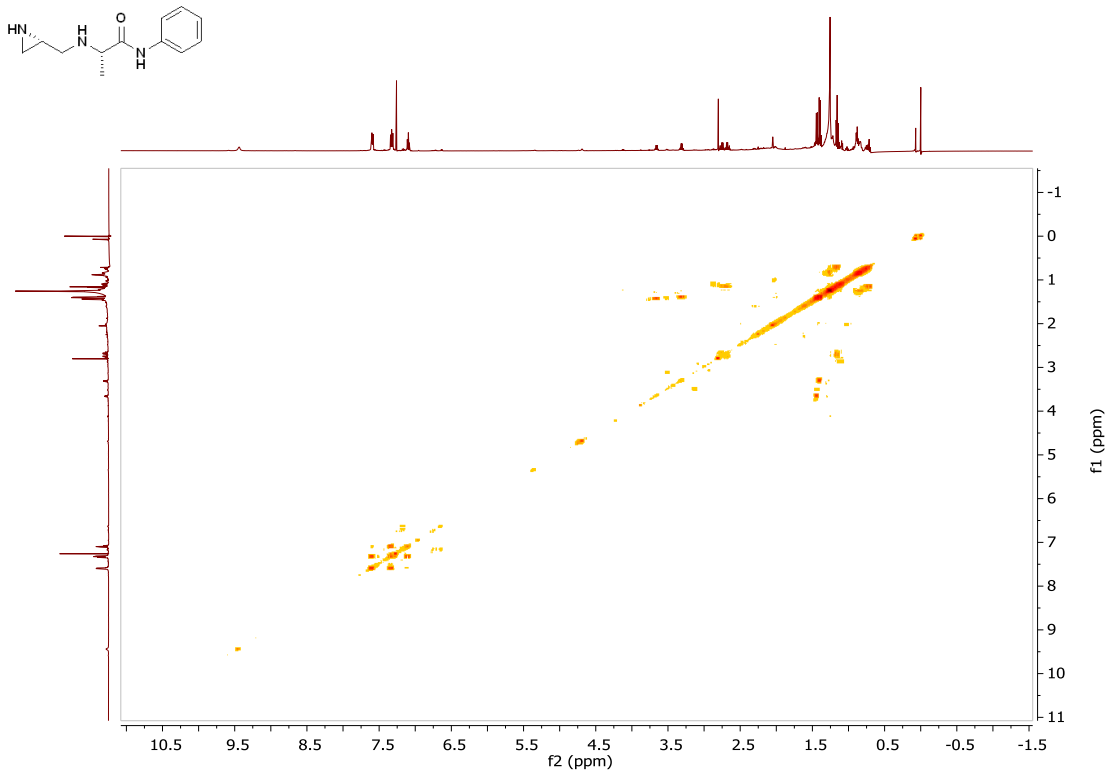

7.62 7.61 7.61 7.60 7.60 7.59 7.59 7.59 7.58 7.35 7.34 7.34 7.33 7.33 7.32 7.32 7.31 7.30 7.30 7.26 7.12 7.11 7.11 7.10 7.09 7.09 7.08 7.08 7.07 4.15 4.13 4.11 4.09 3.65 3.63 3.61 3.59 2.80 2.04 1.60 1.60 1.60 1.59 1.58 1.44 1.43 1.42 1.42 1.28 1.27 1.26 1.24 0.97 0.96 0.88 0.87
1.69 1.99 0.85 0.90 0.69 3.00
f1 (ppm)

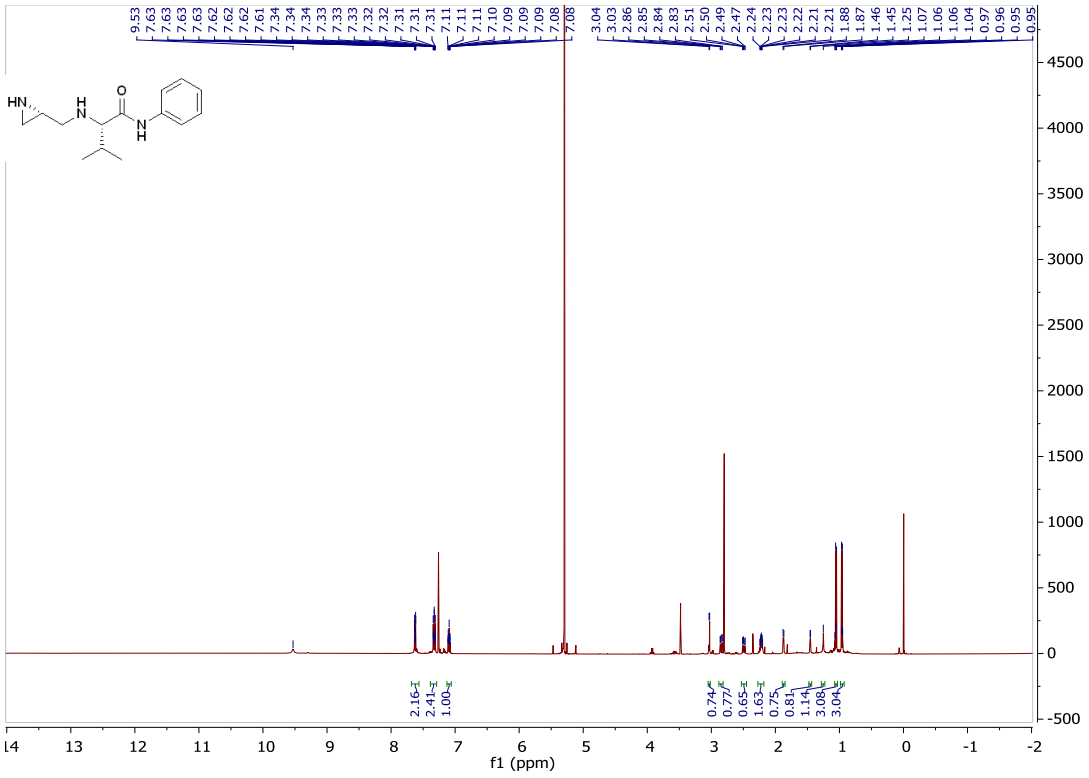

f2 (ppm)
f1 (ppm)

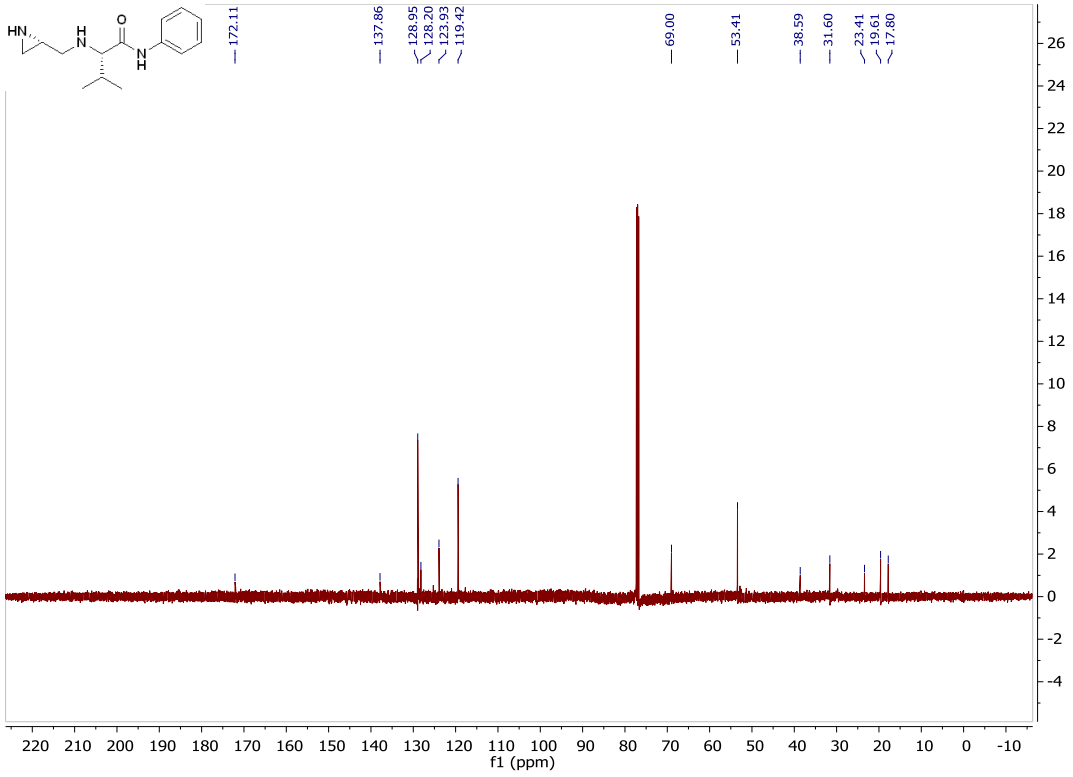

9.50
7.62
7.62
7.61
7.61
7.61
7.60
7.60
7.59
7.59
7.59
7.59
7.35
7.34
7.34
7.33
7.32
7.32
7.32
7.32
7.31
7.30
7.30
7.26
7.11
7.11
7.11
7.09
7.09
7.09
7.09
7.08
7.07
7.07
3.38
3.37
2.47
2.46
2.45
2.45
2.44
2.43
1.06
1.05
1.04
1.04
0.89
0.88
0.87
0.77
1.74
1.81
0.88
0.95
0.94
3.00
3.01
f1 (ppm)

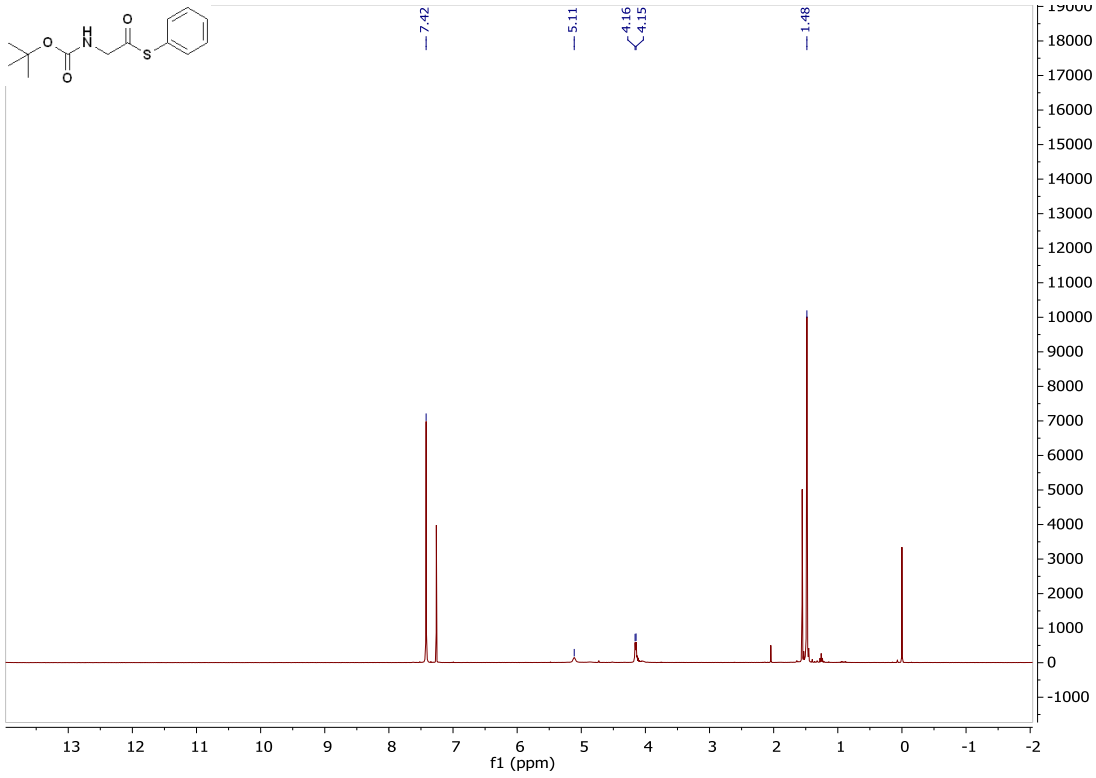

172.58
137.81
128.96
123.98
119.42
60.45
38.62
30.77
19.83
15.96
f1 (ppm)

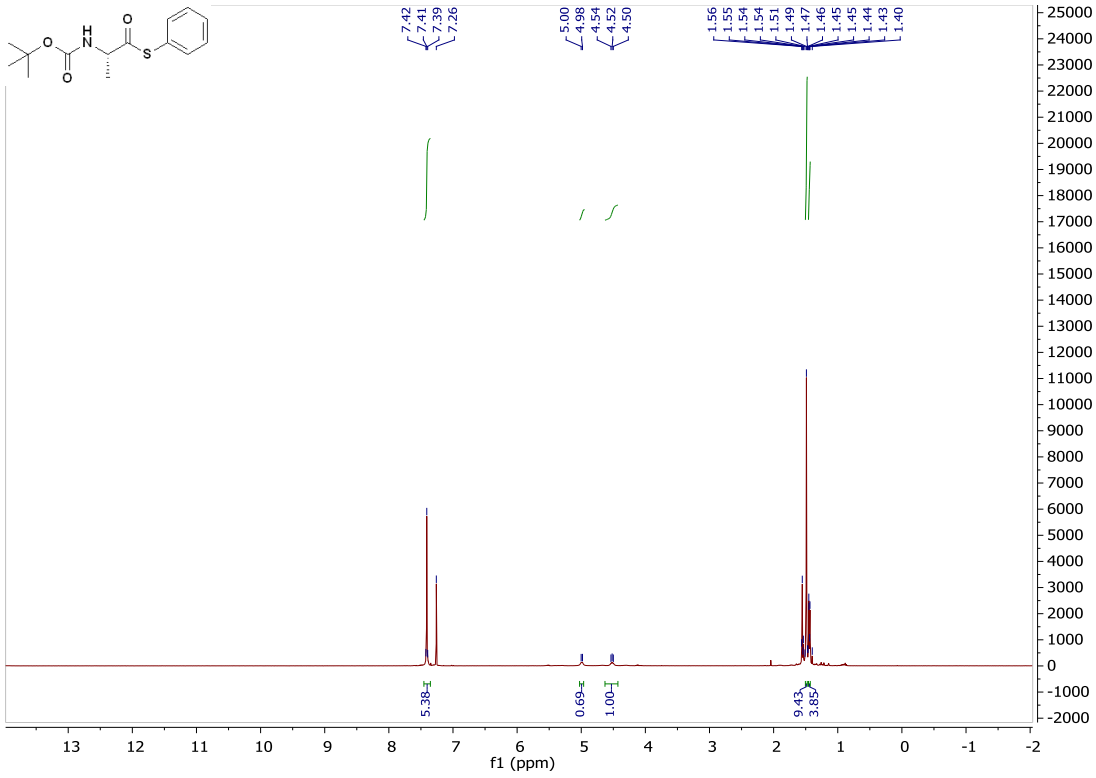

f1 (ppm)

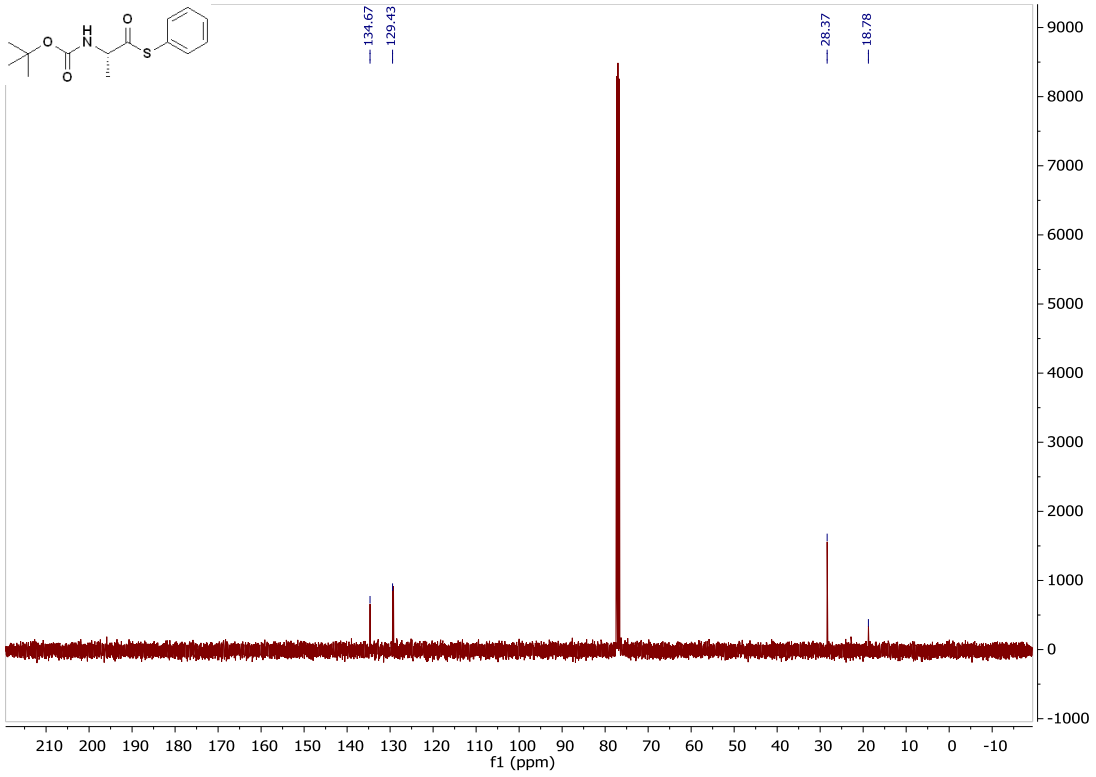

f2 (ppm)
f1 (ppm)

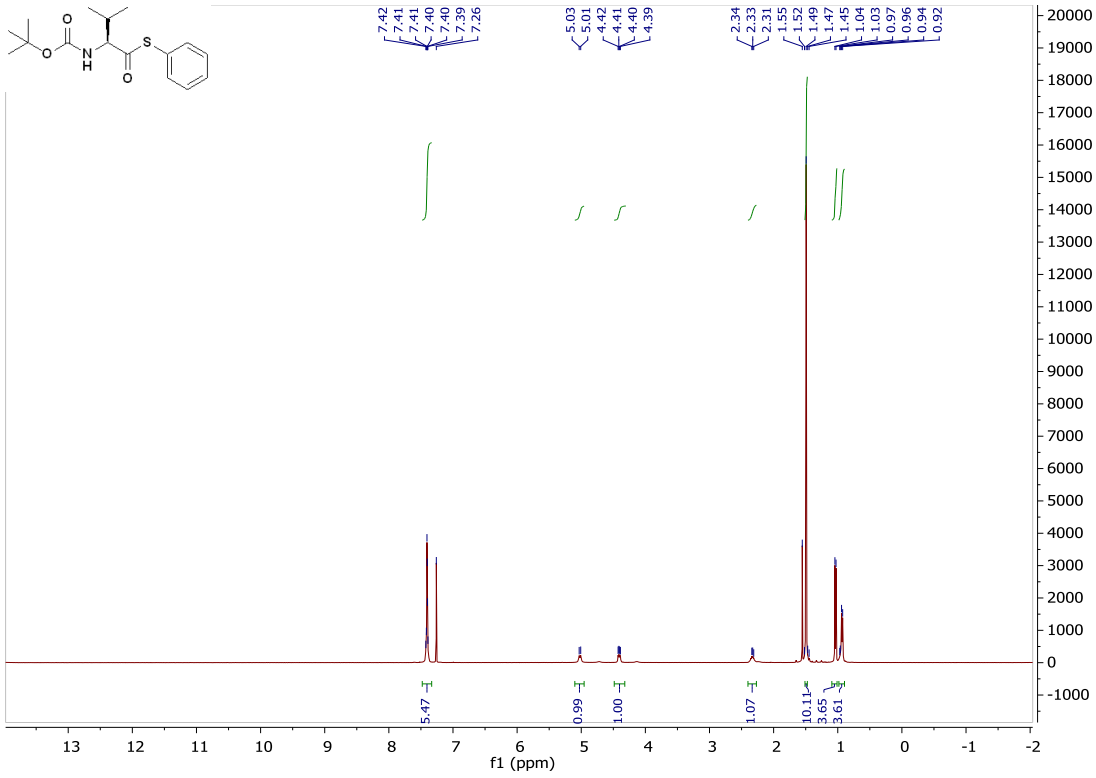

HN
H
N
O
N
H
9.53
7.63
7.62
7.61
7.34
7.33
7.32
7.31
7.11
7.10
7.09
7.08
3.04
3.03
2.86
2.85
2.84
2.83
2.51
2.50
2.49
2.47
2.24
2.23
2.22
2.21
1.88
1.87
1.46
1.45
1.25
1.07
1.06
1.04
0.97
0.96
0.95
2.16
2.41
1.00
0.74
0.77
0.65
1.63
0.75
0.81
1.14
3.08
3.04
f1 (ppm)

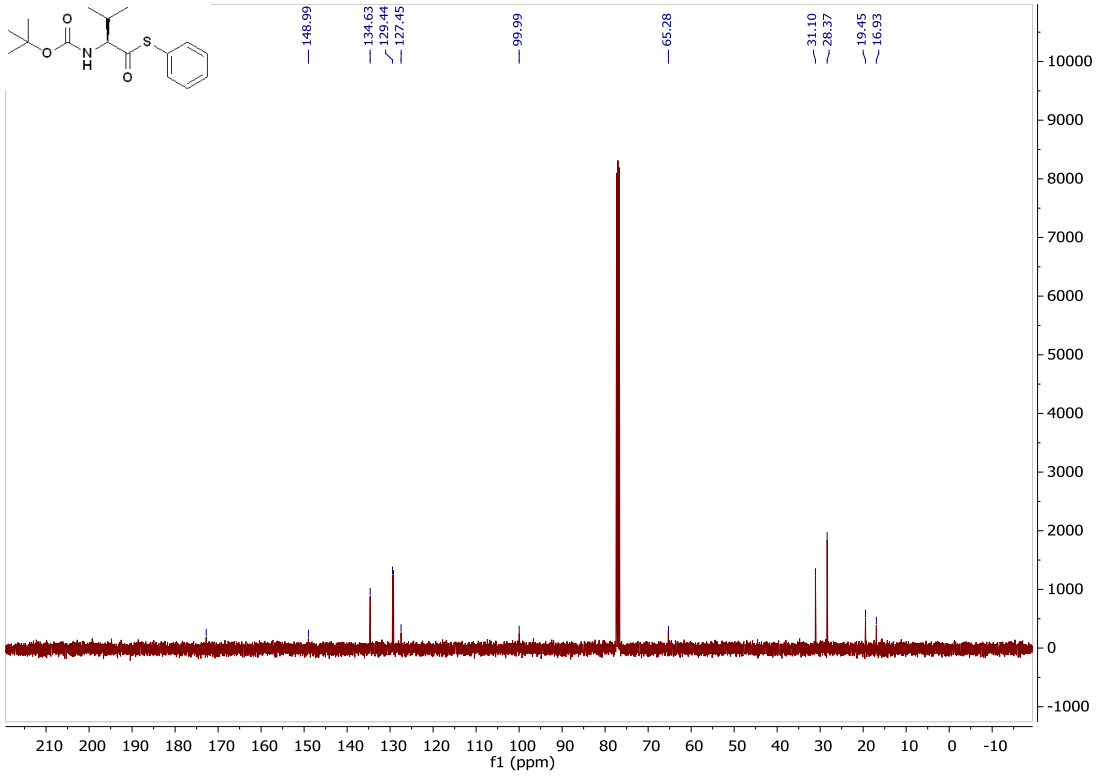

HN
H
N
O
N
H
172.11
137.86
128.95
128.20
123.93
119.42
69.00
53.41
38.59
31.60
23.41
19.61
17.80
f1 (ppm)

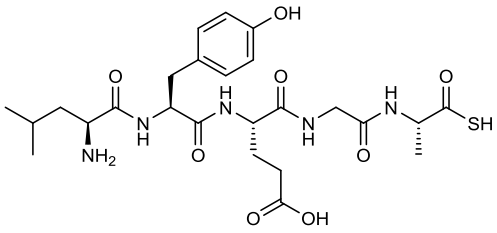

7.42
5.11
4.16
4.15
1.48
f1 (ppm)

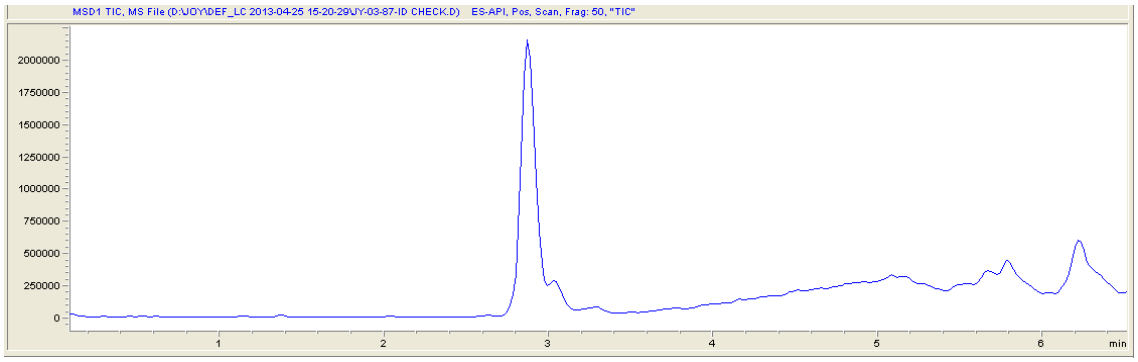

7.42
7.41
7.39
7.26
5.00
4.98
4.54
4.52
4.50
1.56
1.55
1.54
1.54
1.51
1.49
1.47
1.46
1.45
1.45
1.44
1.43
1.40
5.38
0.69
1.00
9.43
3.85
f1 (ppm)

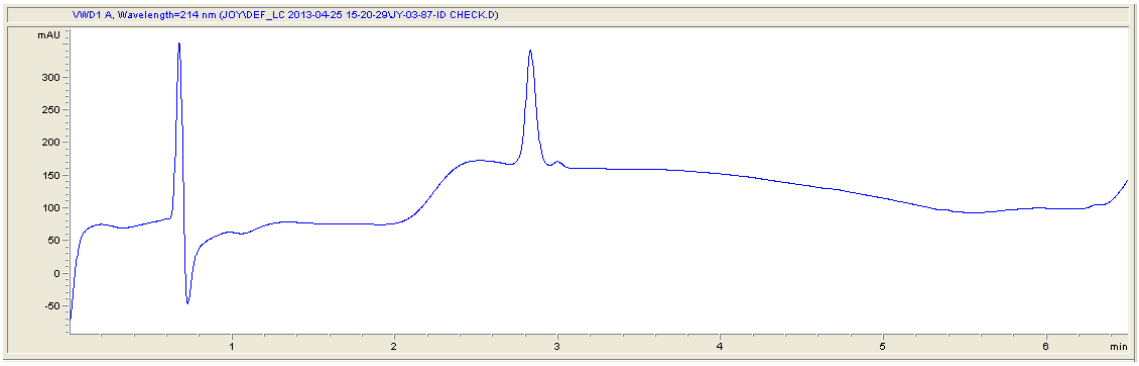

134.67
129.43
28.37
18.78
f1 (ppm)

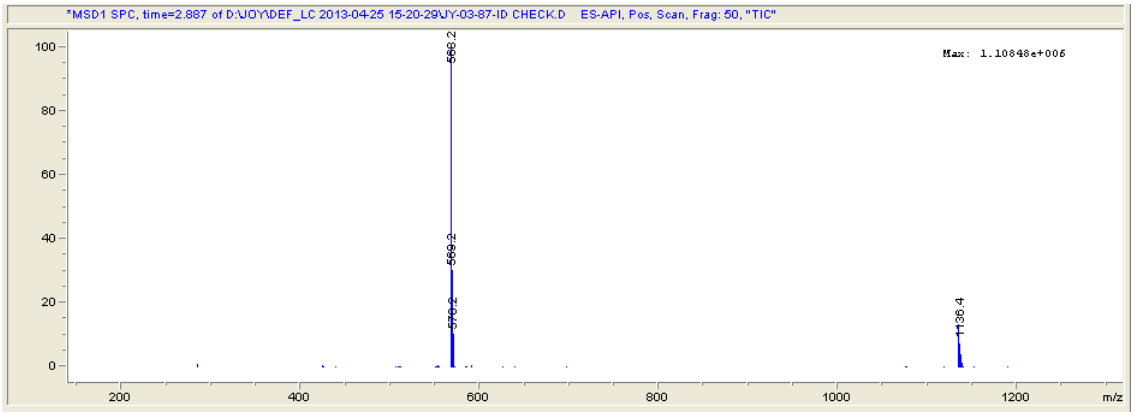

7.42
7.41
7.41
7.40
7.40
7.39
7.26
5.03
5.01
4.42
4.41
4.40
4.39
2.34
2.33
2.31
1.55
1.52
1.49
1.47
1.45
1.04
1.03
0.97
0.96
0.94
0.92
5.47
0.99
1.00
1.07
10.11
3.65
3.61
f1 (ppm)

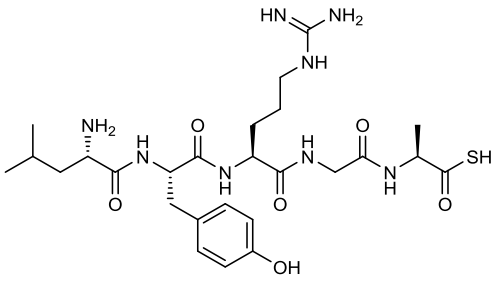

148.99
134.63
129.44
127.45
99.99
65.28
31.10
28.37
19.45
16.93
f1 (ppm)

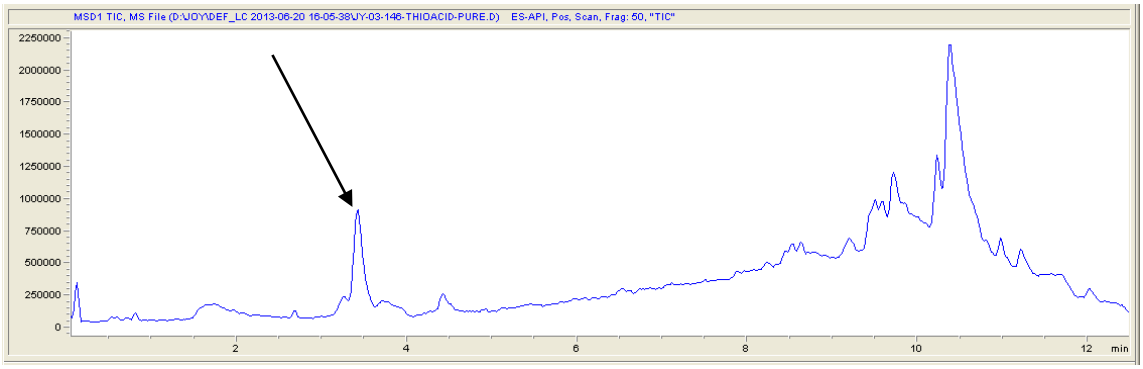

OH
O
N
H
O
NH2
O
N
H
O
N
H
O
SH
O
OH

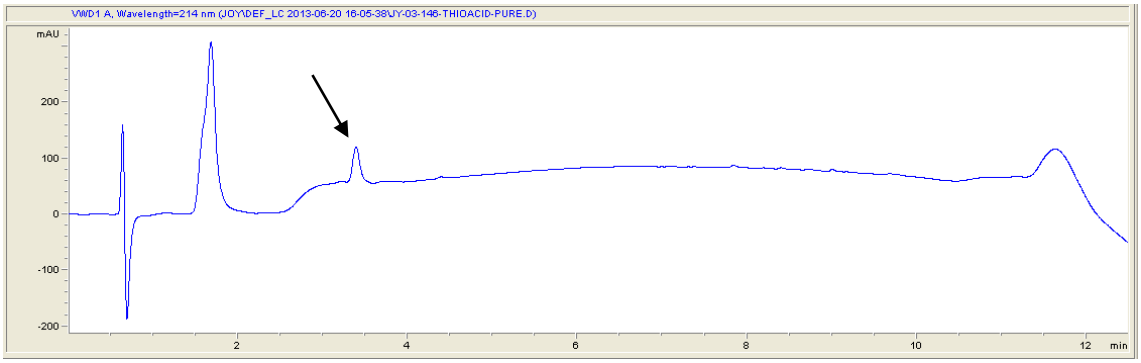

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-04-25 15-20-29\JY-03-87-ID CHECK.D) ES-API, Pos, Scan, Frag: 50, "TIC"
2000000
1750000
1500000
1250000
1000000
750000
500000
250000
0
1
2
3
4
5
6
min

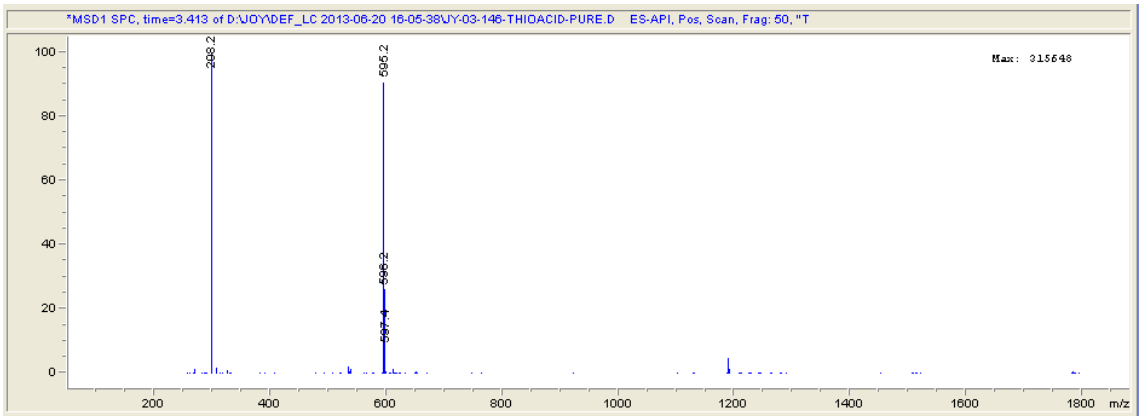

VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-04-25 15-20-29\JY-03-87-ID CHECK.D)
mAU
300
250
200
150
100
50
0
-50
1
2
3
4
5
6
min

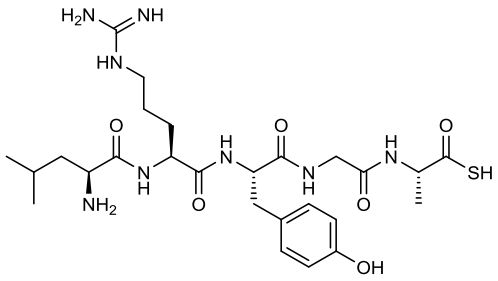

"MSD1 SPC, time=2.887 of D:\JOY\DEF_LC 2013-04-25 15-20-29\JY-03-87-ID CHECK.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 1.10848e+006
566.2
569.2
576.2
1136.4
100
80
60
40
20
0
200
400
600
800
1000
1200
m/z

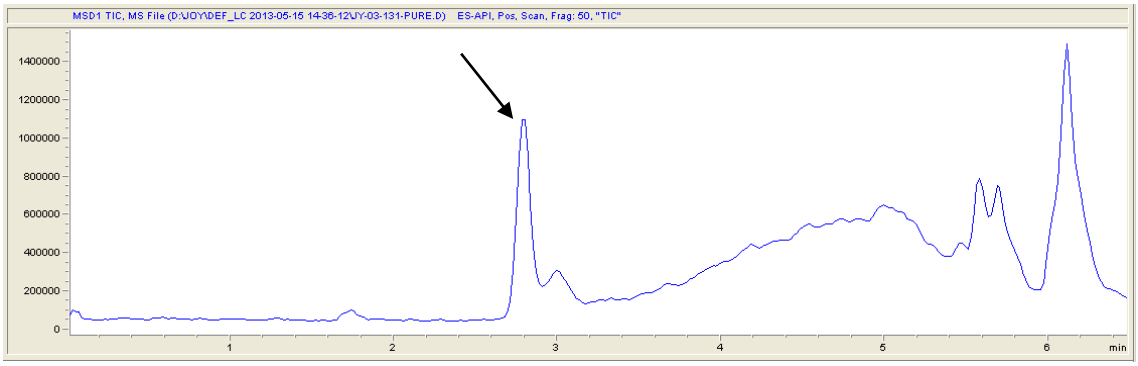

HN
NH2
NH
NH2
H
N
O
N
H
O
H
N
O
N
H
SH
O
O
O
OH

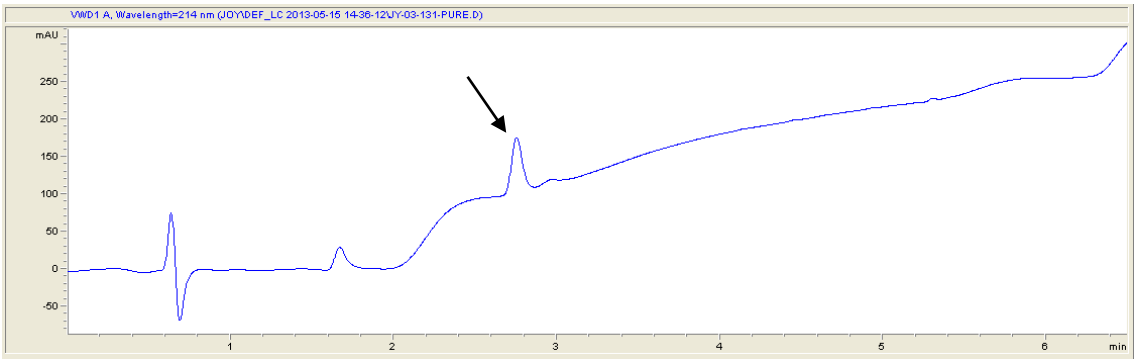

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-06-20 16-05-38\JY-03-146-THIOACID-PURE.D) ES-API, Pos, Scan, Frag: 50, "TIC"
2250000
2000000
1750000
1500000
1250000
1000000
750000
500000
250000
0
2
4
6
8
10
12
min

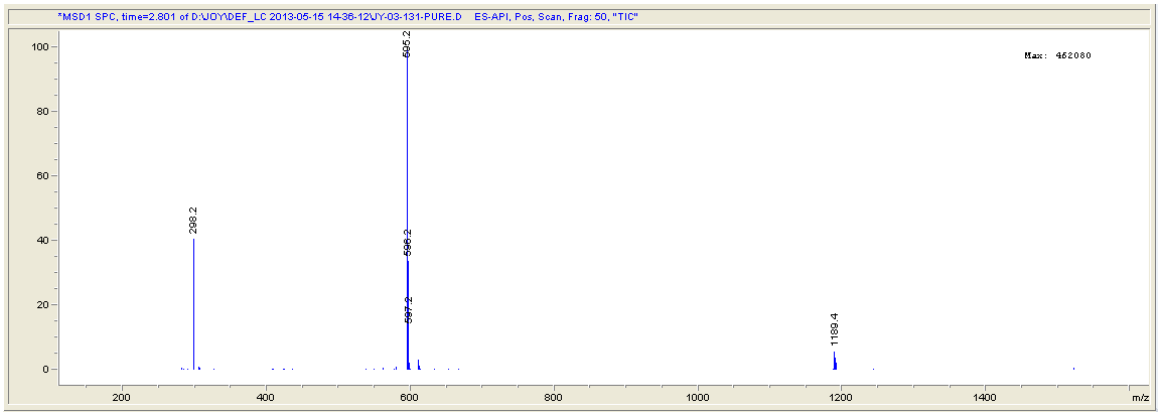

VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-06-20 16-05-38\JY-03-146-THIOACID-PURE.D)
mAU
200
100
0
-100
-200
2
4
6
8
10
12
min

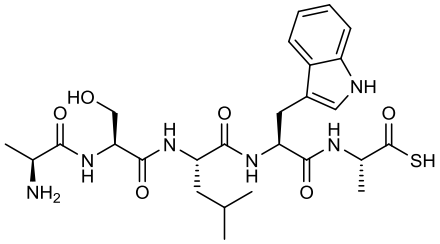

*MSD1 SPC, time=3.413 of D:\JOY\DEF_LC 2013-06-20 16-05-38\JY-03-146-THIOACID-PURE.D ES-API, Pos, Scan, Frag: 50, "T
Max: 315648
298.2
595.2
596.2
597.4
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
m/z

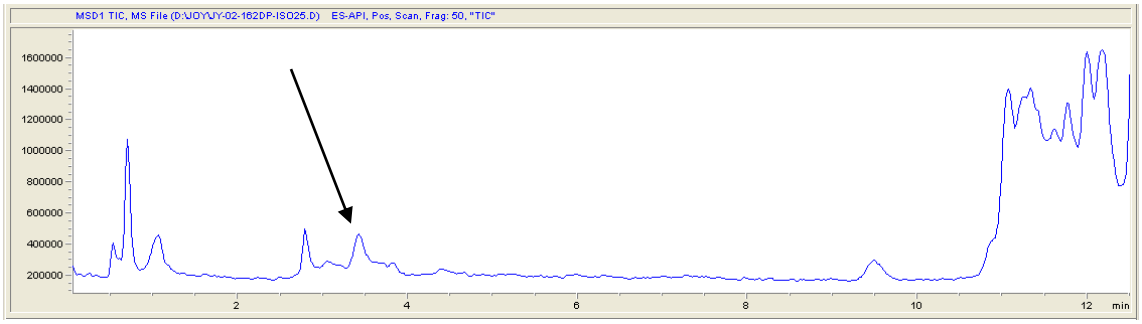

H2N
NH
HN
O
N
H
NH2
O
H
N
O
N
H
O
H
N
O
SH
OH

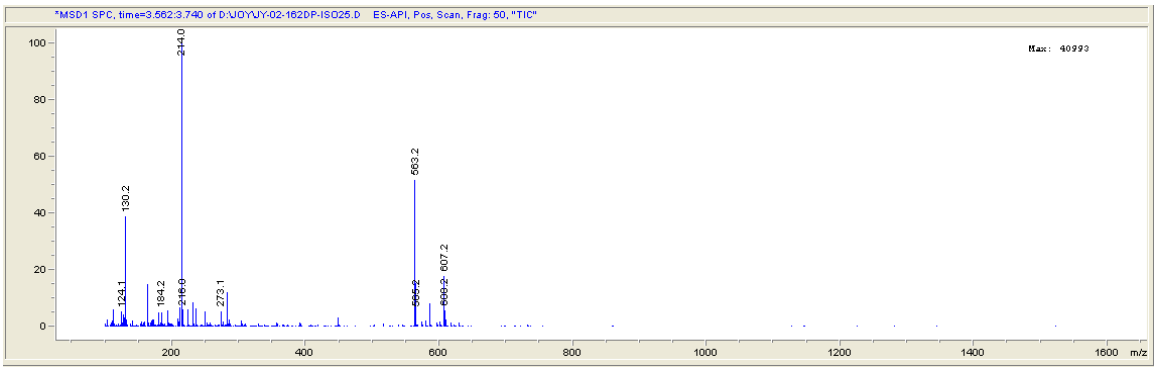

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-05-15 14-36-12\JY-03-131-PURE.D) ES-API, Pos, Scan, Frag: 50, "TIC"
1400000
1200000
1000000
800000
600000
400000
200000
0
1
2
3
4
5
6
min

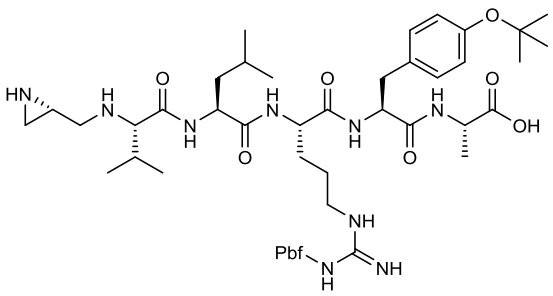

VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-05-15 14-36-12\JY-03-131-PURE.D)
mAU
250
200
150
100
50
0
-50
1
2
3
4
5
6
min

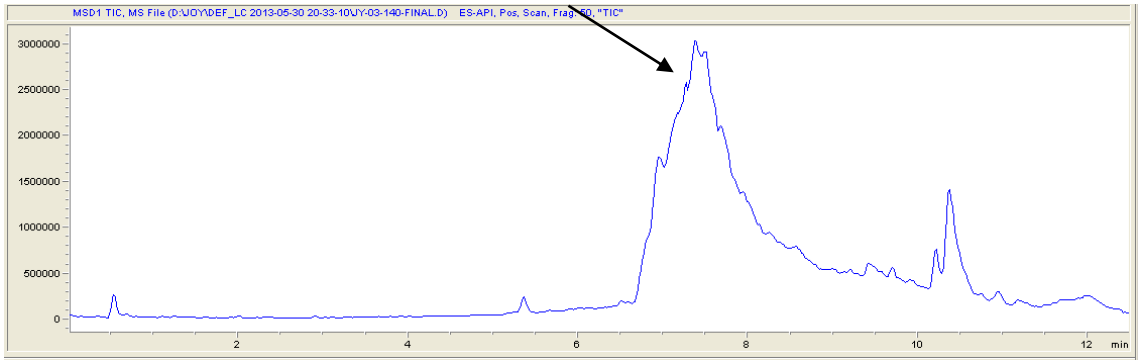

"MSD1 SPC, time=2.801 of D:\JOY\DEF_LC 2013-05-15 14-36-12\JY-03-131-PURE.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 462080
100
80
60
40
20
0
298.2
665.2
666.2
667.2
1188.4
200
400
600
800
1000
1200
1400
m/z

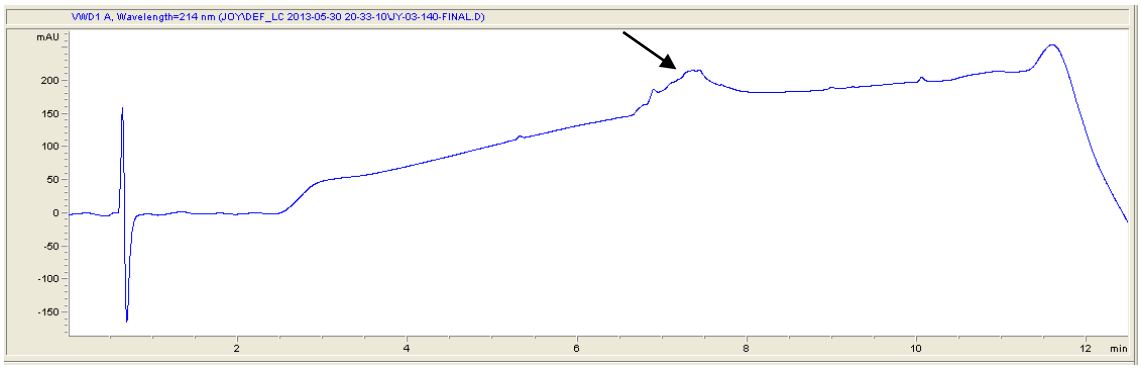

HO
O
NH
O
H
N
O
H
N
O
H
N
O
SH
NH2
O
N
H
O

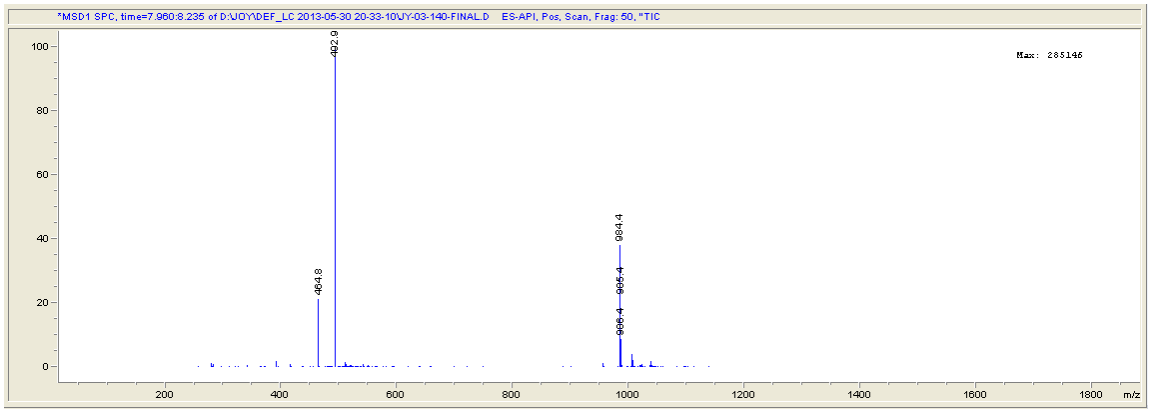

MSD1 TIC, MS File (D:\JOY\JY-02-162DP-ISO25.D) ES-API, Pos, Scan, Frag: 50, "TIC"
1600000
1400000
1200000
1000000
800000
600000
400000
200000
2
4
6
8
10
12
min

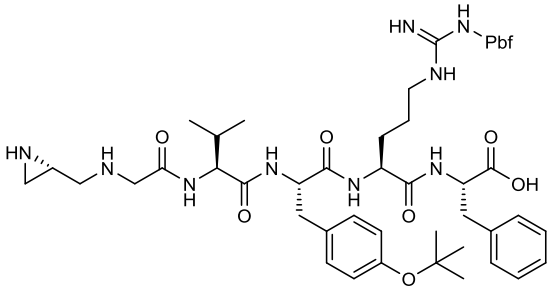

"MSD1 SPC, time=3.562:3.740 of D:\JOY\JY-02-162DP-ISO25.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 40993
100
80
60
40
20
0
214.0
563.2
130.2
607.2
124.1
184.2
216.0
273.1
565.2
609.2
200
400
600
800
1000
1200
1400
1600
m/z

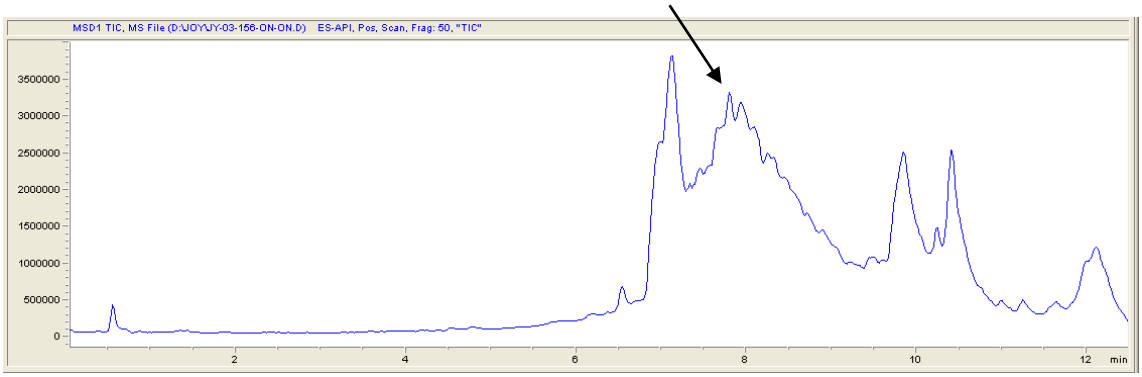

HN
H
N
O
N
H
O
H
N
O
N
H
O
H
N
O
OH
NH
Pbf
N
H
NH

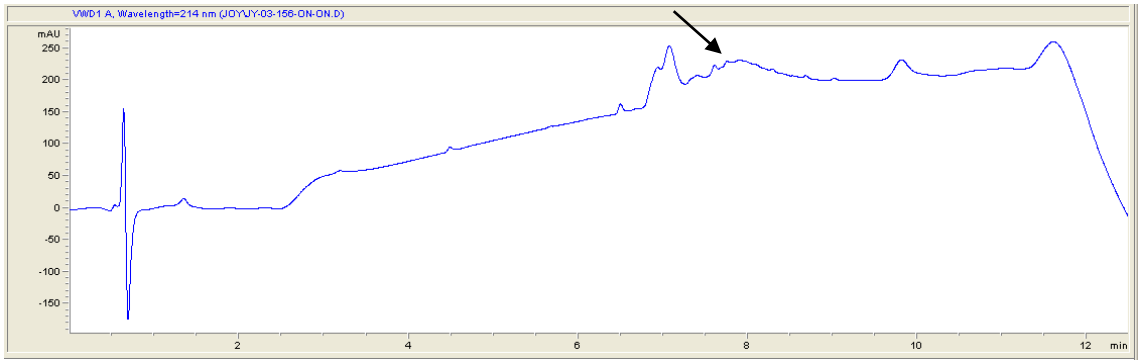

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-05-30 20-33-10\JY-03-140-FINAL.D) ES-API, Pos, Scan, Frag: 50, "TIC"
3000000
2500000
2000000
1500000
1000000
500000
0
2
4
6
8
10
12
min

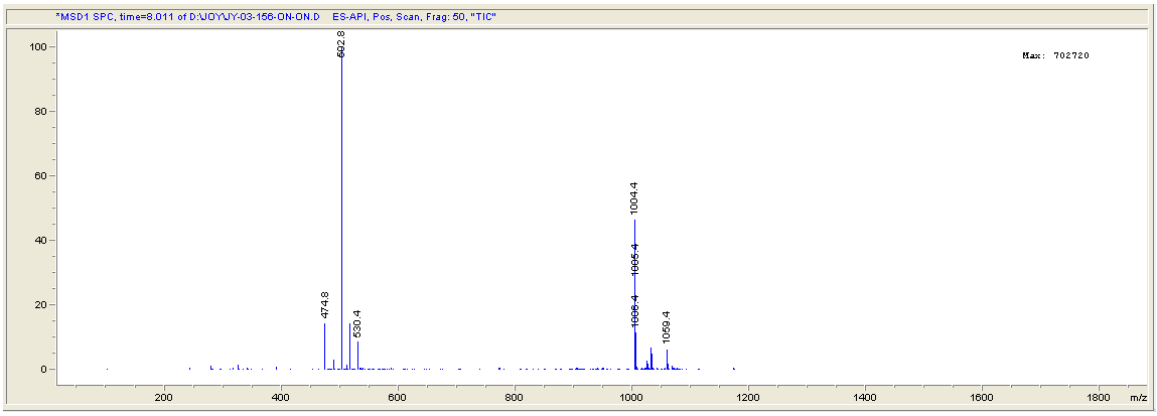

VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-05-30 20-33-10\JY-03-140-FINAL.D)
mAU
200
150
100
50
0
-50
-100
-150
2
4
6
8
10
12
min

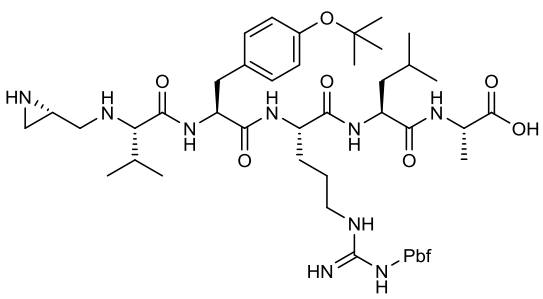

*MSD1 SPC, time=7.960:8.235 of D:\JOY\DEF_LC 2013-05-30 20-33-10\JY-03-140-FINAL.D ES-API, Pos, Scan, Frag: 50, "TIC
Max: 285146
492.9
484.8
984.4
986.4
986.4
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
m/z

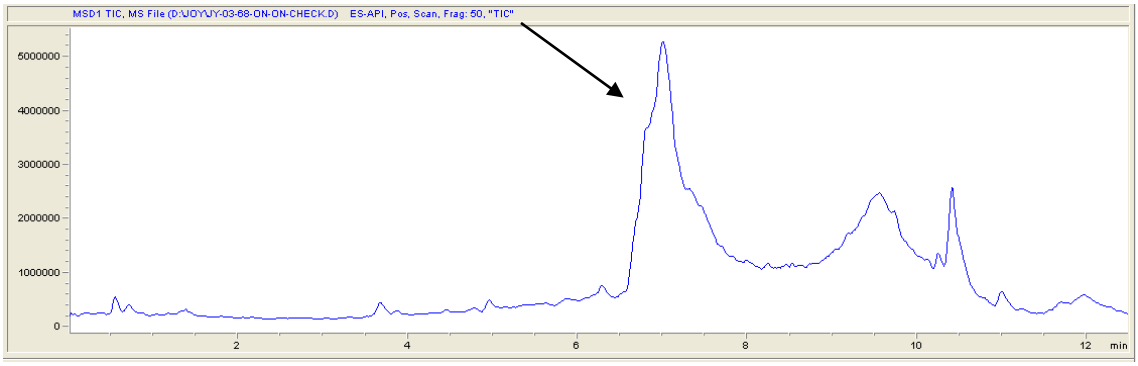

HN
H
N
Pbf
NH
HN
H
N
O
N
H
O
H
N
O
N
H
O
H
N
O
OH
O

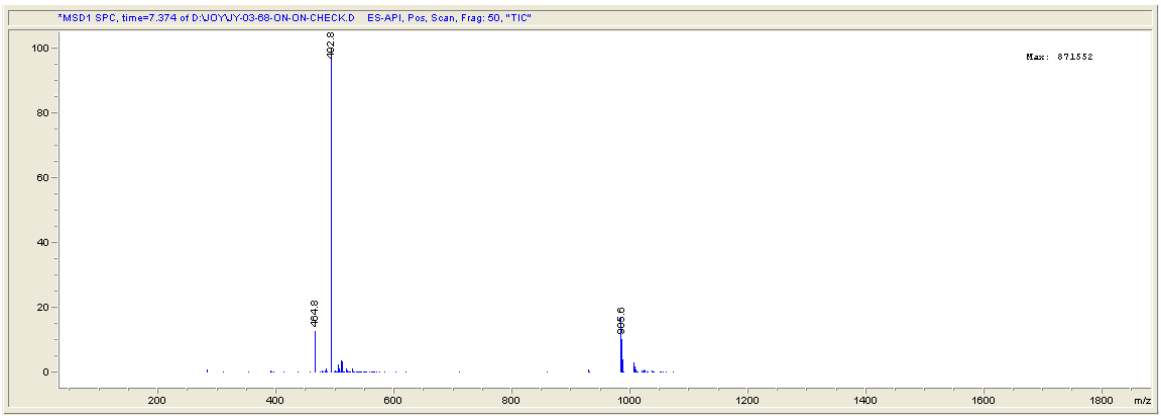

MSD1 TIC, MS File (D:\JOY\JY-03-156-ON-ON.D) ES-API, Pos, Scan, Frag: 50, "TIC"
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
2
4
6
8
10
12
min

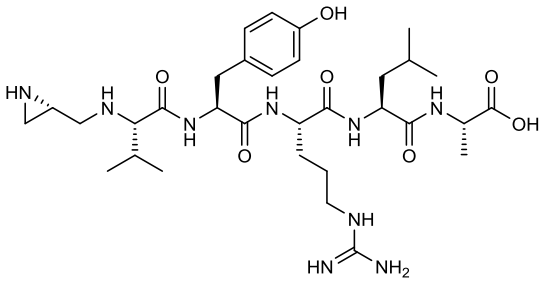

VWD1 A, Wavelength=214 nm (JOY\JY-03-156-ON-ON.D)
mAU
250
200
150
100
50
0
-50
-100
-150
2
4
6
8
10
12
min

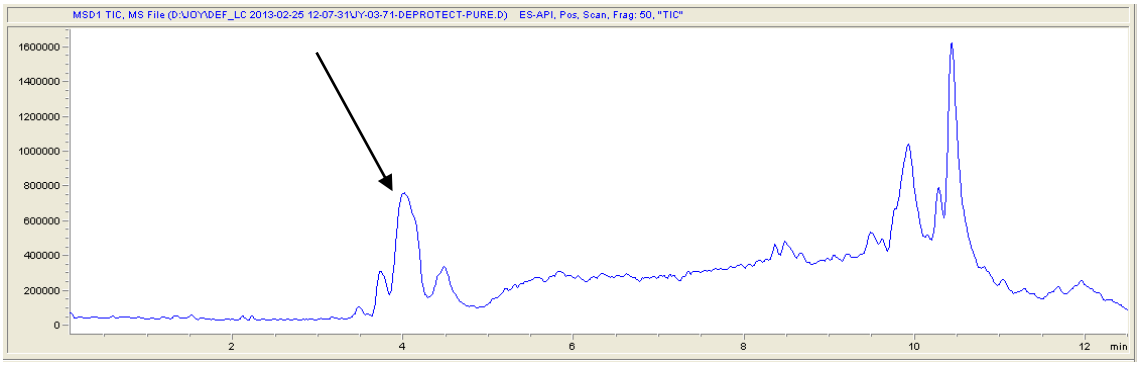

"MSD1 SPC, time=8.011 of D:\JOY\JY-03-156-ON-ON.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 702720
602.8
1004.4
995.4
474.8
530.4
1006.4
1059.4
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
m/z

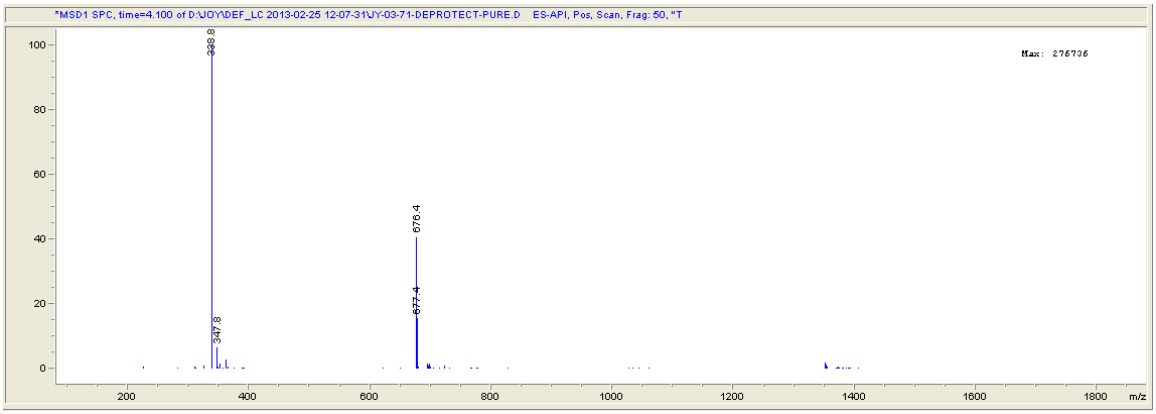

HN
H
N
O
N
H
O
H
N
O
N
H
O
H
N
O
OH
NH
HN
N
H
Pbf

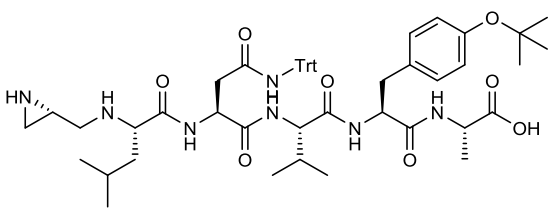

MSD1 TIC, MS File (D:\JOY\JY-03-68-ON-ON-CHECK.D) ES-API, Pos, Scan, Frag: 50, "TIC"
5000000
4000000
3000000
2000000
1000000
0
2
4
6
8
10
12
min

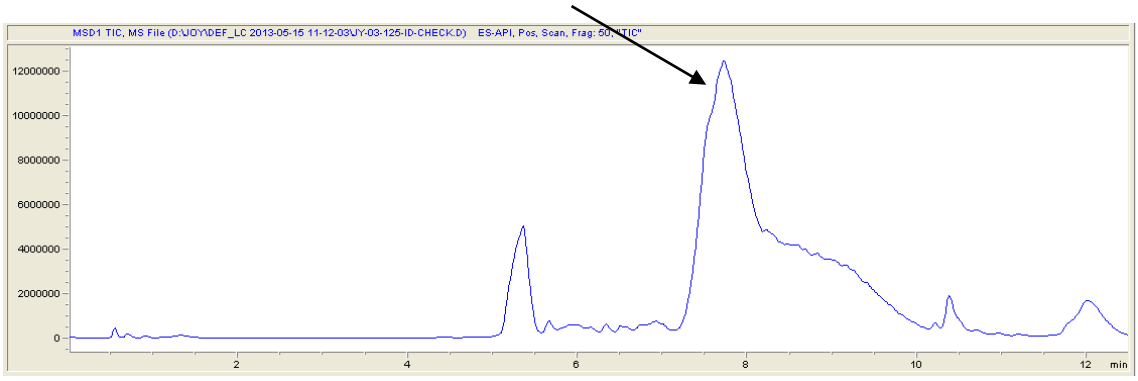

"MSD1 SPC, time=7.374 of D:\JOY\JY-03-68-ON-ON-CHECK.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 871552
492.8
464.8
995.6
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
m/z

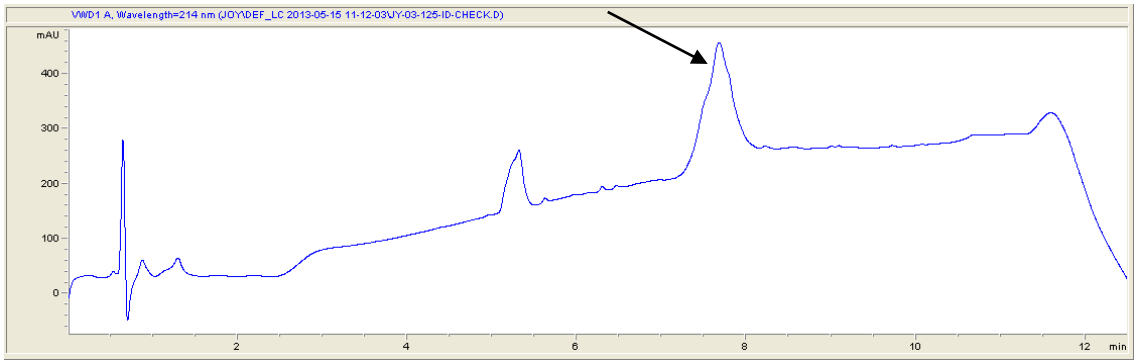

OH
HN
H
N
O
N
H
O
N
H
O
N
H
O
OH
NH
HN
NH2

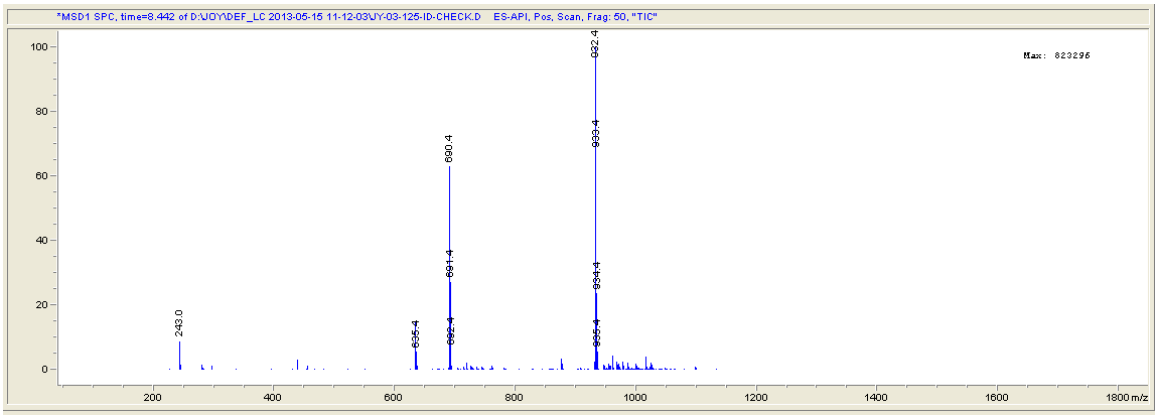

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-02-25 12-07-31\JY-03-71-DEPROTECT-PURE.D) ES-API, Pos, Scan, Frag: 50, "TIC"
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
2
4
6
8
10
12
min

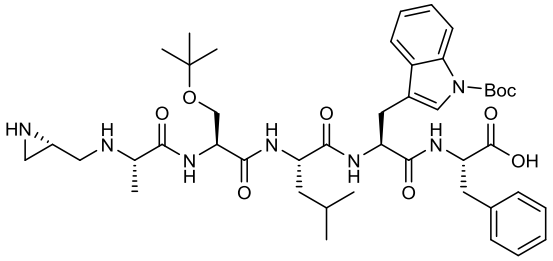

*MSD1 SPC, time=4.100 of D:\JOY\DEF_LC 2013-02-25 12-07-31\JY-03-71-DEPROTECT-PURE.D ES-API, Pos, Scan, Frag: 50, "T
Max: 276736
100
80
60
40
20
0
348.8
678.4
677.4
347.8
200
400
600
800
1000
1200
1400
1600
1800
m/z

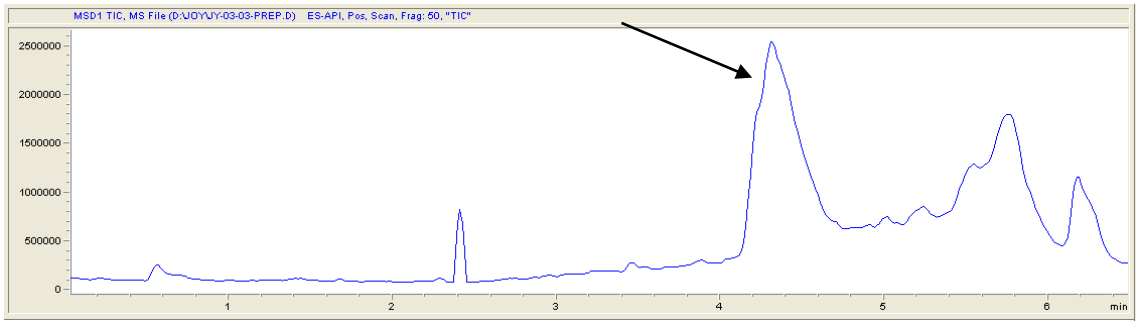

HN
H
N
O
N
H
O
N
Trt
O
N
H
O
O
H
N
O
O
OH

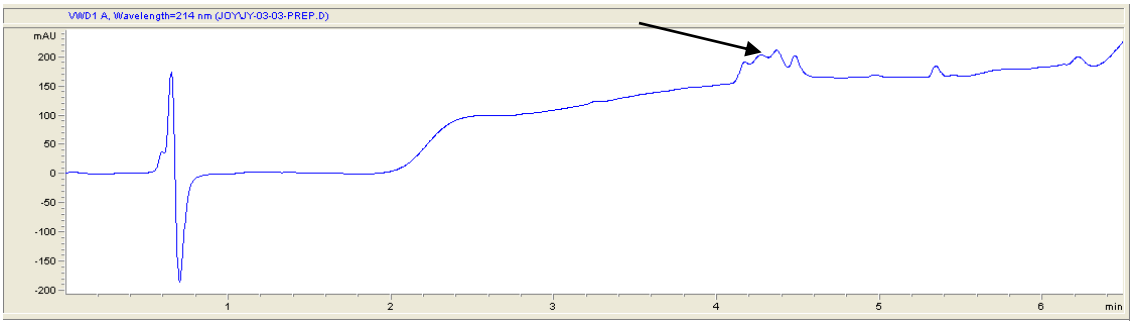

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-05-15 11-12-03\JY-03-125-ID-CHECK.D) ES-API, Pos, Scan, Frag: 50, "TIC"
12000000
10000000
8000000
6000000
4000000
2000000
0
2
4
6
8
10
12
min

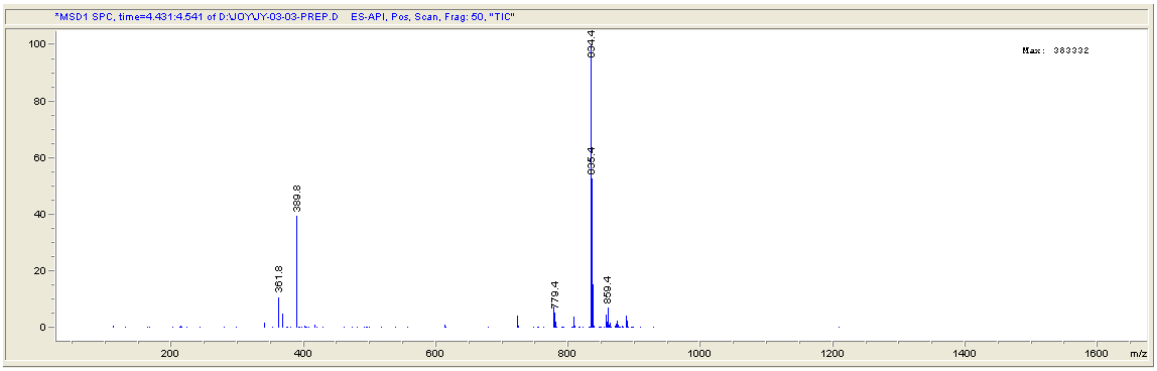

VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-05-15 11-12-03\JY-03-125-ID-CHECK.D)
mAU
400
300
200
100
0
2
4
6
8
10
12
min

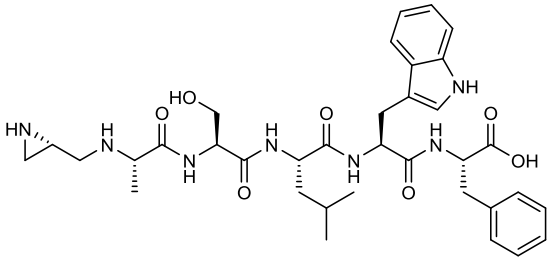

"MSD1 SPC, time=8.442 of D:\JOY\DEF_LC 2013-05-15 11-12-03\JY-03-125-ID-CHECK.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 823296
100
80
60
40
20
0
243.0
635.4
690.4
691.4
692.4
932.4
933.4
934.4
935.4
200
400
600
800
1000
1200
1400
1600
1800 m/z

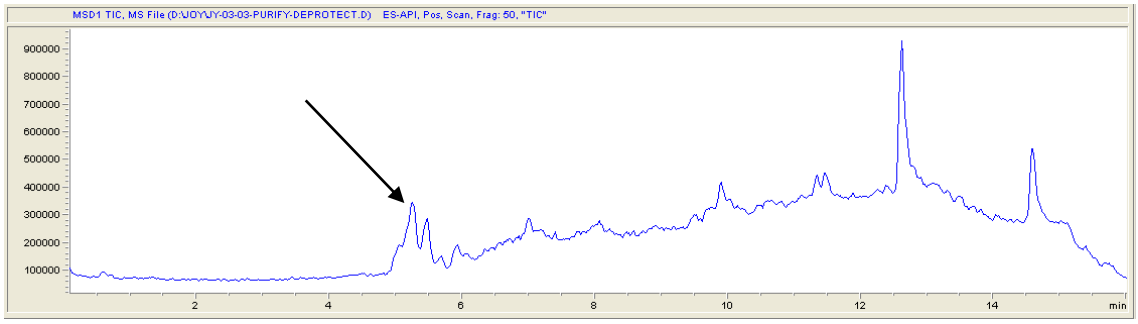

HN
H
N
O
N
H
O
H
N
O
N
H
O
H
N
O
OH
O
N
Boc

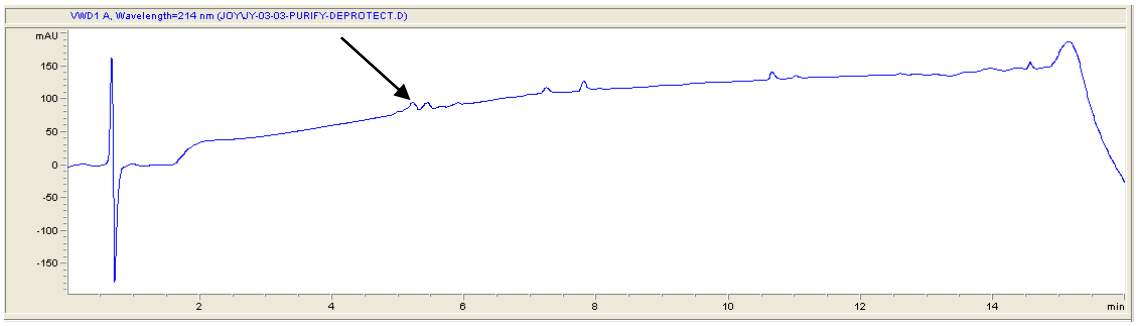

MSD1 TIC, MS File (D:\JOY\JY-03-03-PREP.D) ES-API, Pos, Scan, Frag: 50, "TIC"
2500000
2000000
1500000
1000000
500000
0
1
2
3
4
5
6
min

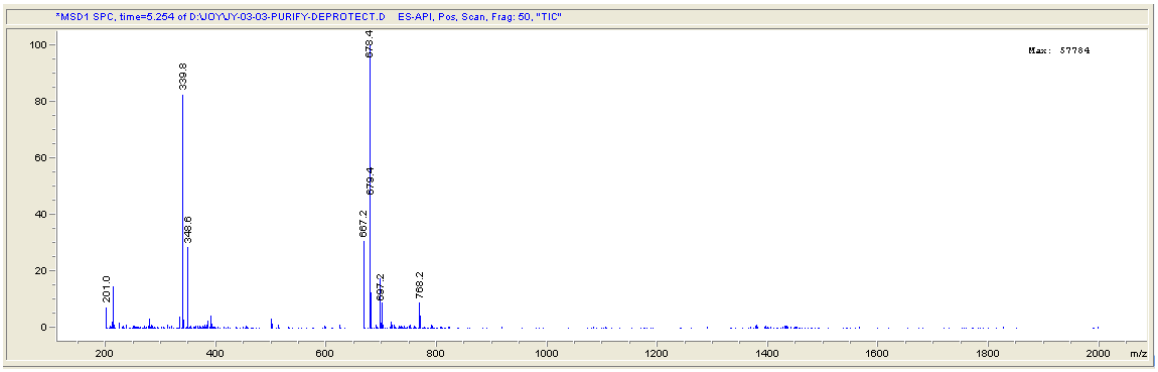

VWD1 A, Wavelength=214 nm (JOY\JY-03-03-PREP.D)
mAU
200
150
100
50
0
-50
-100
-150
-200
1
2
3
4
5
6
min

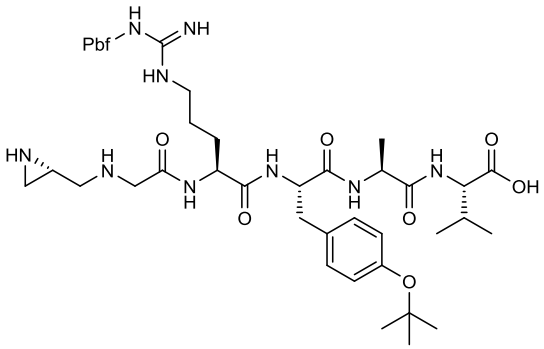

"MSD1 SPC, time=4.431:4.541 of D:\JOY\JY-03-03-PREP.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 383332
100
80
60
40
20
0
694.4
695.4
389.8
361.8
779.4
859.4
200
400
600
800
1000
1200
1400
1600
m/z

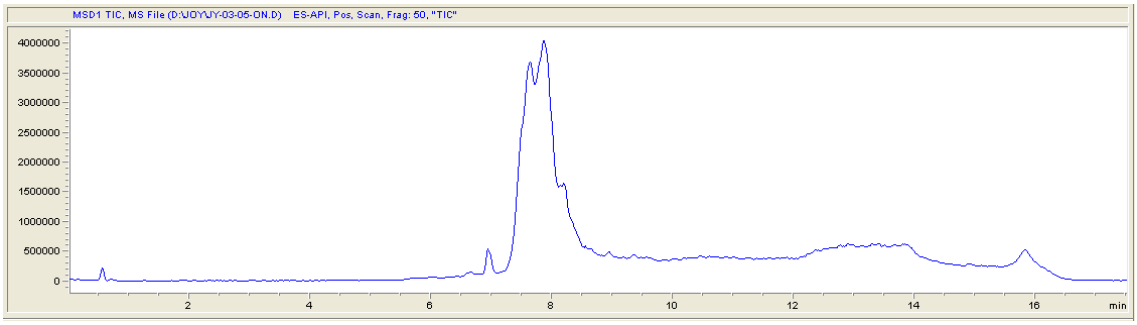

HN
H
N
O
HO
N
H
O
H
N
O
N
H
O
H
N
O
NH
OH

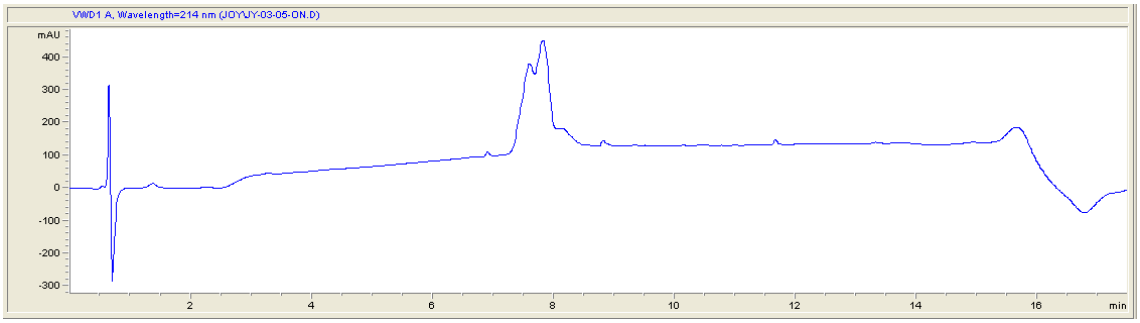

MSD1 TIC, MS File (D:\JOY\JY-03-03-PURIFY-DEPROTECT.D) ES-API, Pos, Scan, Frag: 50, "TIC"
900000
800000
700000
600000
500000
400000
300000
200000
100000
2
4
6
8
10
12
14
min

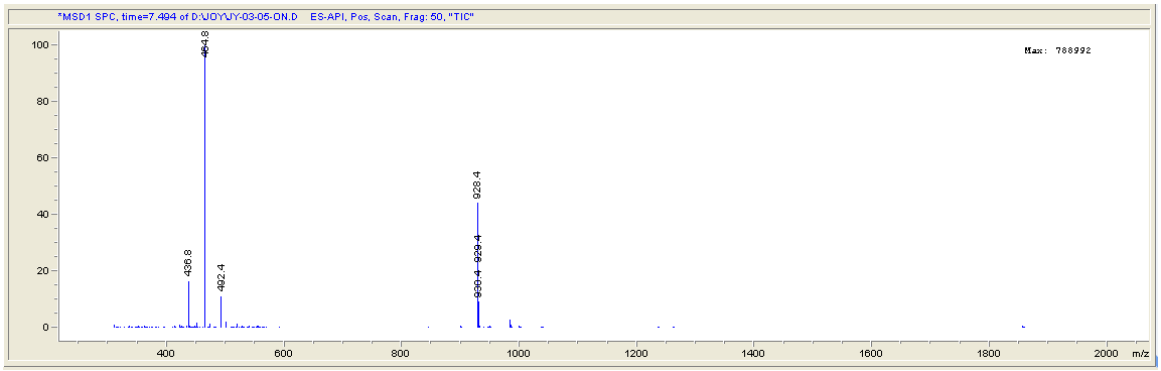

VWD1 A, Wavelength=214 nm (JOY\JY-03-03-PURIFY-DEPROTECT.D)
mAU
150
100
50
0
-50
-100
-150
2
4
6
8
10
12
14
min

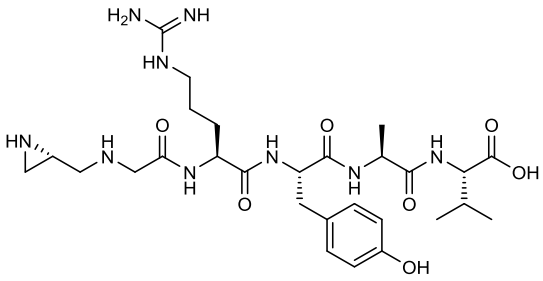

*MSD1 SPC, time=5.254 of D:\JOY\JY-03-03-PURIFY-DEPROTECT.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 57784
100
80
60
40
20
0
339.8
348.6
678.4
679.4
667.2
697.2
768.2
201.0
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

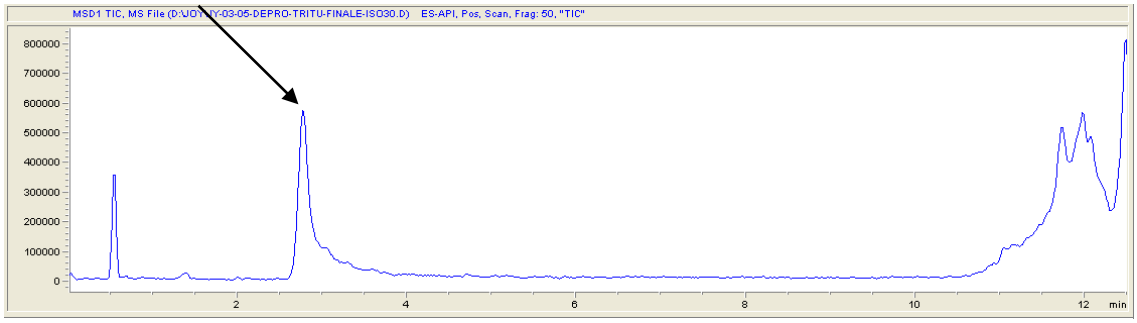

Pbf
H
N
NH
HN
HN
H
N
O
N
H
O
H
N
O
N
H
O
H
N
O
OH
O

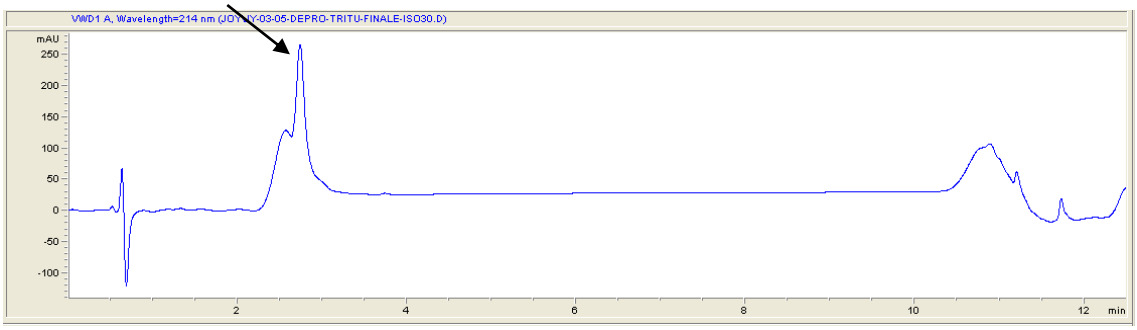

MSD1 TIC, MS File (D:\JOY\JY-03-05-ON.D) ES-API, Pos, Scan, Frag: 50, "TIC"
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
2
4
6
8
10
12
14
16
min

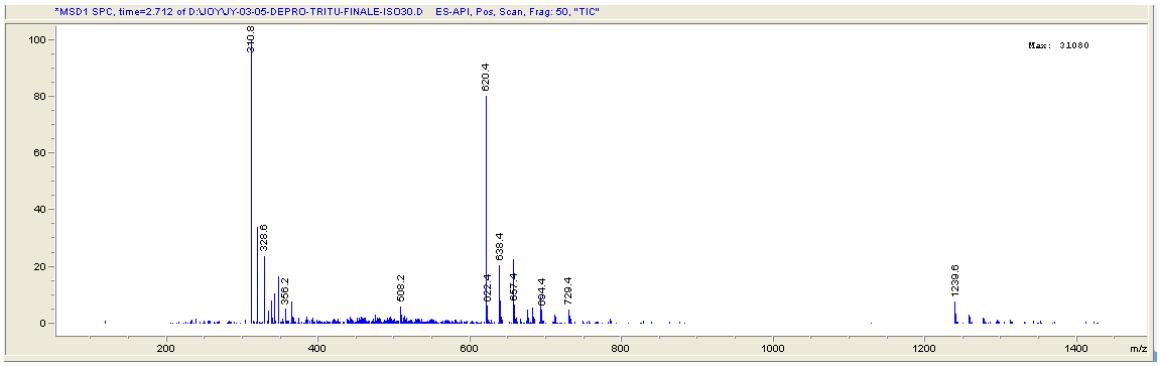

VWD1 A, Wavelength=214 nm (JOY\JY-03-05-ON.D)
mAU
400
300
200
100
0
-100
-200
-300
2
4
6
8
10
12
14
16
min

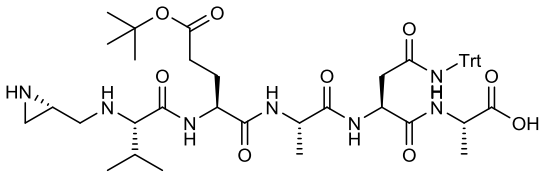

*MSD1 SPC, time=7.494 of D:\JOY\JY-03-05-ON.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 788992
464.8
928.4
929.4
930.4
438.8
402.4
100
80
60
40
20
0
400
600
800
1000
1200
1400
1600
1800
2000
m/z

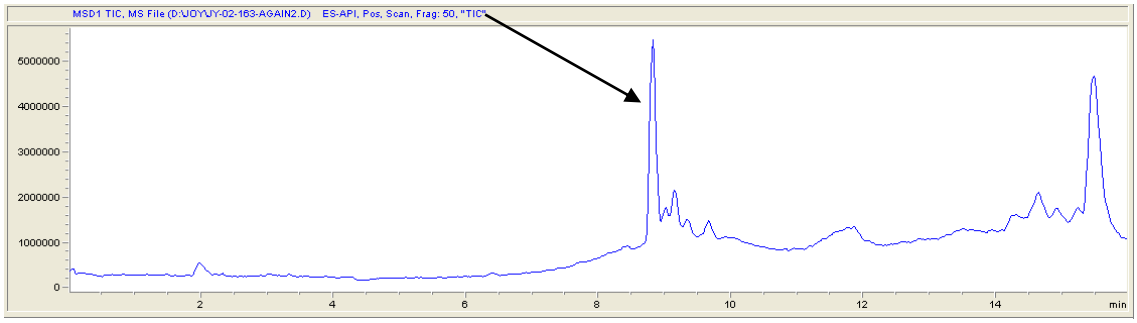


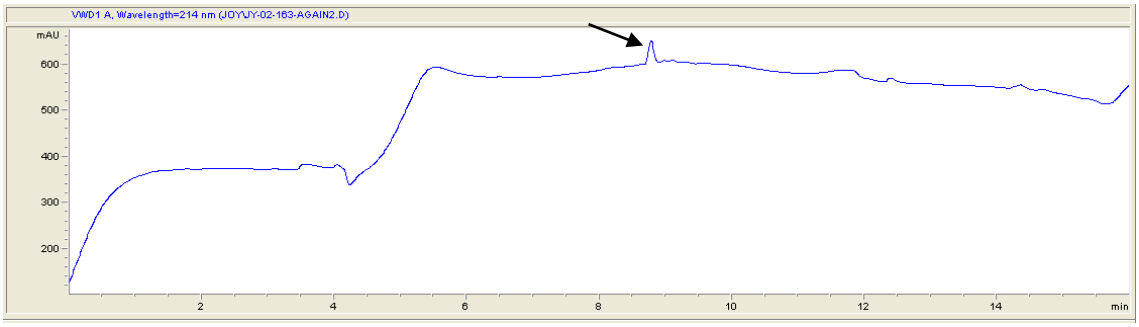

MSD1 TIC, MS File (D:\JOYUJY-03-05-DEPRO-TRITU-FINALE-ISO30.D) ES-API, Pos, Scan, Frag: 50, "TIC"
800000
700000
600000
500000
400000
300000
200000
100000
0
2
4
6
8
10
12
min

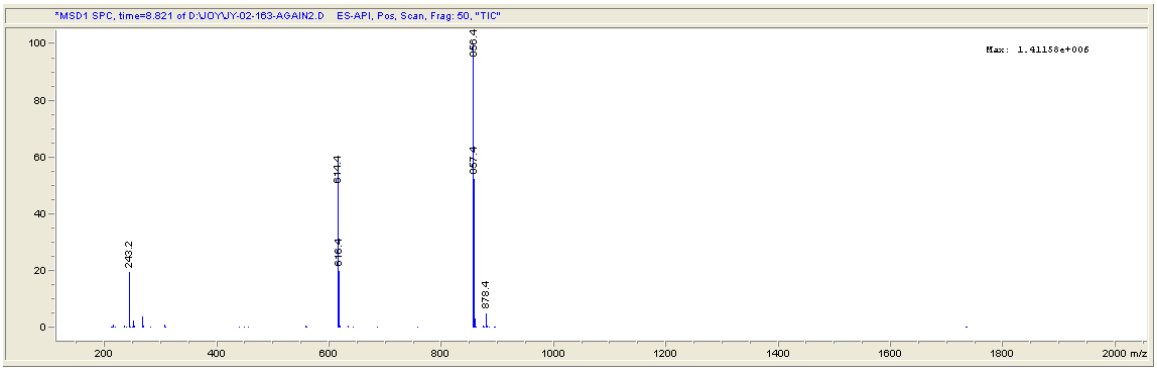

VWD1 A, Wavelength=214 nm (JOYUJY-03-05-DEPRO-TRITU-FINALE-ISO30.D)
mAU
250
200
150
100
50
0
-50
-100
2
4
6
8
10
12
min

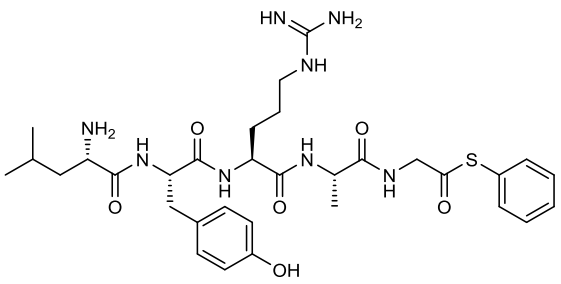

*MSD1 SPC, time=2.712 of D:\JOYUJY-03-05-DEPRO-TRITU-FINALE-ISO30.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 91080
340.8
620.4
328.6
638.4
356.2
508.2
622.4
657.4
664.4
729.4
1239.6
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
m/z

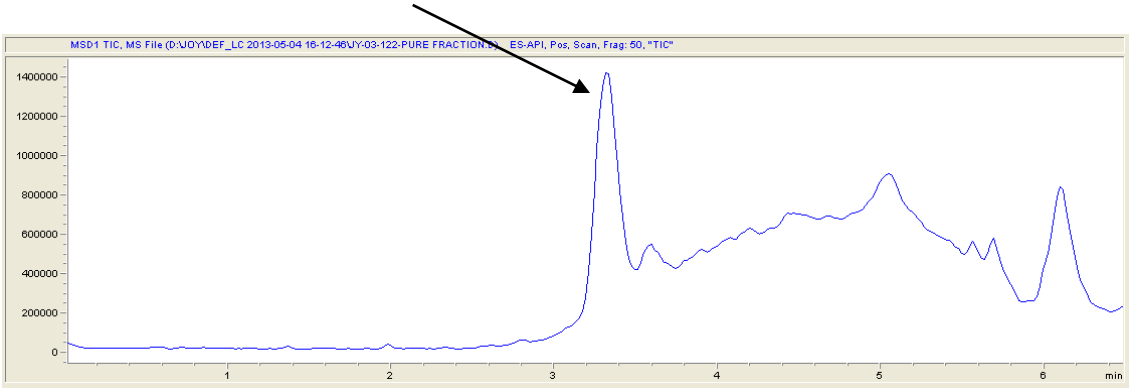

HN
O
O
O
H
N
O
N
H
O
H
N
O
N
H
O
N
Trt
O
N
H
O
OH

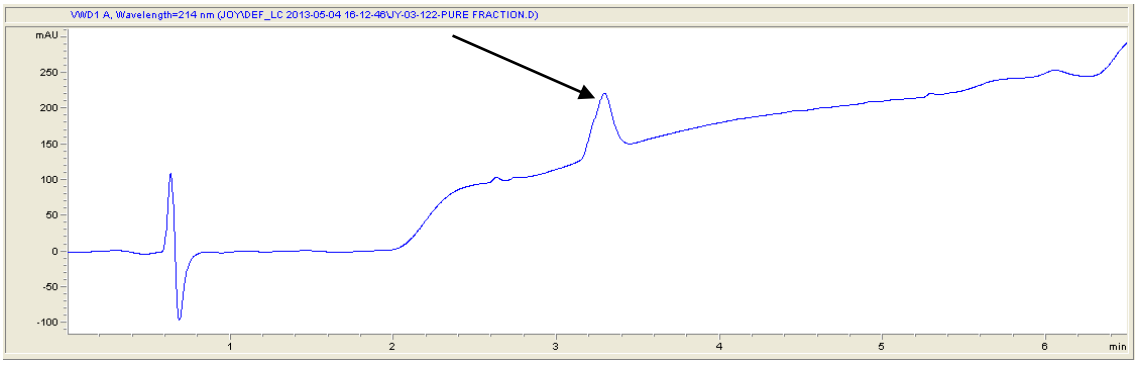

MSD1 TIC, MS File (D:\JOY\JY-02-163-AGAIN2.D) ES-API, Pos, Scan, Frag: 50, "TIC"
5000000
4000000
3000000
2000000
1000000
0
2
4
6
8
10
12
14
min

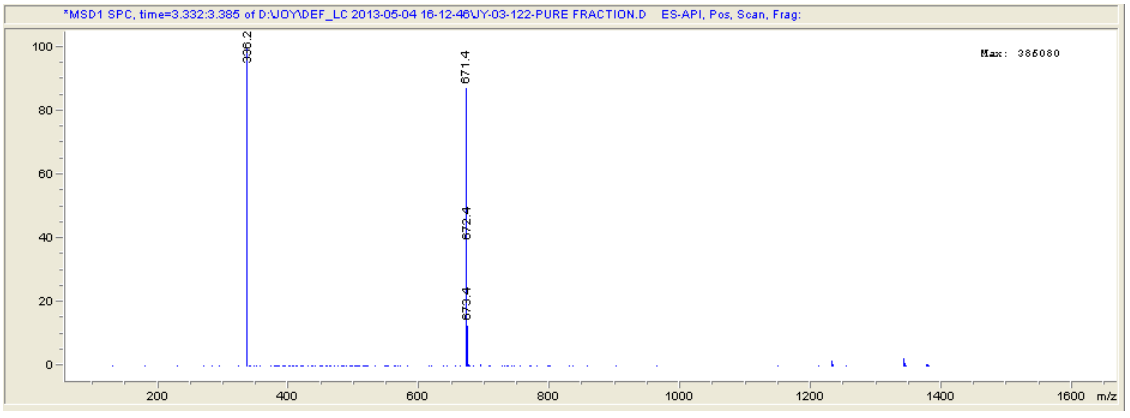

VWD1 A, Wavelength=214 nm (JOY\JY-02-163-AGAIN2.D)
mAU
600
500
400
300
200
2
4
6
8
10
12
14
min

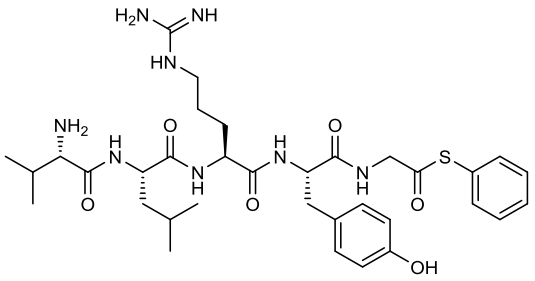

"MSD1 SPC, time=8.821 of D:\JOY\JY-02-163-AGAIN2.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 1.41158e+006
100
80
60
40
20
0
243.2
614.4
646.4
657.4
660.4
678.4
200
400
600
800
1000
1200
1400
1600
1800
2000 m/z

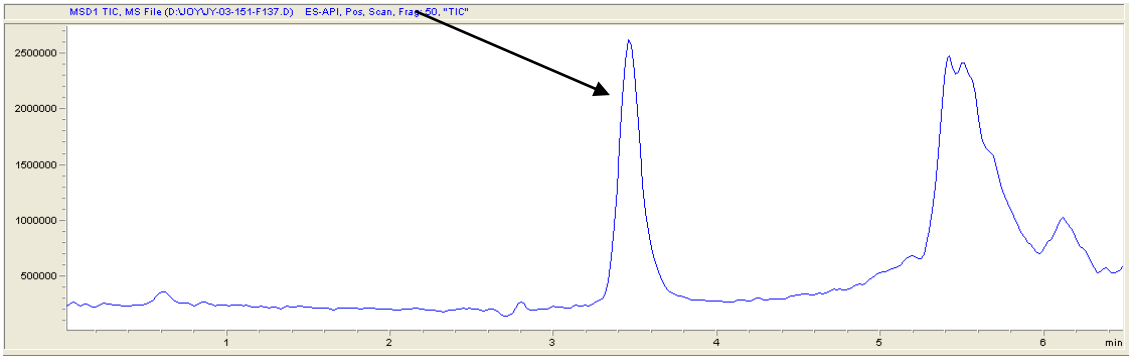

HN
NH2
NH
NH2
H
N
O
N
H
O
H
N
O
N
H
S
O
O
O
OH

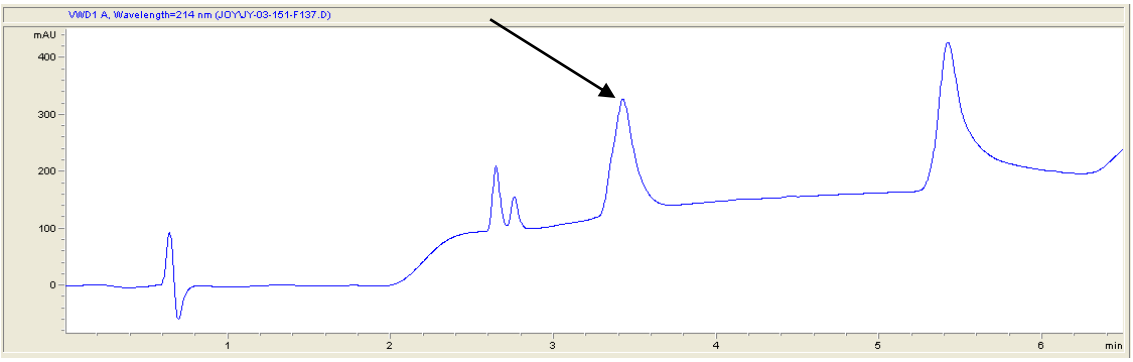

MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-05-04 16-12-46\JY-03-122-PURE FRACTION.D) ES-API, Pos, Scan, Frag: 50, "TIC"
1400000
1200000
1000000
800000
600000
400000
200000
0
1
2
3
4
5
6
min

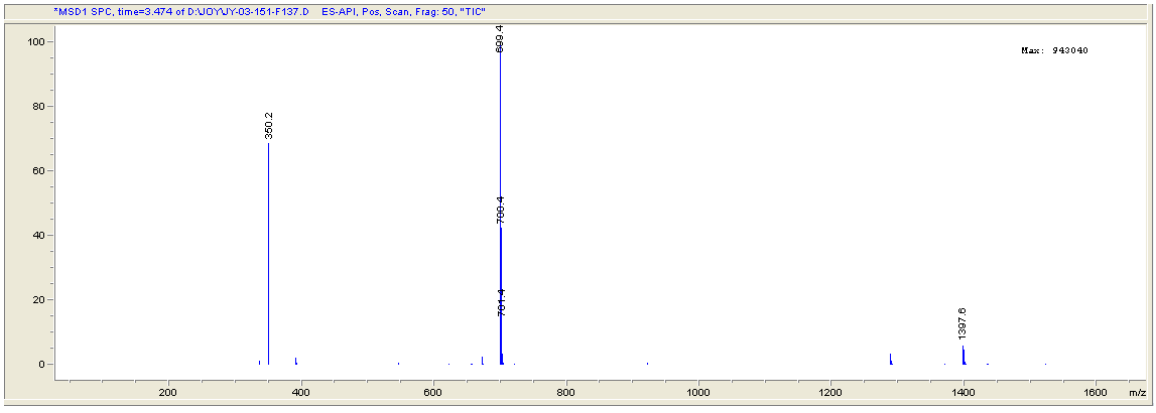

VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-05-04 16-12-46\JY-03-122-PURE FRACTION.D)
mAU
250
200
150
100
50
0
-50
-100
1
2
3
4
5
6
min

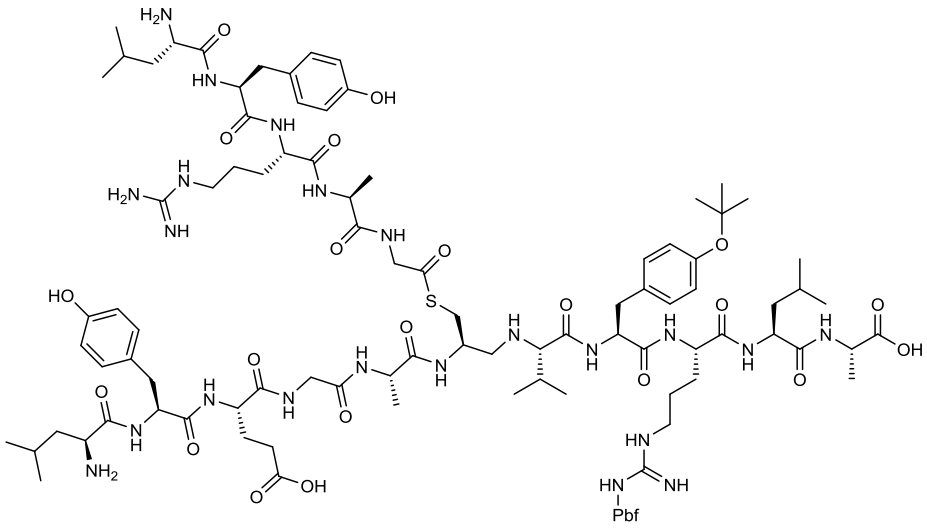

*MSD1 SPC, time=3.332:3.385 of D:\JOY\DEF_LC 2013-05-04 16-12-46\JY-03-122-PURE FRACTION.D ES-API, Pos, Scan, Frag:
Max: 385080
396.2
671.4
672.4
673.4
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
m/z

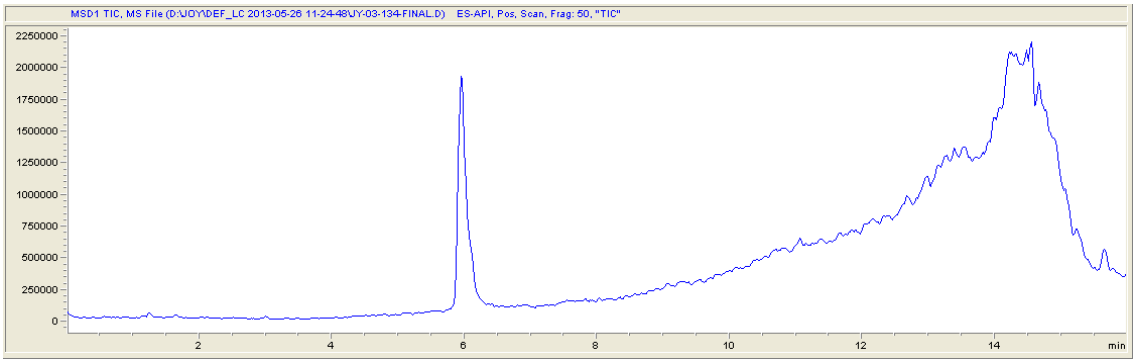


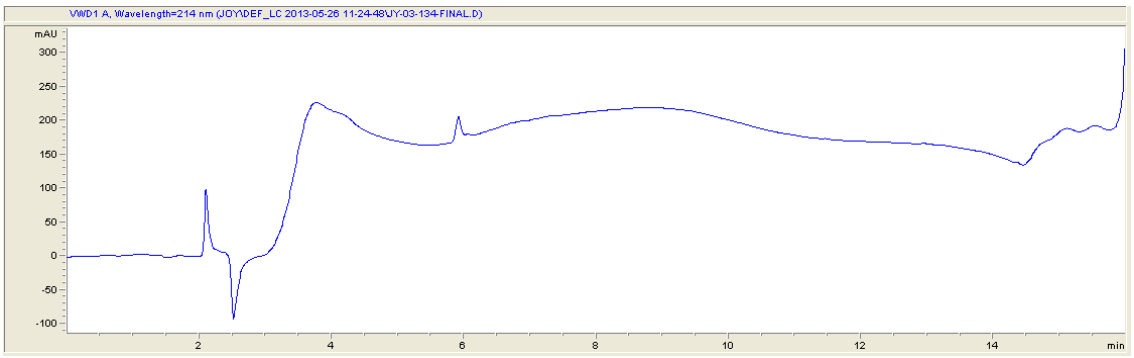

MSD1 TIC, MS File (D:\JOY\JY-03-151-F137.D) ES-API, Pos, Scan, Frag: 50, "TIC"
2500000
2000000
1500000
1000000
500000
1
2
3
4
5
6
min

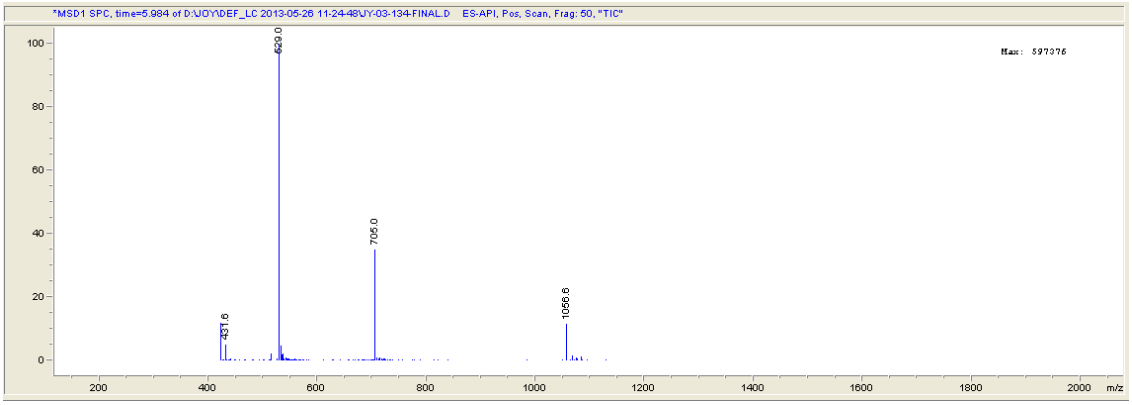

VWD1 A, Wavelength=214 nm (JOY\JY-03-151-F137.D)
mAU
400
300
200
100
0
1
2
3
4
5
6
min


*MSD1 SPC, time=3.474 of D:\JOY\JY-03-151-F137.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 943040
100
80
60
40
20
0
350.2
699.4
700.4
701.4
1397.6
200
400
600
800
1000
1200
1400
1600
m/z




MSD1 TIC, MS File (D:\JOY\DEF_LC 2013-05-26 11-24-48\JY-03-134-FINAL.D) ES-API, Pos, Scan, Frag: 50, "TIC"
2250000
2000000
1750000
1500000
1250000
1000000
750000
500000
250000
0
2
4
6
8
10
12
14
min


VWD1 A, Wavelength=214 nm (JOY\DEF_LC 2013-05-26 11-24-48\JY-03-134-FINAL.D)
mAU
300
250
200
150
100
50
0
-50
-100
2
4
6
8
10
12
14
min


"MSD1 SPC, time=5.984 of D:\JOY\DEF_LC 2013-05-26 11-24-48\JY-03-134-FINAL.D ES-API, Pos, Scan, Frag: 50, "TIC"
Max: 597376
529.0
705.0
1056.6
431.6
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z