NOVEL CONOPEPTIDES FROM CONUS TESSULATUS

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Novel Conopeptides from *Conus tessulatus*

By Paula Borges

A Thesis Submitted to the Faculty of

The Charles E. Schmidt College of Science

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, Fl

August 2005

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Frank Mari, Department of Chemistry and Biochemistry, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Acknowledgements

First and foremost, I would like to thank Dr. Frank Mari for giving me this great opportunity to work with him and his group on this very exciting project. Because of him, I was exposed to this stimulating new field that I wish to continue to learn more about and expand. Dr. Mari's guidance and advice has helped me to grow academically and to narrow the scope of my educational pursuits. I am enormously grateful to him for these reasons.

I would also like to thank Dr. Keith Brew and Dr. Deborah Louda for their time, help, advice, and efforts as members of my advisory committee.

Furthermore, I would like to profoundly thank Aldo Franco for mentoring me and for teaching me all that I needed to know in order to conduct this research. Without him, I would not have accomplished any of this. Thank you also to Dr. Ana Maria Urdaneta, Carolina Moller, Herminsul Cano, Jose Riviera-Ortiz, Sanaz Rahmankhah, Simon Bully and all the other members of Dr. Mari's research group for having guided me through all my questions and mistakes, and for making my experience in the group remarkable and enjoyable.

I also thank my mother deeply for giving her all into teaching me the importance of education, and for guiding and supporting me through everything.

Abstract

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Institution:	Florida Atlantic University
Thesis Advisor:	Dr. Frank Mari
Degree:	Master of Science
Year:	2005

Cone snails are predatory marine mollusks that utilize their peptide rich venom to capture prey, deter competitors and defend themselves. Each of the 1000 known species expresses over 100 conotoxins with little overlap between species. Most of these conotoxins selectively target a specific neuronal ion-channel or receptor. Because of their unprecedented diversity and specificity, they hold enormous potential as neuropharmacological agents, and as neuroscience research tools. In this study, the venom of a common shallow water cone snail that thrives in the Indo-Pacific to the Panamic region, *Conus tessulatus*, was analyzed; conopeptide components of the venom were isolated and investigated by high performance liquid chromatography, nuclear magnetic resonance, mass spectrometry, and automated Edman degradation sequencing. Five new peptide sequences are herein reported, among which there are three members of the M superfamily, one α conotoxin, and a conophan. The novel peptides comprise a partial peptide library of this particular cone.

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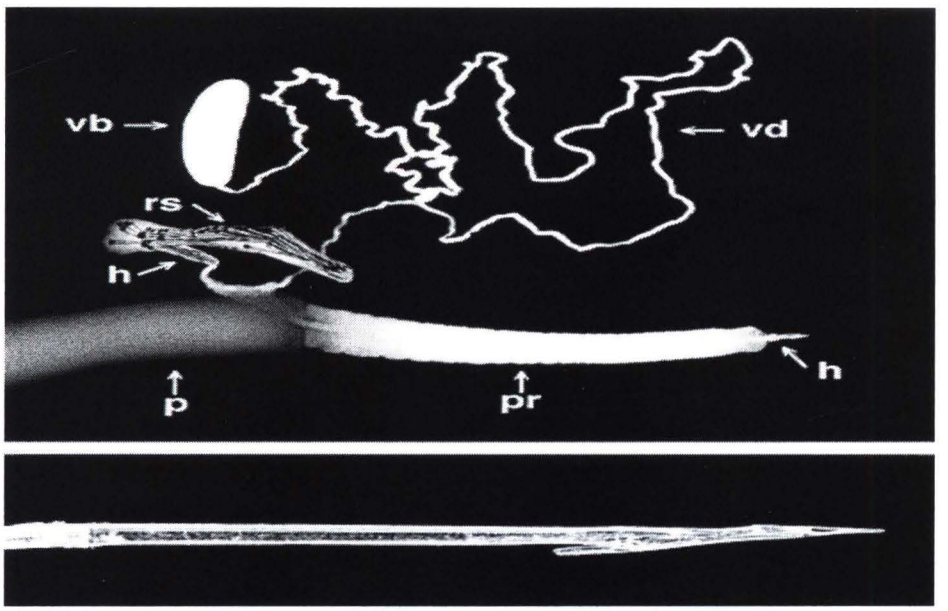
Introduction

Cone snails within the genus *Conus* are venomous marine gastropods that utilize modified peptide toxins in an effective biochemical campaign to capture prey, deter competitors, defend against predators, and possibly in other biological functions (Terlau and Olivera 2004). These modified peptides (conopeptides) that make up the venom of the snails are fast acting neuroactive compounds that immobilize the target by affecting the victims' neurophysiology. When envenomed, a cone's prey is rapidly subdued by the concerted, high-affinity binding of the venom's protein and peptide toxins to voltage and ligand gated ion channels essential for the proper function of the prey's nervous and muscular systems (Newcomb and Miljanich 2001). Since the first conopeptides were isolated a few decades ago, extensive systematic investigation has been conducted on these promising ion channel targets. This research has shown that each species among the hundreds of species has its own distinct complex cocktail of 20-200 peptides that comprises its venom.

Every *Conus* species captures their prey by envenomation via a highly efficient venom apparatus and delivery system (Figure 1). The venom apparatus in all cone snails consists of a venom bulb (vb) which pushes the venom out; the venom duct (vd) where the venom is synthesized by epithelial cells lining the duct and stored; a radula sac (rs) where the harpoon-like teeth (h) are kept; proboscis (pr), which is used to deliver the harpoon and

venom to the prey. At the end of the secretory pathway, which is mainly unknown, conotoxins are packed into secretory granules that are secreted into the duct lumen (Cruz et al. 1992). Each harpoon is used only once and they act as disposable hypodermic needles to eject the venom (Olivera 1997).

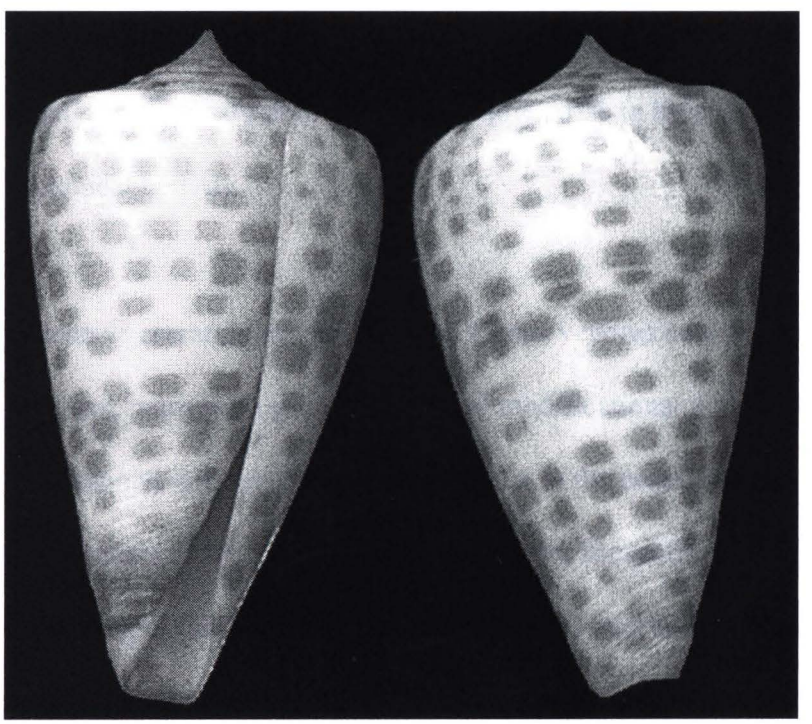
Figure 1- Stucture of a Typical Conus Venom Apparatus from *Conus purpurascens* (Olivera 1997)



Cone snails can be broadly characterized by their choice of prey. Mulluscivorous *Conus* prey on other gastropods, vermivorous *Conus* feed on small polychaete worms, and piscivorous *Conus* prefer small fish. Because they live in a hostile marine environment and exhibit a general lack of mobility, the need for fast acting venom to paralyze their agile prey becomes a demanding necessity in order to quickly immobilize their target (Terlau and Olivera 2004). Hence, the major components of the cone snail venom have been found to target key cell surface-signaling components of nervous systems such as ion channels and receptors.

Conus tessulatus is a common Indo-Pacific cone snail that feeds on polychaete worms, and whose venom scarcely affects mollusks and small fishes. All *Conus* predatory gastropods hunt prey from one of five different phyla, and all of these preys have cholinergic synapses (Cartier et al. 1996). Vermnivorous cones have not been well charachterized to date. *Conus tessulatus* is from the class gastropoda, order caenogastropoda, superfamily conacea, and family conidae. It is a nocturnal shallow water species found buried in sand with grass. They thrive in intertidal and subtidal regions usually up to 40 meters. The cone resides in the entire Indo-Pacific and also in the East Pacific Region (Rockel et al., 1995). Figure 2 shows a photograph of a *Conus tessulatus* shell.

Figure 2 – Picture of a *Conus tessulatus* Shell from http://www.active.net.gr/aegeanshells/conus/c-tessulatus.htm



Conus Venom Diversity

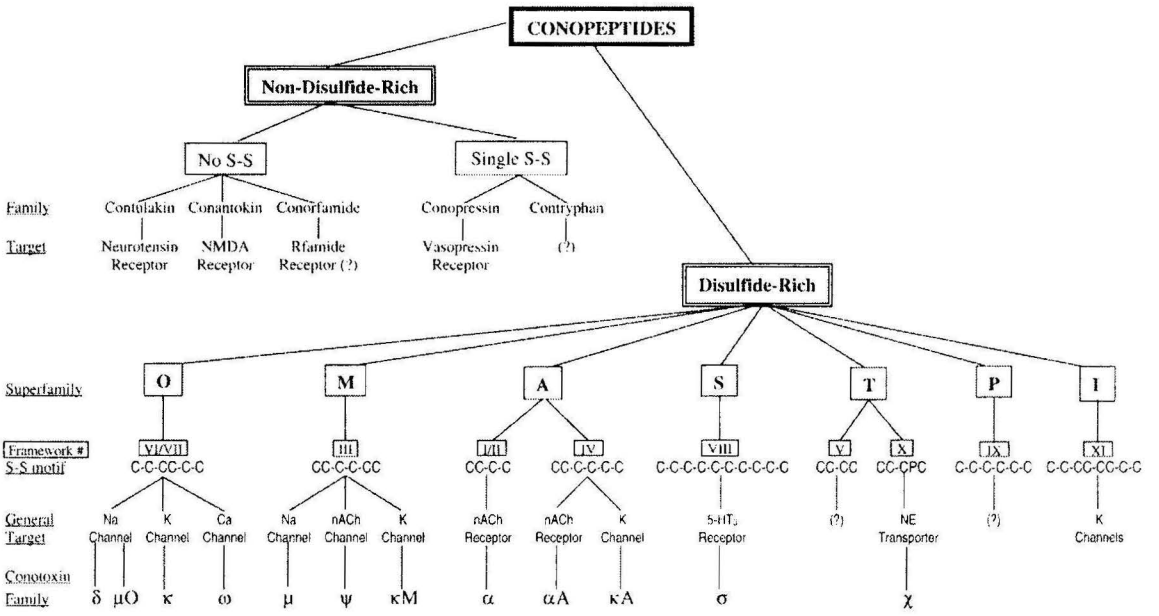
The genus *Conus* is one of the largest and most successful living genuses of marine animals, comprised of about 1000 different species (Mari and Fields 2003). Each species of *Conus* exhibits its own distinct repertoire of conopeptides, different from those of all other species. This difference is thought to arise from divergent biotic interactions within the tropical marine habitats in which cone snails thrive, that give rise to a corresponding divergence of cone snail venoms. Each *Conus* species has a unique set of biotic interactions characteristic of that species that helps to rationalize why each one has a different stock of 2–200 venom peptides (Bulaj et al. 2003). Although the specific detailed interactions between any *Conus* species with other animals within its environment is unknown, the fact that there are about 100 conopeptides per species and thus over 50,000 unique active conotoxins remains clear (Terlau and Olivera 2004). Moreover, each species is believed to express only a subset of all of its peptides at one time. This diversity in the venom has given rise to much interest in their highly selective biological activity.

Extensive research has shown these *Conus* venoms contain various small structured peptides, many with post translational modifications, which specifically target different ion channels and receptors. Conopeptides is the broad definition given to all peptide components of the venom. Conopeptides are biosynthesized as larger precursor peptides, consisting of the N-terminal signal sequence, intervening propeptide, and a mature toxin (Garrett et al. 2005). There are two major classes of conopeptides: non-disulfide rich and disulfide rich, a term indicating the relative number of cystine amino acids within the

primary sequence. Conophans and contryphans are linear peptides with no disulfide bridges, while conopressins have one disulfide bride due to the presence of two cysteines. The general term conotoxins refers to disulfide rich peptides that contain two or more disulfide bridges. The conotoxins are sub-divided into super families in accordance with their cysteine residue backbone arrangement. The precursors of super family members share a highly conserved signal sequence in their precursors and a characteristic cysteine backbone in mature peptides. The cysteines form the disulfide bridges which stabilize and structure the conotoxins.

Many super families have already been identified and named, such as the O, M, A, S, T, P, and I superfamilies, and many others have been found but remain nameless to date. Sub classifications within superfamilies are based on relative arrangement and number of cysteine residues, the number of residues between these cysteines, and on the particular molecular target of the family (Figure 3).

Figure 3 – Classification of Conopeptides by Molecular Target and Loop Pattern (Terlau and Olivera 2004)



Another characteristic feature of conotoxins that yields even more diversity is the remarkable number of post-translational modifications, such as O-glycosylation, bromination of tryptophan, γ-carboxylation of glutamate residues, hydroxylation of prolines, or L- to D-epimerization that appears in many of the peptides (Craig et al. 1999). Some of these modifications are quite common while others are unusual, and some were first discovered in *Conus* peptides.

Neurophysiology and Neuropharmacology of Venom

Due to the diverse nature of cone snail peptides, they exhibit a huge assortment of pharmacological activity even within a given superfamily. The diversity is ascribed to differences in primary structure of the peptides, which give rise to variability in charge, structure and characteristics of each unique peptide. Molecular targets are functionally

diverse and include G-protein coupled receptors, neurotransmitter transporters, ligand – gated ion channels, and voltage – gated ion channels, the latter two being the better known categories. Over the last 50 years, the concept of Na and K channels as components of action potential generation has given way to an intricate molecular complexity characterized by the fact that the K channel actually consists of over 80 genes that can arrange in a variety of combinations to yield an overwhelming diversity of tetrameric isoforms of K channels. These diverse ion channel substrates have been effective molecular targets for conopeptides because of the very diversity of targets that yield so many biological activities.

Although there are a variety of different peptides in the venom of any given cone, each peptide acts synergistically with the other peptides in order to accomplish some goal. For example, the first set of peptides injected into the prey acts to immediately immobilize the victim by inhibiting voltage gated Na channel inactivation and blocking K channels. This type of activity leads to mass depolarization of axons in the vicinity of the site of venom injection resulting in a tetanic state. The second set of peptides works to completely inhibit neuromuscular transmission. These peptides must be transported to sites, such as neuromuscular junctions, that are away from the site of venom injection, and are therefore more slow acting. They inhibit presynaptic Ca channels, postsynaptic nicotinic receptors, and Na channels involved in the muscle action potential (Terlau and Olivera 2004).Table 1 shows a listing of molecular targets and therapeutic potentials of different classes of peptides.

Table 1 – Targets and Therapeutic Potential of Different Classes of Conopeptides (Alonso et al. 2003)

Class	Target	Therapeutic Potential
Contulakins - Linear	Neurotensin receptors	Neuropathic pain
Conatokins - Linear	NMDA receptors	Epilepsy, Parkinson's
Conopressins - CC	Vasopressin receptors	Regulates blood pressure
χ-Conotoxins - CC-CPC	Neuropathic pain	Neuropathic pain
ω-Conotoxins - C-C-CC-C-C	Ca channels	Analgesic, Stroke
κ-Conotoxins - C-C-CC-C-C	K channels	Arrhythmia, Hypertension
μ-Conotoxins – CC-C-C-CC	Skeletal muscle Na channels	Neuromuscular block
ψ-Conotoxins - CC-C-C-CC	Skeletal muscle nACh channels	Analgesic, Parkinson's, Hypertension
α-Conotoxins - CC-C-C	Skeletal muscle nACh receptor	Analgesic, Parkinson's, Hypertension

From the above described actions of the different peptides in the venom, one can clearly see that the venom requires a variety of peptides with different targets and activities to achieve these means. This type of highly assorted toxin repertoire is a perfect biochemical strategy to advance the needs of the snail that has generated an enormous conopeptide library with unfathomable potential for neuroscience. One of the first of these conopeptides to be used therapeutically was Ziconotide, a ω-conotoxin now known as Prialt™. This toxin was isolated from *Conus magus* and has obtained FDA-approval and is currently one of the strongest analgesics on the market (Heading 2002).Other therapeutic toxins being tested to date include the following as shown in Table 2 (Alonso et al, 2003).

Table 2 – Potential Therapeutic Conopeptides (Alonso et al. 2003, Mari and Fields 2003)

Name of Toxin	Class of Toxin	Species	Therapeutic Interest	Mode of Action	Company	Stage of Development
MrIA/B	χ	C. marmoreus	Neuropathic pain	Targets noradrenaline transporter	Xenome	Preclinical
AM336	ω	C. cactus	Morphine resistant pain	Inhibit Ca channel	Amrad	Phase II
ACV1	α	C. victoriae	Neuropathic pain/ Nerve injury recovery	Nicotinic receptor agonist	Metabolic	Preclinical
Contulakin-G	Contulakin	C. geographus	Chronic pain	Targets neurotensin receptor	Cognetix	Phase II
Conatokin-G	Conatokin	C. geographus	Antiepileptic agent	NMDA receptor antagonist	Cognetix	Preclinical

This study was undertaken because of this huge diversity in potential uses of conopeptides. These marine drugs may one day be the key to elucidating several neuronal processes and to cure or treat a variety of neurological disorders. The goal of this study was to isolate and characterize several conopeptides from this previously untapped cone in order to develop a partial peptide library to be later submitted for biological assays to determine function and uses.

Materials and Methods

Specimen Collection

To initiate the study, living samples of *Conus tessulatus* were obtained from their natural habitat. In order to acquire the specimens, a shallow water dive into the Red Sea was conducted at night, and several cones were obtained from the rocks, coral or sand. Once the cones were obtained, they were immediately placed in a salt water tank where they were kept while being fed worms. The cones were kept in this condition until further use. In preparation for the extraction of the venom, the cones were placed in a -80°C freezer and stored there until further use.

Extraction of Crude Venom

The frozen specimens were thawed, removed from their shell, and dissected in order to acquire the venom ducts, where the crude venom is located. A small needle was inserted lengthwise between the shell and body and gently turned in the direction of the shell opening and the body removed. The duct is highly coiled and found embedded within the soft tissues. It is easily dissected from the rest of the body, with dissecting pins or needles, under a magnifying lens. The ducts were then uncoiled and measured for length. This process was done on ice to protect the contents of the ducts. The venom ducts were subsequently lyophilized. The venom was extracted from the lyophilized ducts with a

0.1% trifluoroacetic acid (TFA) solution to maximize dissolution. The extracts were centrifuged at 10,000 x g for 20 min, at 4°C, and the pellets were washed three times with 0.1% TFA and re-centrifuged in the same manner. The supernatants with the soluble peptides were pooled, lyophilized, and stored at -80°C until further use. The extracted venom was then subject to various purification steps. Approximately 65.4 mg of crude venom was extracted from eight cones.

Purification of Peptides

The first purification step was conducted via a pre-equilibrated size exclusion high performance liquid chromatography (HPLC) column (Pharmacia Superdex-30, 2.5 x 100 cm from Thermo Separation Products) to separate the venom components based on size. Elution of compounds was done with a 0.1 M NH_4HCO_3 mobile phase at 1.5 ml/min in an isocratic manner. These fractions were monitored at wavelengths of 220, 250 and 280 nm. The range for all three wavelengths was 2 in most cases unless otherwise specified.

Collected fractions from each peak were pooled, lyophilized, and stored at -40°C until further use. Fractions containing lower molecular weight components characteristic of peptides were chosen for further separation in another pre-equilibrated size exclusion HPLC column, (Tricorn Superdex™ Peptide 10/300 GL). Lyophilized fractions were dissolved in 0.1 M NH_4HCO_3 and elution was done with a 0.1 M NH_4HCO_3 mobile phase at a rate of 0.3 ml/min. Again, fractions were pooled, lyophilized and stored at -40°C until further use.

Fractions coming from the Superdex peptide column were then subject to separation in a reverse-phase HPLC column to separate the peptides according to hydrophobicity. The first reverse-phase HPLC column, a C18 semi-preparative column (Vydac, 218TP510, 10 x 250 mm; 5μm particle diameter; 300 Å pore size) equipped with a C18 guard column (Upchurch Scientific, AC-43 4.6 mm), allowed for better separation of single components and analysis of relative quantity. The lyophilized fractions were dissolved in 0.1% TFA and eluted with a linear gradient of 0.1% TFA (buffer A) and 0.1% TFA in 60% acetonitrile (buffer B) at a flow rate of 3.5 ml/min with a 1% buffer B increase/min. Absorbance was monitored at wavelengths of 220 and 280 nm. Major peaks were lyophilized, stored at -40°C, then re-dissolved in 0.1% TFA for further purification in an analytical reverse-phase C18 column (Vydac, 238TP54, 4.6 x 250 mm; 5μm particle diameter; 300 Å pore size), with a flow rate of 1 ml/min. Elution was also done with the same buffers as described for the semi-preparative column, and absorbance was monitored likewise. Fractions were manually collected, lyophilized and kept at -40°C until further use. The purity and molecular weight of the separated peptides were analyzed using mass spectrometry. Impure samples were subject to an additional analytical run when necessary. Pure samples were subject to other experiments.

Determination of Molecular Mass

Molecular mass was determined by positive ion matrix laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry as detected by a Voyager-DE STR (Applied Biosystems). Samples were dissolved in 0.1% TFA, 50% acetonitrile, and applied between two layers of a α-cyano-4-hydroxycinnamic acid matrix (Acros Organics) onto a

magnetic plate. Spectra were acquired in either linear or reflector mode. Calmix 1 and Calmix 2 (Applied Biosystems) were used as external calibration standards.

Nuclear Magnetic Resonance Spectra

Pure samples were subject to one dimensional NMR experiments to determine relative peptide concentration, and to identify characteristics of the peptide. The NMR spectra were obtained by a Varian Inova 500 MHz instrument equipped with pulse field gradients, three radiofrequency channels and waveform generators. Pure peptide samples were dissolved in high purity water (Fisher) containing 38 pmol of trimethyl silyl propionic acid (TSP) (Aldrich) as an internal reference and 10% D_2O (Aldrich). The total volume of samples, 40 µl at a pH of ~3.6, was placed in 1.7 mm NMR capillary tube (Wilmad), and spectra were acquired at 25°C and 0 °C. If the sample had a high enough concentration, then two dimensional NMR experiments, namely NOESY and TOCSY, were performed which aided in confirming the presence of certain amino acids in sequence.

Reduction and Alkylation of Peptides

Once the NMR experiments were concluded, part of each sample was subject to reduction and alkylation of disulfide bonds (if any are present). This procedure allows for determination of the number of cysteines in the peptide by mass spectrometry. Samples were lyophilized then re-dissolved in 0.1 M Tris-HCl (pH 6.2), and reduced with 20 mM dithiothreitol (DTT). Upon a 30 minute incubation period at 60°C, the peptides were

alkylated with 50 mM Iodoacetamide (IAM) and 3 μl of NH_4OH (pH 10.5) for one hour at room temperature in the dark. The reduced and alkylated peptides were recovered and purified using a pre-equilibrated Zip Tip (C18, size P10, Millipore) with a 0.1% TFA in 60% acetonitrile solution and 0.1% TFA solution.

Peptide Sequencing

The reduced and alkylated peptides were subsequently subject to sequencing by automated Edman Degradation. Alkylated peptides were adsorbed onto Biobrene-treated glass fiber filters and subsequently sequenced by Edman degradation using an Applied Biosystems Procise model 491A Sequencer equipped with a micro gradient delivery system, model 610A model 785A UV detector, and data analysis software model 140C. This model causes the N-terminal amino acid to react with phenylisothiocyanate (PITC) in basic conditions forming a phenylthiocarbamyl derivative (PTC-protein).The first amino acid is then cleaved by trifluoroacetic acid forming its anilinothialinone derivative (AZT-amino acid) and leaving behind the next amino acid for degradation in the next cycle. The newly formed AZT-amino acid is extracted with N-butyl chloride and converted to a phenylthiohydantoin derivative (PTH-amino acid) that is transferred to a reverse-phase HPLC C-18 column for detection at 270 nm. For cross referencing, a standard mixture of PTH-amino acids is injected onto the column for separation and detection, providing a standard elution profile for comparison with unknowns. The sequences are then confirmed by matching the expected molecular weight of the peptide with the molecular weight obtained via mass spectrometry.

Results and Discussion

Purification of Peptides

The first step in the purification process of the crude venom was the dissolution of the lyophilized crude venom into 1000μL of 0.1 M NH_4HCO_3. The sample was then eluted through the Superdex 30 column for 266 minutes. This initial separation by size exclusion yielded 9 fractions that showed absorbance at 220 nm, six fractions showed absorbance at 280 nm suggesting the presence of aromatic amino acids, and six fractions showed absorbance at 250 nm signaling the presence of cysteine bonds, all fractions showing clear overlap among absorbance spectra. Moreover, the profiles show the possibility of overlap between adjacent peaks. Figure 4 and table 3 shows the elution profile and retention times for this run. Each run was recorded at range 2 due to the speculated quantities of crude venom.

Tessulatus_B was the designation given to this batch of crude venom, and Tes_B is the abbreviation for this designation. The nomenclature assigned from here on in is as follows: Superdex 30 fractions are labeled as numbers in numerical order such as Tes_B 01, Tes_B 02, and Tes_B 03 and so on. Superdex Peptide fractions are labeled as consecutive letters in front of the number from the Superdex 30 fractions such as Tes_B 02a, Tes_B 02b, and so on. The semi-preparative fractions then take a number after the letter from the Superdex Peptide aliquots such as Tes_B 02b04, Tes_B 02b05 and so on.

The analytical fractions take a letter after the semi-preparative column number such as Tes_B 02b04a, Tes_B 02b04b and so on.

Figure 4 – Elution Profile of Tessulatus_B Crude Venom in the Superdex 30

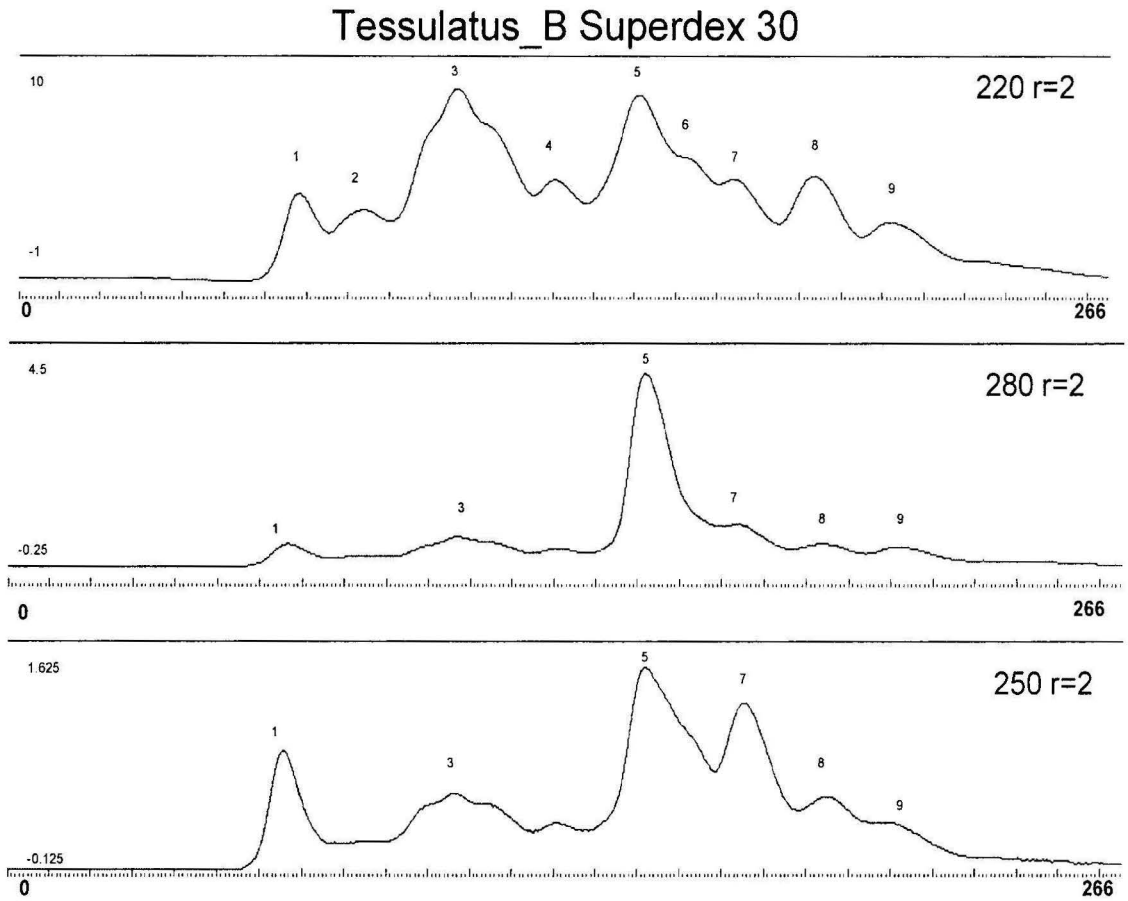


Table 3 – Elution Times of Tes_B Crude Venom Fractions from Superdex 30

Peak #	Elution Time (Minutes)
1	56.53 – 75.50
2	75.50 – 90.30
3	90.30 – 125.21
4	125.21 – 138.22
5	138.22 – 163.03
6	163.03 – 171.24
7	171.24 – 185.30
8	185.30 – 204.29
9	204.29 – 229.73

Fractions 5, 6, and 7 were subsequently pooled together since there was much overlap between the peaks in order to assure that like peptides were kept together in order to obtain higher purified quantities. These peaks were the first to be chosen for further purification since they were the three major peaks that fell within the molecular weight range of peptides (as determined by their elution times relative to the other peaks in the size exclusion column).The combined peaks were renamed Tes_B 05, lyophilized, then re-dissolved in two batches of 500 µL of 0.1 M NH_4HCO_3 for further separation in the Superdex Peptide column. Figure 5 and table 4 shows the chromatography profile and retention times of this run. Each run of both batches was recorded at range 2 and 280 nm (also 220 nm for batch 2).

Figure 5 – Elution Profile of Tes_B 05, 06, 07 Combined Fractions (renamed 05) in Superdex Peptide

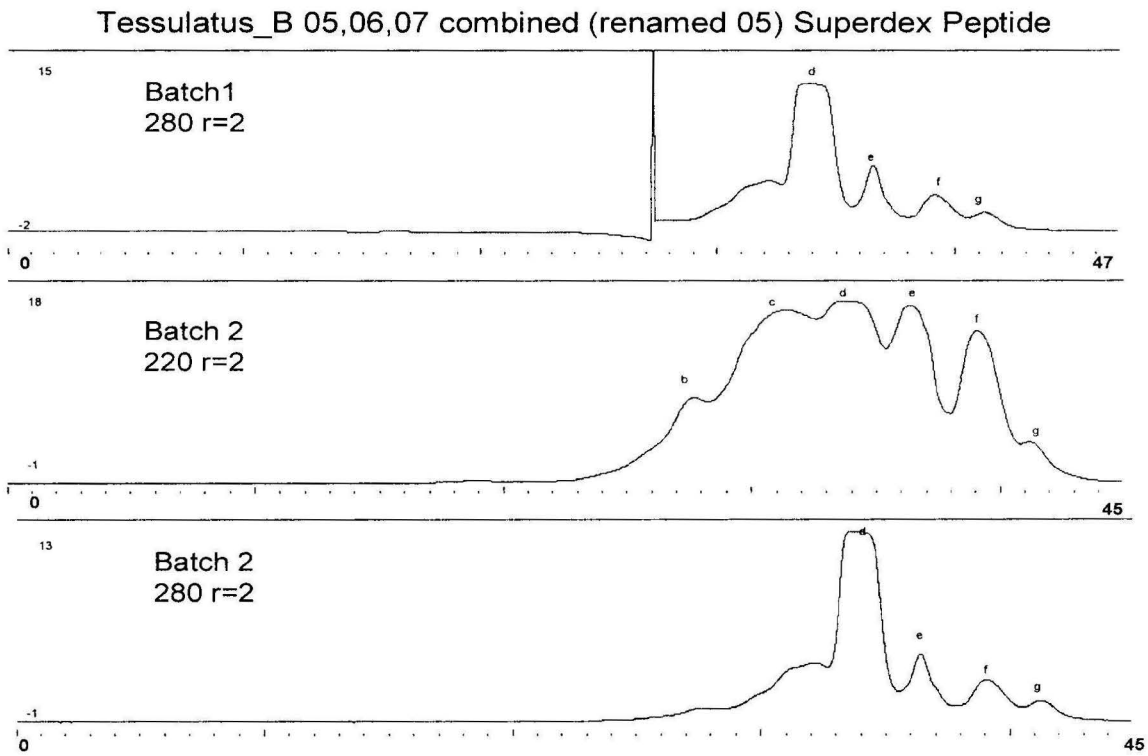


Table 4 – Elution Times of Tes_B 05, 06, 07 Combined Fractions (renamed 05) in Superdex Peptide

Batch 1

Peak #	Elution Time (minutes)
a	24.30
b	28.60
c	31.81
d	32.90
e	35.60
f	38.15
g	40.90

Batch 2

Peak #	Elution Time (minutes)
a	17.01
b	24.36
c	30.70
d	33.00
e	35.40
f	38.00
g	41.10

During the run of batch 1, the column exhibited several problems and the data for the run was not stored. Only the run monitored at 280 nm was salvaged. Fortunately, batch 2 ran smoothly and the two profiles closely resembled one another, therefore the batch 1 fractions were still used by pooling with the batch 2 fractions. Batch 1 ran for 47 minutes while batch 2 ran for 45 minutes. Approximately seven peaks were present in both batches, and in both wavelengths of 220 nm and 280 nm. Peak Tes_B 05d was chosen for further separation as it was the major peak. Upon being lyophilized, the sample was dissolved in 1000 μL of a 0.1% TFA solution, then subject to separation in the semi-preparative column. Figure 6 shows the elution profile of this run which ran for 87 minutes in batch one and for 98 minutes in batch 2, and yielded 11 collected peaks in batch 1 and 14 collected peaks in batch 2 under monitoring at 220 nm. The run was also

monitored under 280 nm and yielded several peaks at this wavelength. Each run in both batches was recorded at range 2.

Figure 6 – Elution Profile of Tes_B 05d in the Semi-preparative Column

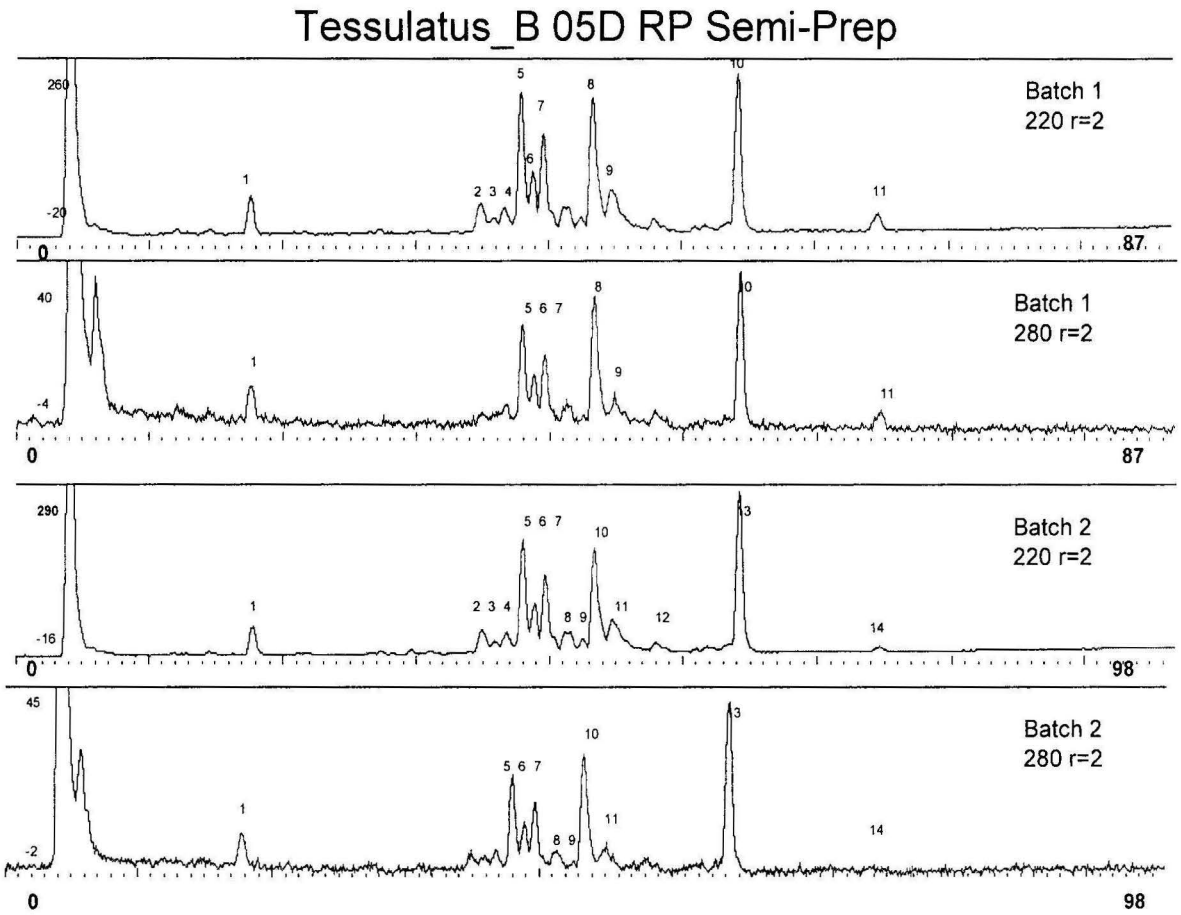


Table 5 – Elution Times of Tes_B 05d in the Semi-preparative Column

Batch 1

Peak #	Elution Time (minutes)
1	17.5
2	35.0
3	36.0
4	36.7
5	37.9
6	38.8
7	39.6
8	43.3
9	44.8
10	54.2
11	64.8

Batch 2

Peak #	Elution Time (minutes)
1	17.80
2	34.95
3	36.10
4	36.80
5	38.00
6	38.90
7	39.70
8	41.30-41.65
9	42.50
10	43.37
11	44.78
12	47.85
13	54.20
14	64.75

Once the major peaks (peaks 2 pooled from both batch 1 and 2, peaks 5, 6, 7 from batch 1 and 2 all pooled together, peak 8 and 9 from batch 1 pooled with peak 10 and 11 from batch 1 (renamed Tes_B 05d1011), and peak 10 from batch 1 and 13 from batch 2 pooled together (renamed Tes_B 05d13)) had been lyophilized, they were re-dissolved in 20 μL of a 0.1% TFA solution and 0.3 μL of the sample was submitted to mass spectroscopy experiments. Figures 7–12 show the mass spectra for these peaks.

Figure 7 – TOF MS of Tes_B 05d02 (MW= 1703.37 Da)

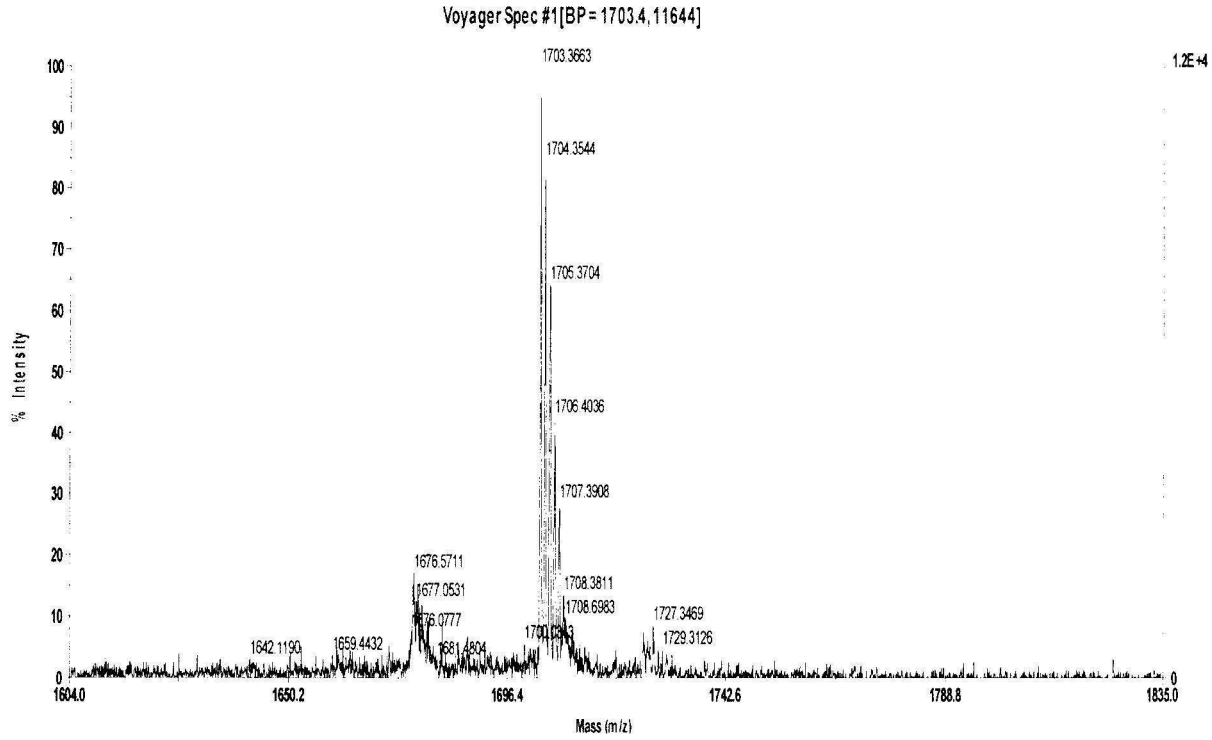


Figure 8 – TOF MS of Tes_B 05d05 (MW= 2435.81Da)

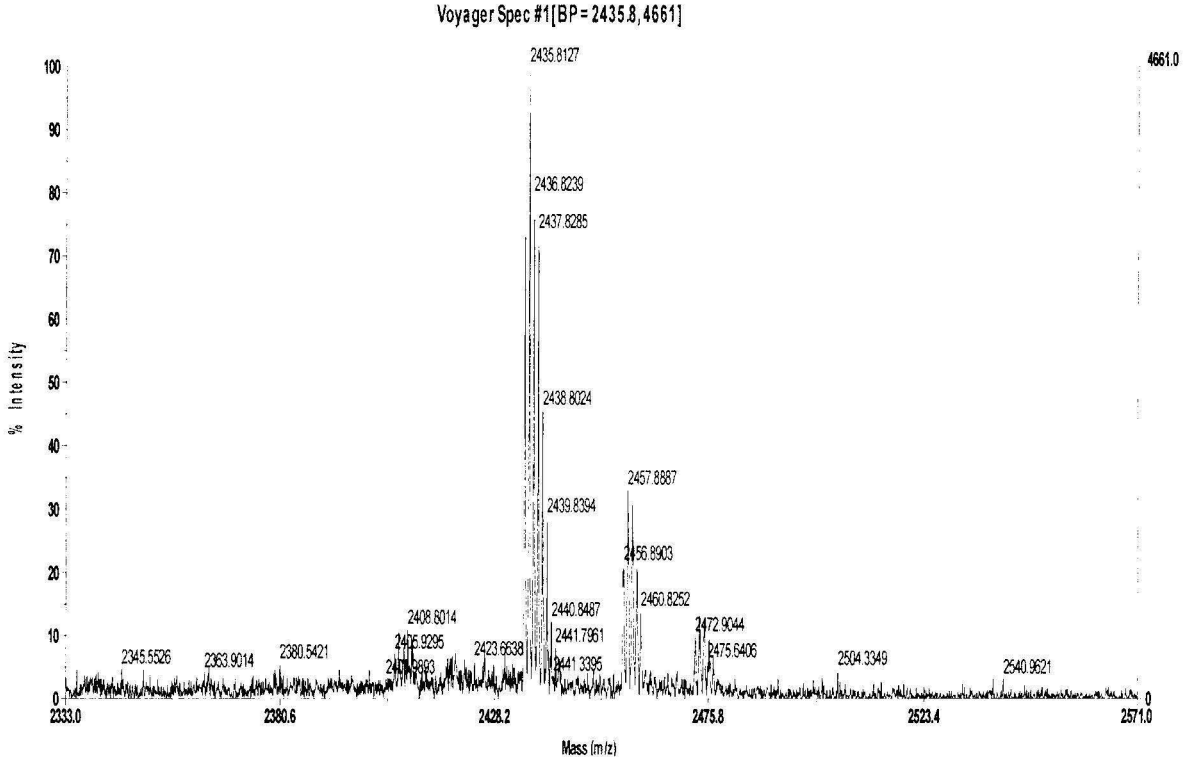


Figure 9 – TOF MS of Tes_B 05d06 (MW= 1531.02 Da)

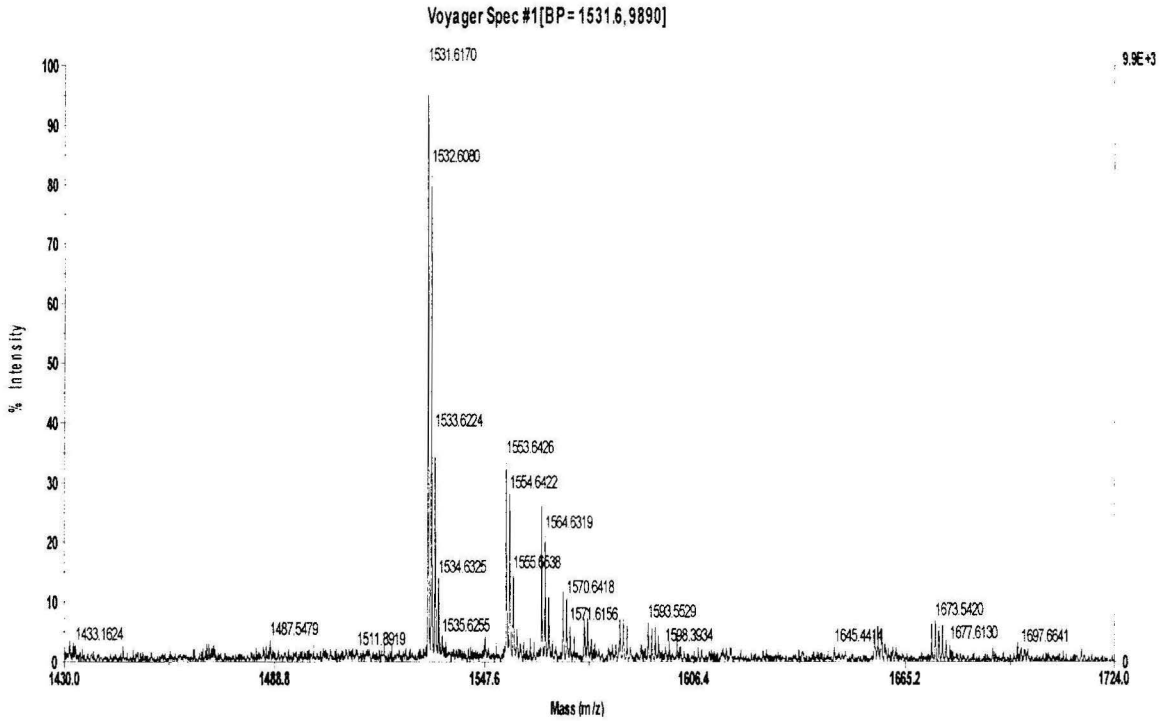


Figure 10 – TOF MS of Tes_B 05d07 (MW= 1655.50 Da)

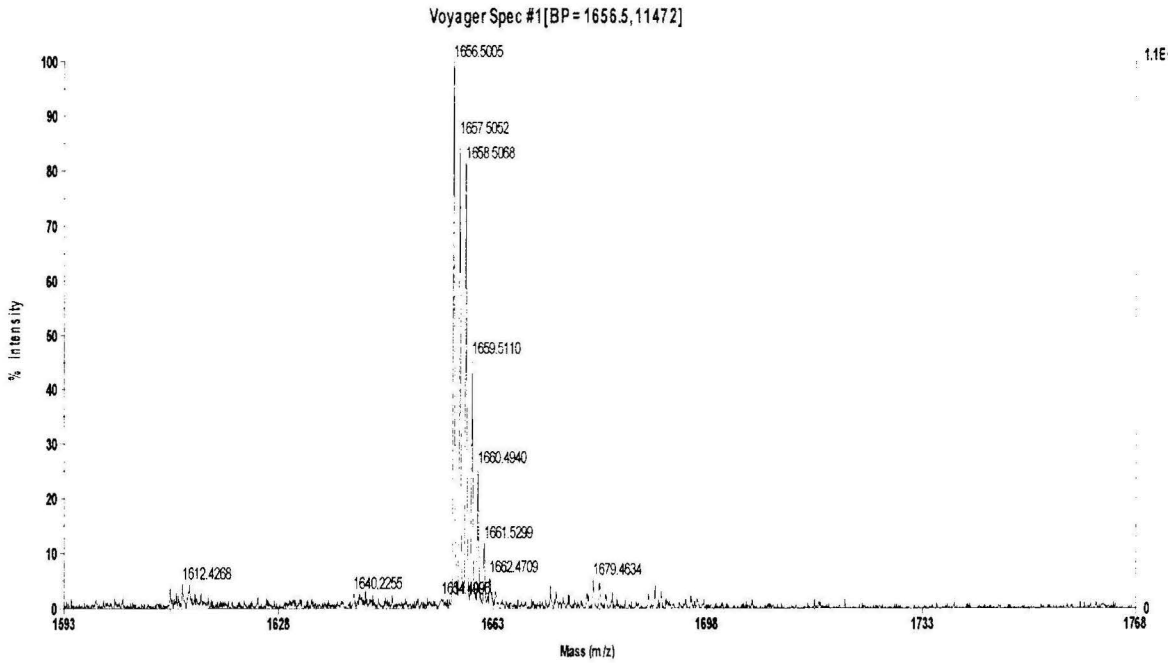


Figure 11 – TOF MS of Tes_B 05d10 (MW= 1809.54 Da)

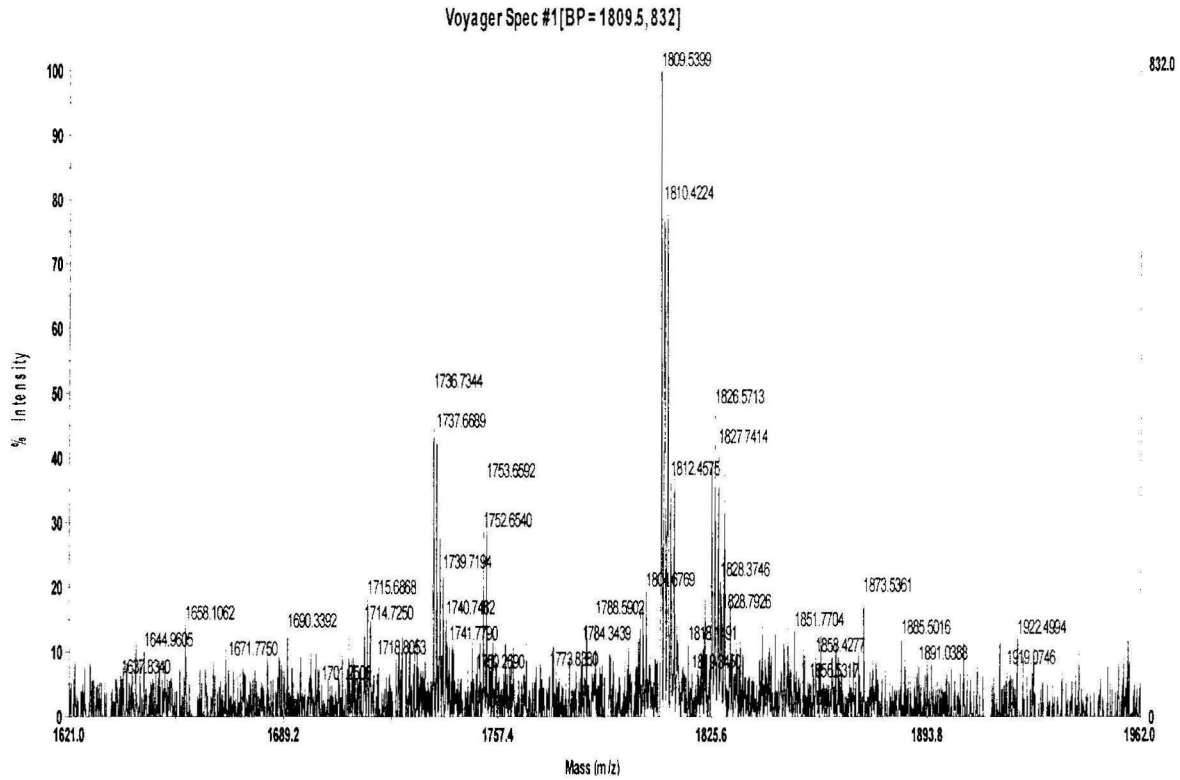
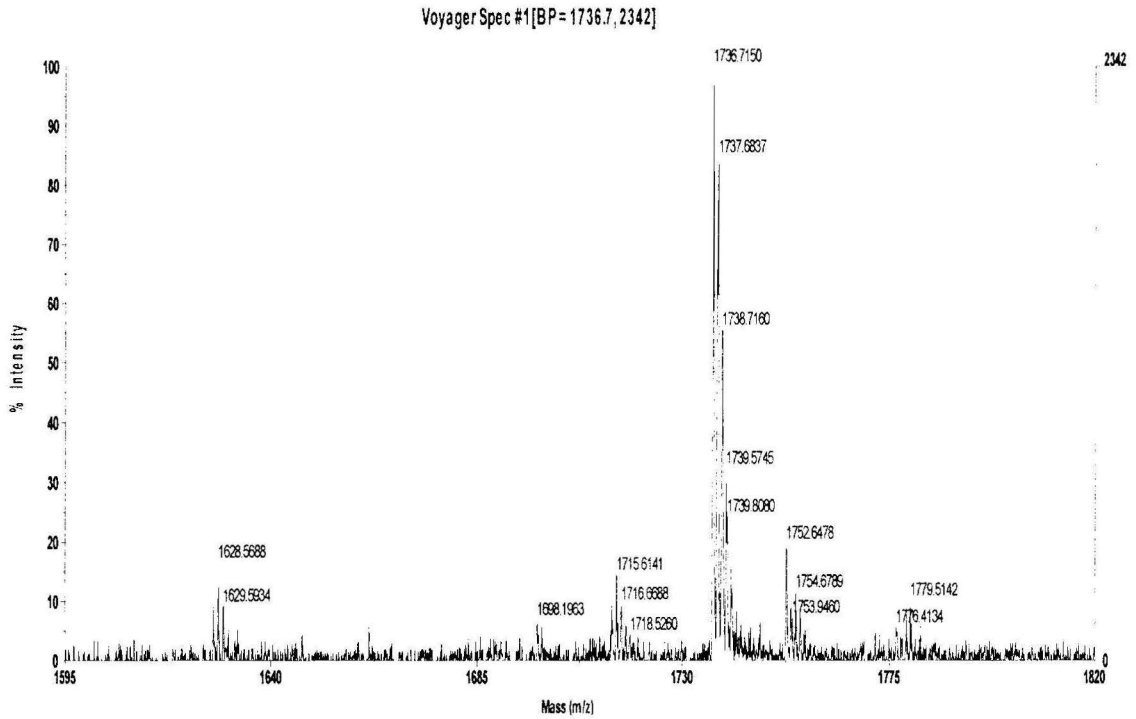
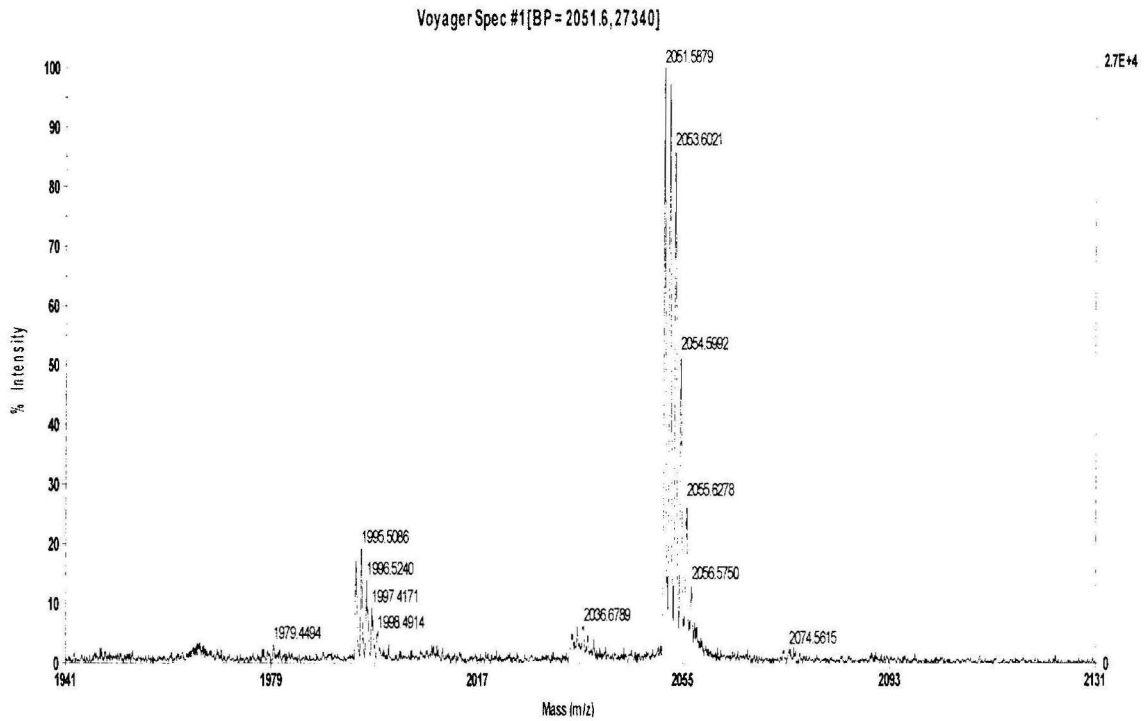


Figure 12 – TOF MS of Tes_B 05d11 (MW= 1736.72 Da)



The first peak to be further processed was peak Tes_B 05d02 since the mass spectra of this sample revealed a potentially pure compound with no need for further purification. The sample was lyophilized then dissolved in an NMR solution as previously described. The sample was thus subject to a 1D NMR experiment. However, a spectrum was not obtained for this sample due to insufficient concentration. Therefore, two thirds of the sample was then reduced and alkylated. The clean product of this reaction was submitted for a mass spectroscopy experiment that revealed the presence of six cysteine residues since the molecular weight of the compound increased from 1703.37 Da to 2051.58 Da. This indicated the presence of six cysteines since the reduction of each disulfide bond, which consists of two cysteines, and subsequent alkylation of each cysteine residue results in a 58 Da increase in molecular weight (2051.58 – 1703.37 = 348.21 and 348.21/58 = 6). Figure 13 shows the mass spectrum of the reduced and alkylated peptide.

Figure 13 – TOF MS of the Reduced and Alkylated Tes_B 05d02 (MW= 2051.58 Da)



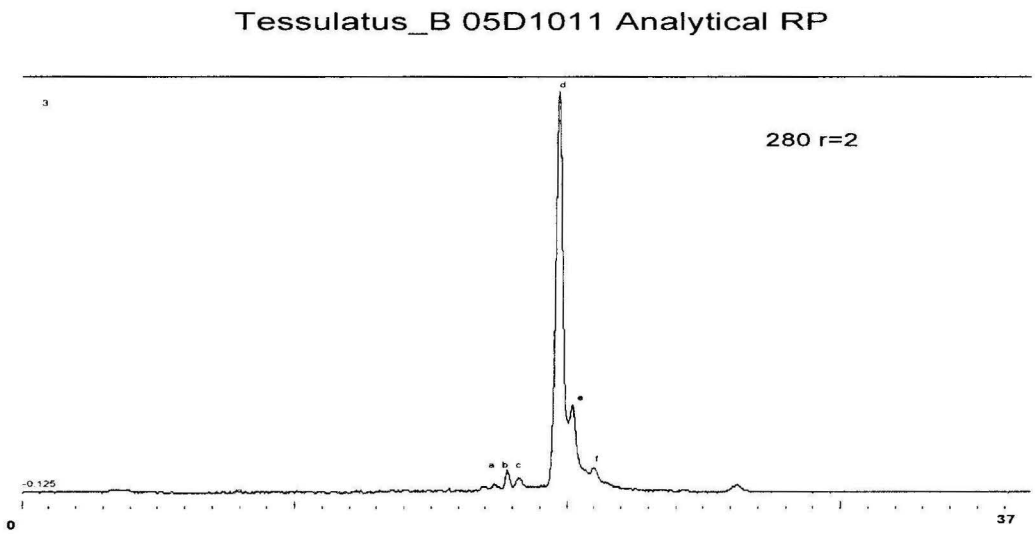
The entire reduced and alkylated sample was then loaded onto the peptide sequencer and yielded the following sequence:

Sequence: CCSQDCRVCIOCCPY

The measured molecular weight of this sequence corresponds to the theoretical molecular weight as determined by Protein Prospector MS-Product software with a 0.76 Da difference. This difference can be attributed to calibration errors or other minor experimental errors. This peptide corresponds to an M-conotoxin of the framework CC-C-C-CC. This type of conotoxin is a M-2 mini-M since the molecular weight is under 2000 Da and there are two residues within the last loop. The target of mini-M's still remains unknown (McDougal et al, 2004).

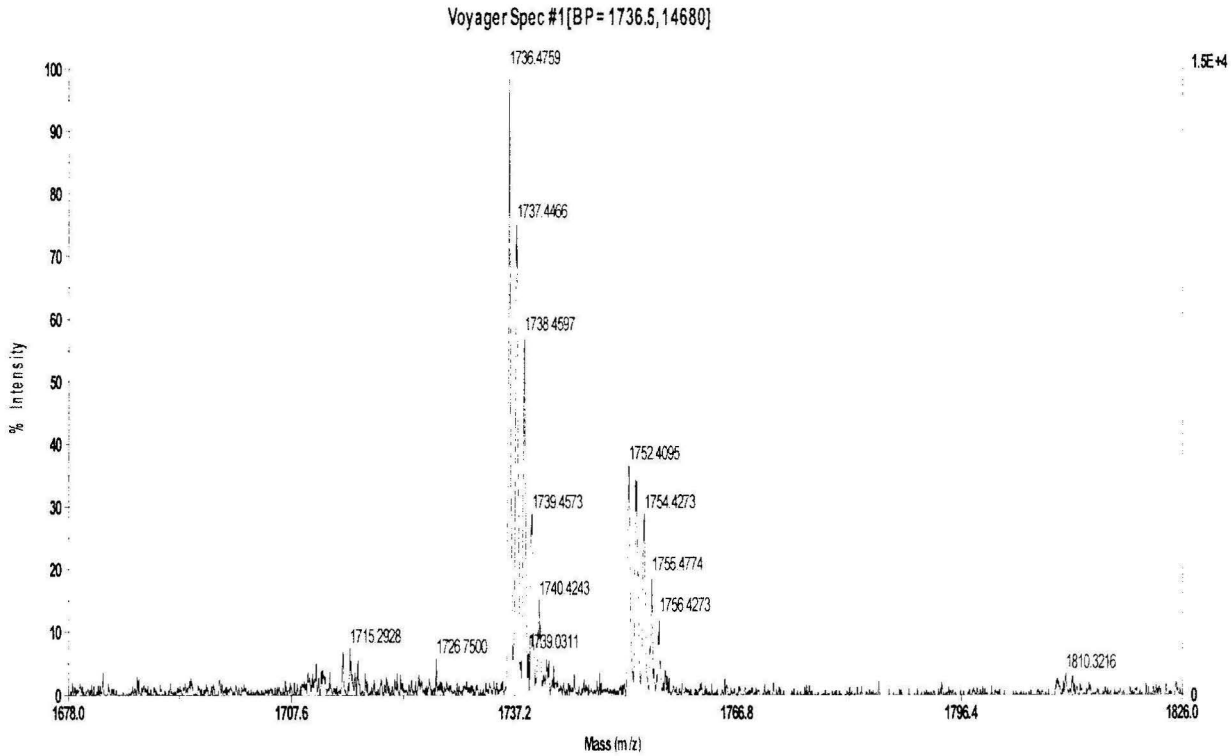
The next peak to be further analyzed from the Tes_B 05d was combined peaks 10 and 11 from batch 2, and 8 and 9 from batch 1 polled together (renamed Tes_B 05d1011). The peak was lyophilized and re-dissolved in 500 μL of a 0.1% TFA and subject to a run in the analytical reverse-phase column. This run yielded the following elution profile (Figure 14), which ran for 37 minutes at range 2 and absorbance of 280 nm (220 was lost).

Figure 14 – Elution Profile of Tes_B 05d1011



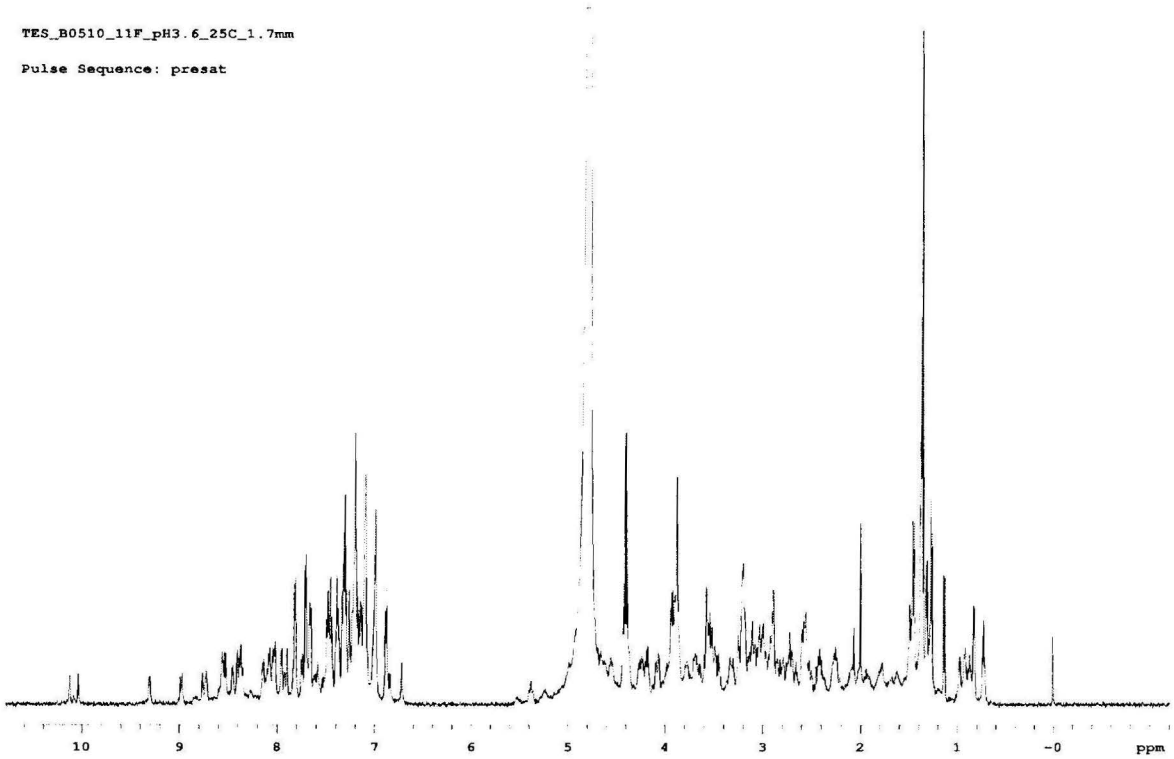
Upon analyzing this chromatogram, extensive work went into attempting to purify the major peak, D. However, this attempt was not successful, and the F fraction seemed to be relatively pure (as indicated by the mass spectrum in figure 15). Therefore, this peak was chosen for further analyses.

Figure 15 – TOF MS of Tes_B 05d1011f (MW= 1736.48 Da)



The sample was lyophilized and re-dissolved into an NMR solution as previously described. The following figure (Figure 16) shows the 1 dimensional NMR spectrum of this sample.

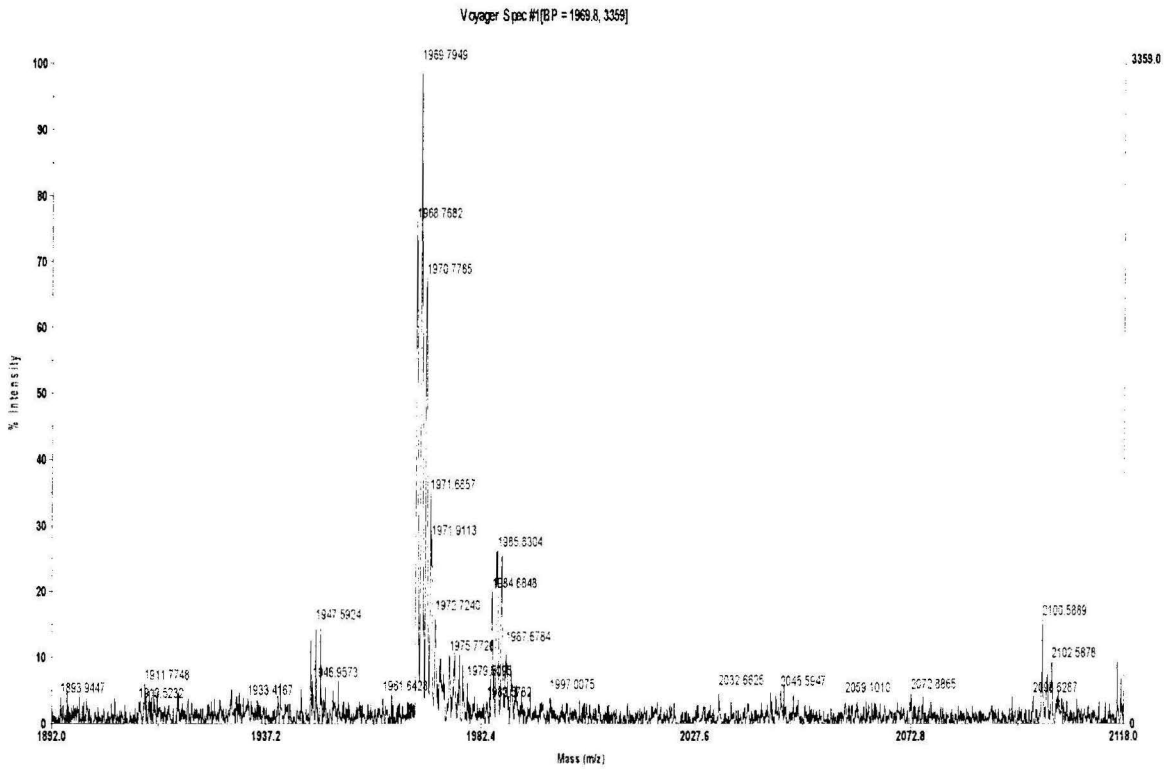
Figure 16 -1-D NMR Spectrum of Tes_B 05d1011f



The NMR spectrum shows several characteristics of the peptide. Initially, the TSP peak is sharp and strong, and smaller than the amino acid hydrogen resonances, indicating that the peptide has a concentration that is suitable for the 1-D experiment, and possibly for 2-D experiments. Moreover, the region between 6-10 ppm indicates that there are aromatic amino acids present. The region between 1 and 1.5 ppm is highly crowded, indicating the presence of aliphatic residues, corresponding to the Ile residue. The doublet of doublets characteristic of Tyr that resonates around 7 ppm is seen here. The spectrum is problematic in that there appears to be two Trp resonating around 10 ppm that do not correspond to the sequence data. The reason for the presence of these peaks is not yet known.

Once the NMR spectrum had been acquired, an attempt was made to obtain a 2-D spectrum. Unfortunately, concentration did not permit, and the spectrum was not acquired. Therefore, one third of the sample was subject to reduction and alkylation (figure 17).

Figure 17 - TOF MS of the Reduced and Alkylated Tes_B 05d1011f (MW= 1968.76 Da)



The molecular weight of the reduced and alkylated peptide signaled the presence of four cysteine residues (1968.73 – 1736.48 = 232.25. 232.25/58 = 4.00). The sample was then subject to sequencing.

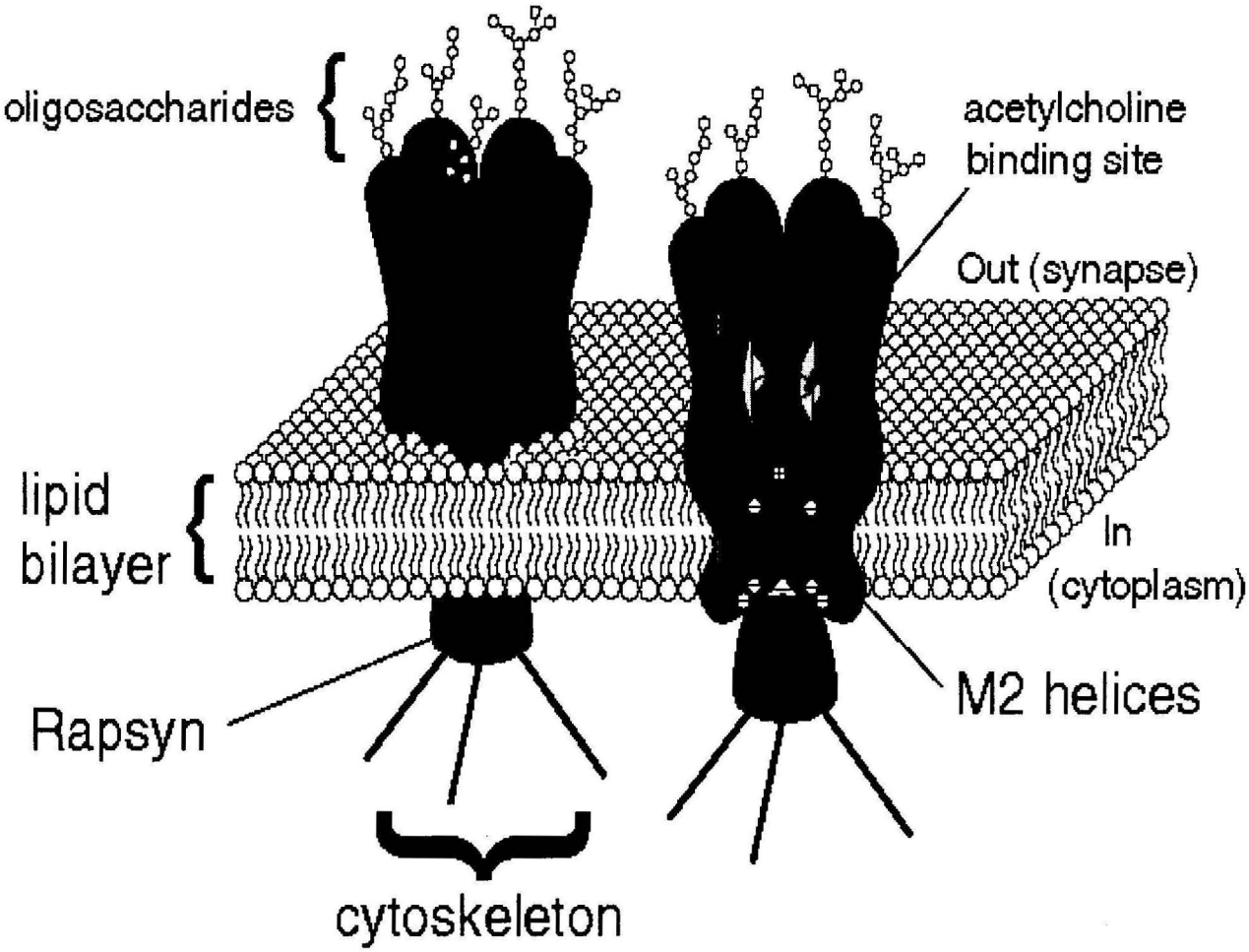
Sequence: GGCCSYOPCIANNPFCG

This sequence yielded a theoretical molecular weight of approximately 1714.65 Da as calculated by Protein Prospector MS-product software. This weight represents an

approximate 21.83 Da difference from the mass spectrum of the peptide, a difference corresponding to an ion of Na with minor experimental errors. Moreover, the framework of this peptide corresponds to the A superfamily as a 4/6 α-conotoxin because of the number of residues in the loop. The peptide has sequences of 4 and 6 non Cys amino acids in the format CC----C------C.

This type of conotoxin is known to be a competitive antagonist of acetylcholine in nicotinic acetylcholine receptors, which are found in the central and peripheral nervous system (neuronal type) and in the neuromuscular junctions (neuromuscular type).These receptors are ligand gated channels embedded in the membrane that have five homologous subunits that allow for the movement of Na and K ions across the membrane. The binding of acetylcholine causes opening of the channel, thus acetylcholine agonists such as the α-conotoxins prevent channel opening. Each α-conotoxin is specific for a different type and subunit of the nACh receptor that it binds. Further work on this compound will be aimed at elucidating the specific target and action of the toxin.

Figure 18 – Theoretical Structure of the nACh Receptor from http://indigo1.biop.ox.ac.uk/graham/work.html



The next sample to be further purified was the Tes_B 05d050607 peak that had previously been pooled together. This peak was re-dissolved in 500 µL of a 0.1% TFA solution and ran on the analytical reverse phase column for 34 minutes at range 1 in both 220 nm and 280 nm. Figure 19 shows this elution profile.

Figure 19 – Elution Profile of Tes_B 05d050607 in the Analytical Column

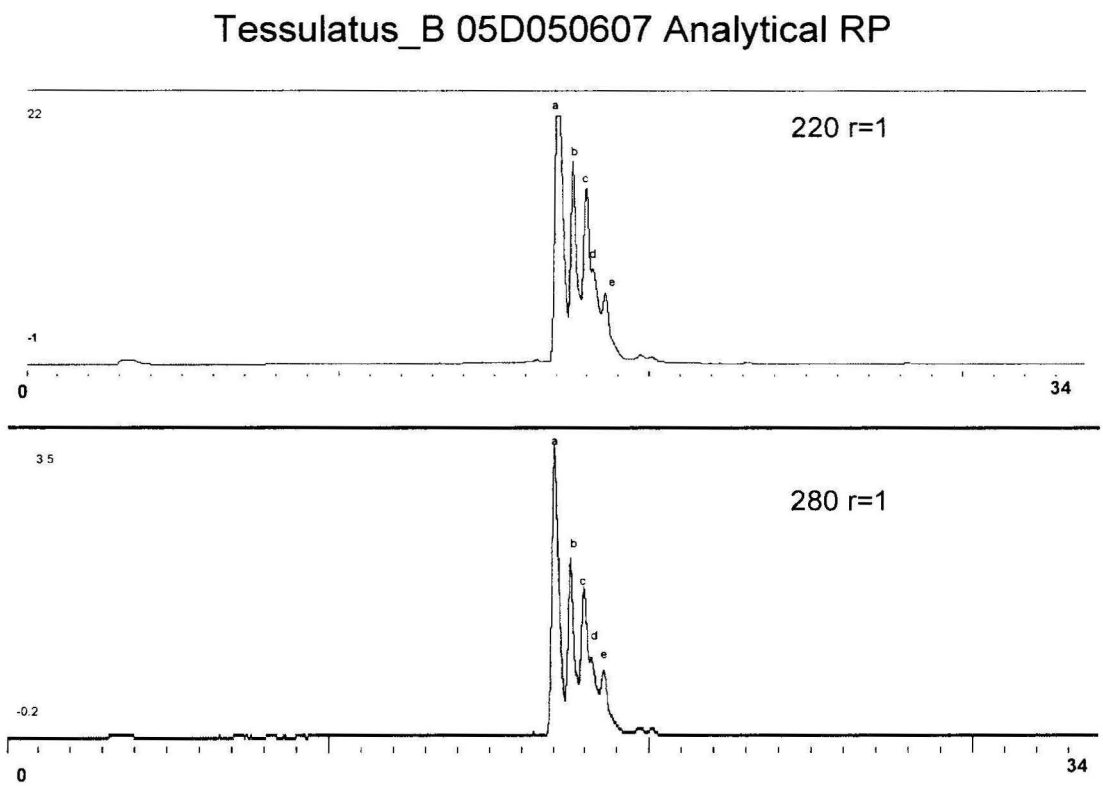
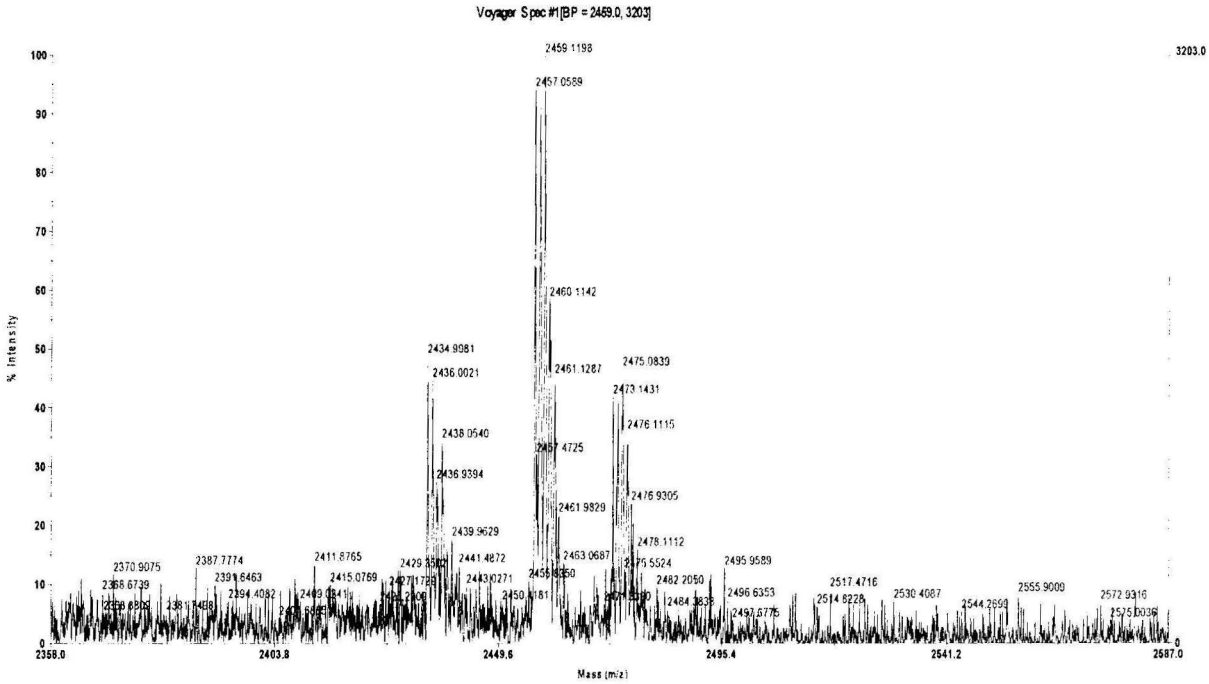


Table 6 – Elution times of Tes_B 05d050607 in the Analytical Column

Peak #	Elution Time (minutes)
a	17.0
b	17.8
c	18.1
d	18.4
e	18.8

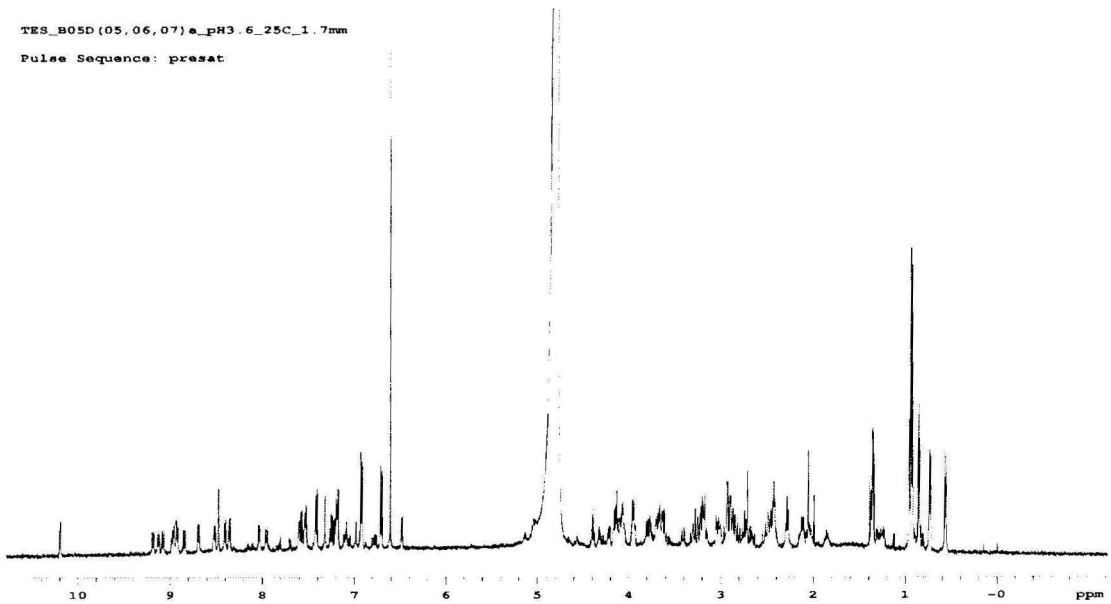
Once again efforts were made to further purify the biggest peak, peak a. The mass spectrum of the native peptide is the following:

Figure 20 – TOF MS of Tes_B 05d050607a (MW = 2435.00 Da)



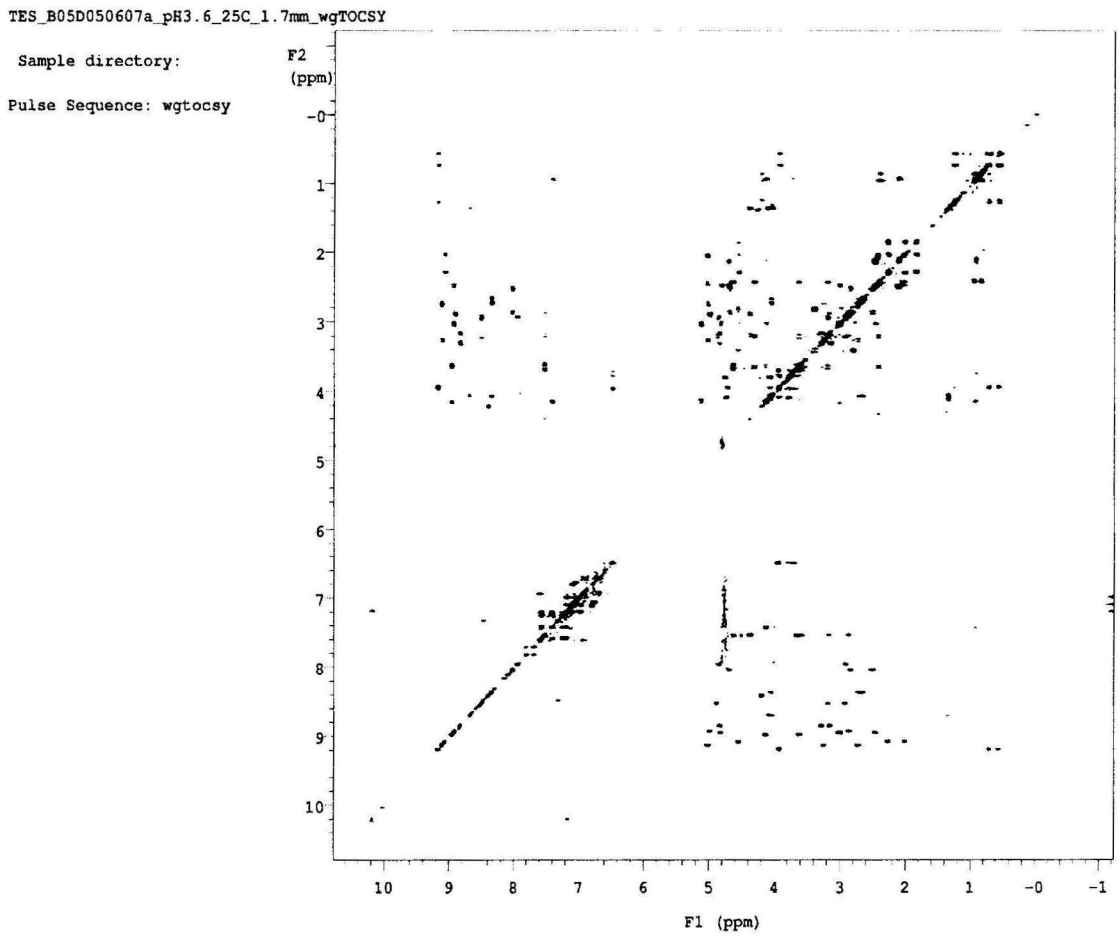
The sample then underwent the following NMR experiments to yield the following results (Figure 21 and 22):

Figure 21 – 1-D NMR Spectrum of Tes_B 05d050607a



The NMR spectrum shows characteristics of several residues and shows that there are sufficient quantities of the peptide to run 2-D NMR experiments. The peptide is actually very concentrated compared to many native samples from this study. The most obvious residue is that of Trp near 10 ppm and the presence of aliphatic residues, maybe Ile or Leu or Val, under 1 ppm. This peptide has several residues, about 22 since there are so many NH peaks between 6 and 10. There appears to be slight impurities as well.

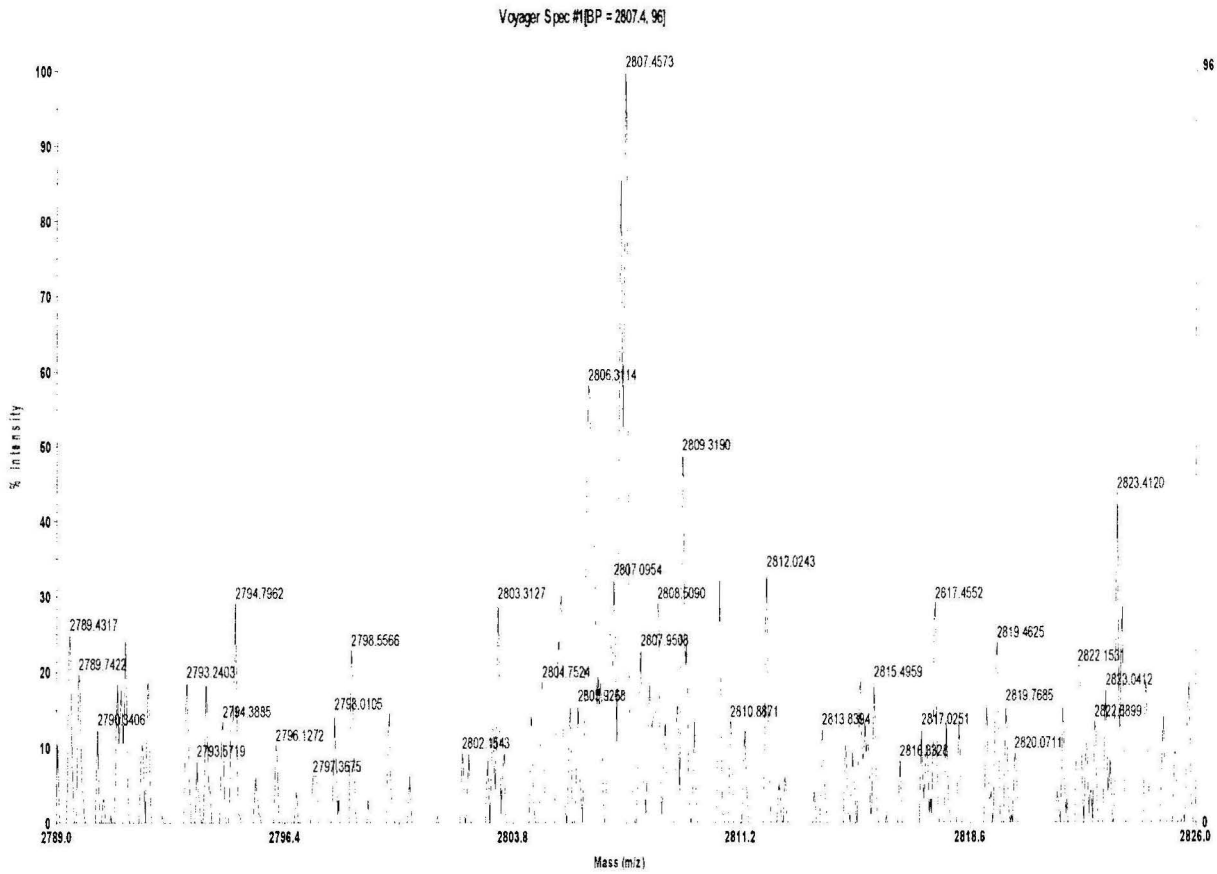
Figure 22 – TOCSY Spectrum of Tes_B 05d050607a



The TOCSY spectrum revealed information about some residues, but the information was not sequence dependent. Namely, the presence of the Tyr residue is confirmed. However, assignments of residues without the sequence information are difficult and futile.

Once the TOCSY spectrum had been acquired attempts to run other 2-D NMR experiments were undertaken but proved unsuccessful due to insufficient quantities. The sample was then reduced and alkylated, yielding the following mass spectrum:

Figure 23 – TOF MS of Reduced and Alkylated Tes_B 05d050607a (MW = 2806.31)



The data suggests that there are probably six cysteine residues in this peptide (2806.31-2435.00 – 22 (Na) = 349.31. 349.31/58 = 6.03). Unfortunately, upon two attempted trials

of sequencing, no sequence data was obtained. Although the reason that this peptide was unable to be sequenced is not known, it is presumably blocked at the N-terminus and therefore not prone to Edman degradation. The peptide is most likely an M-conotoxin, if indeed there are six cysteines present. However, at this point, speculation is futile without a sequence.

Since these efforts were unsuccessful, we chose to further analyze the next major peak, peak b. This peak was once again run in the analytical column yielding the following spectrum at range 0.2 in both wavelengths of 220 nm and 280 nm (figure 24):

Figure 24 - Elution Profile of Tes_B 05d050607b in the Analytical Column

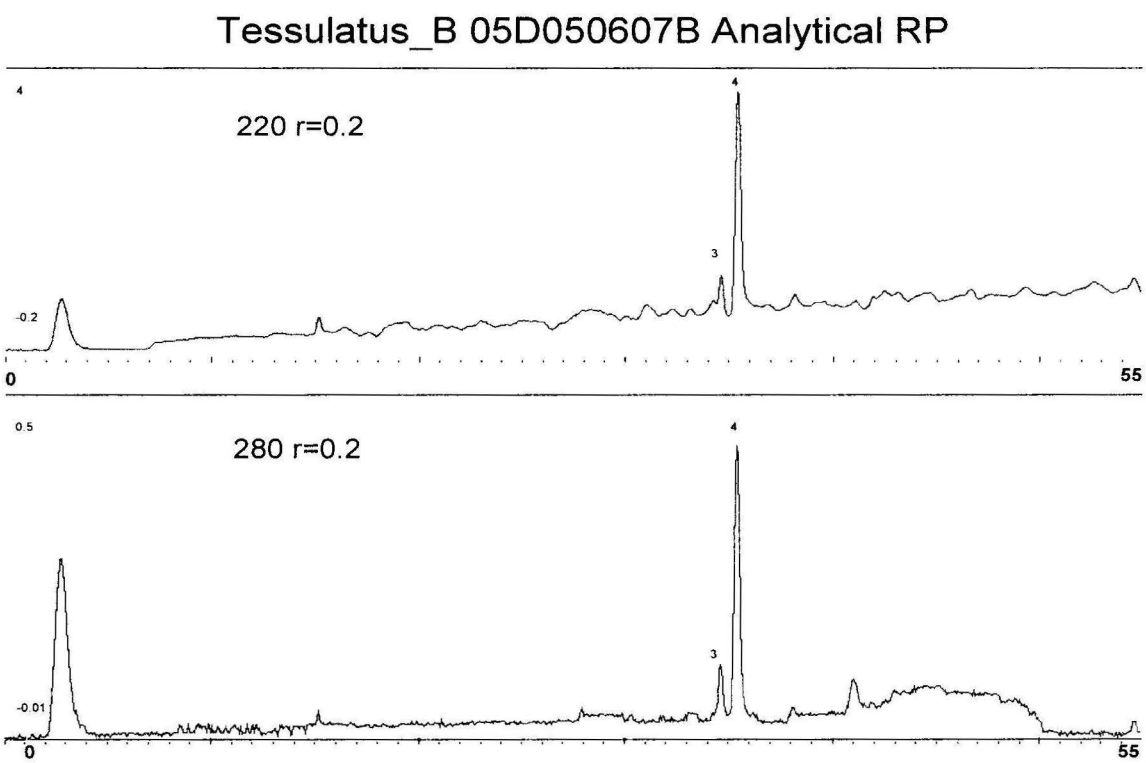
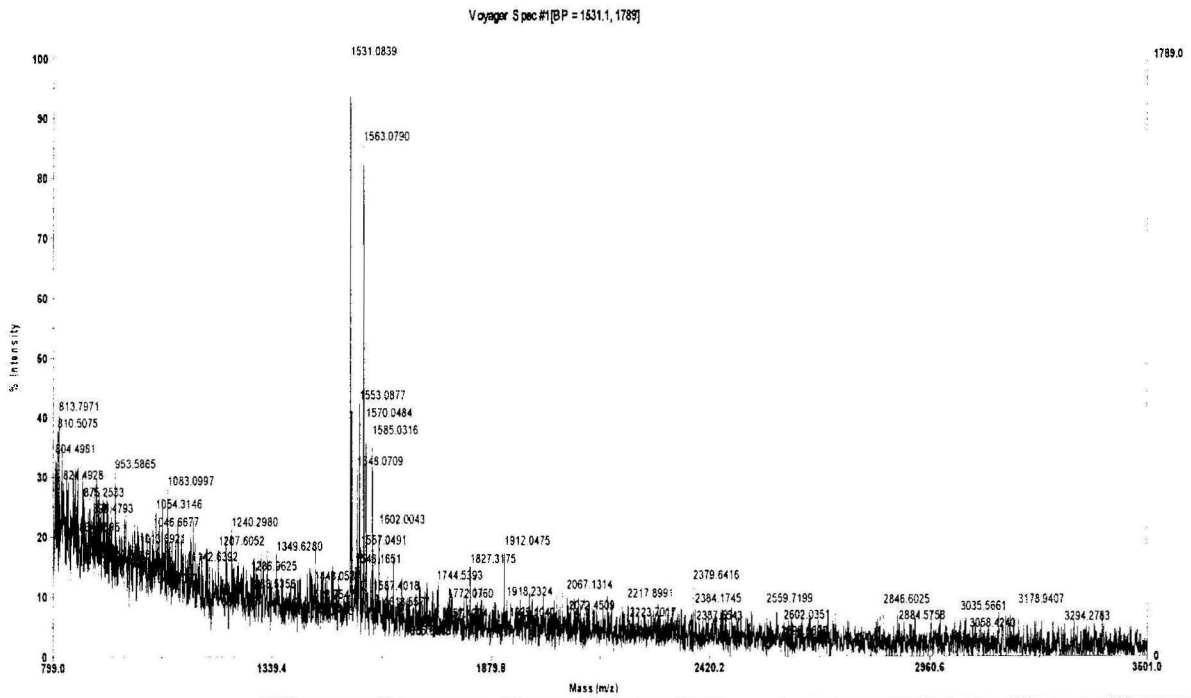


Table 7 – Elution times of Tes_B 05d050607b in the Analytical Column

Peak #	Elution Time (minutes)
1	15.2
2	28.2
3	34.8
4	35.7

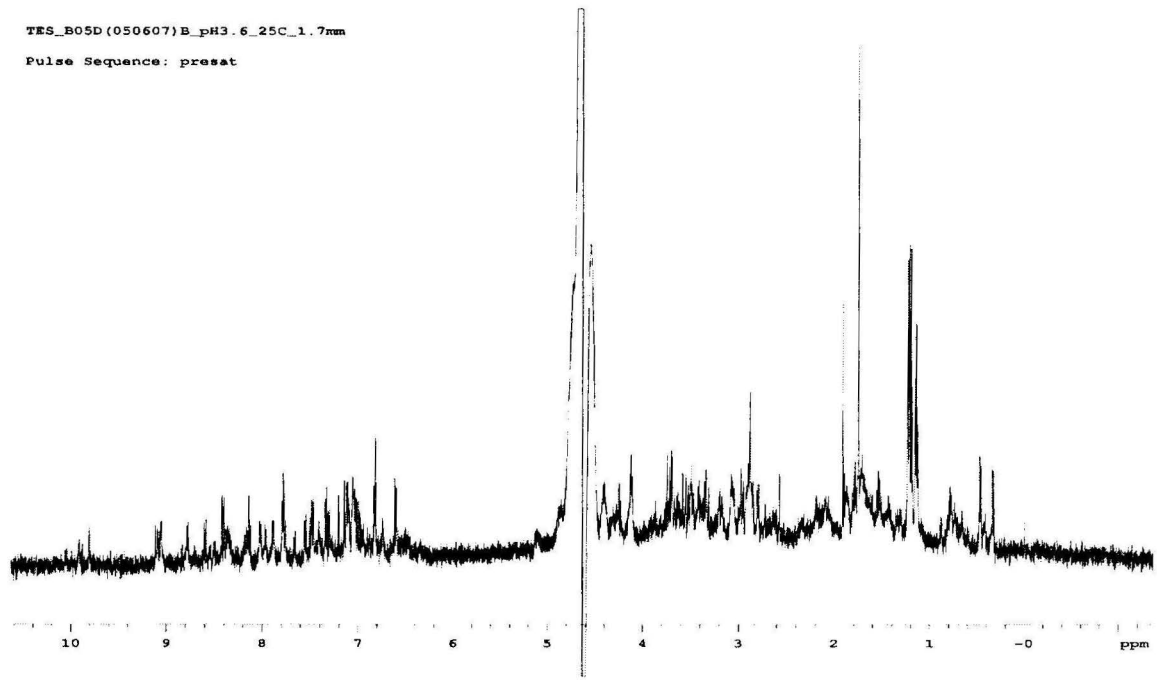
A mass spectrum was acquired for the major peak, peak 4 (figure 25):

Figure 25 - TOF MS of Tes_B 05d050607b4 (MW= 1531.08 Da)



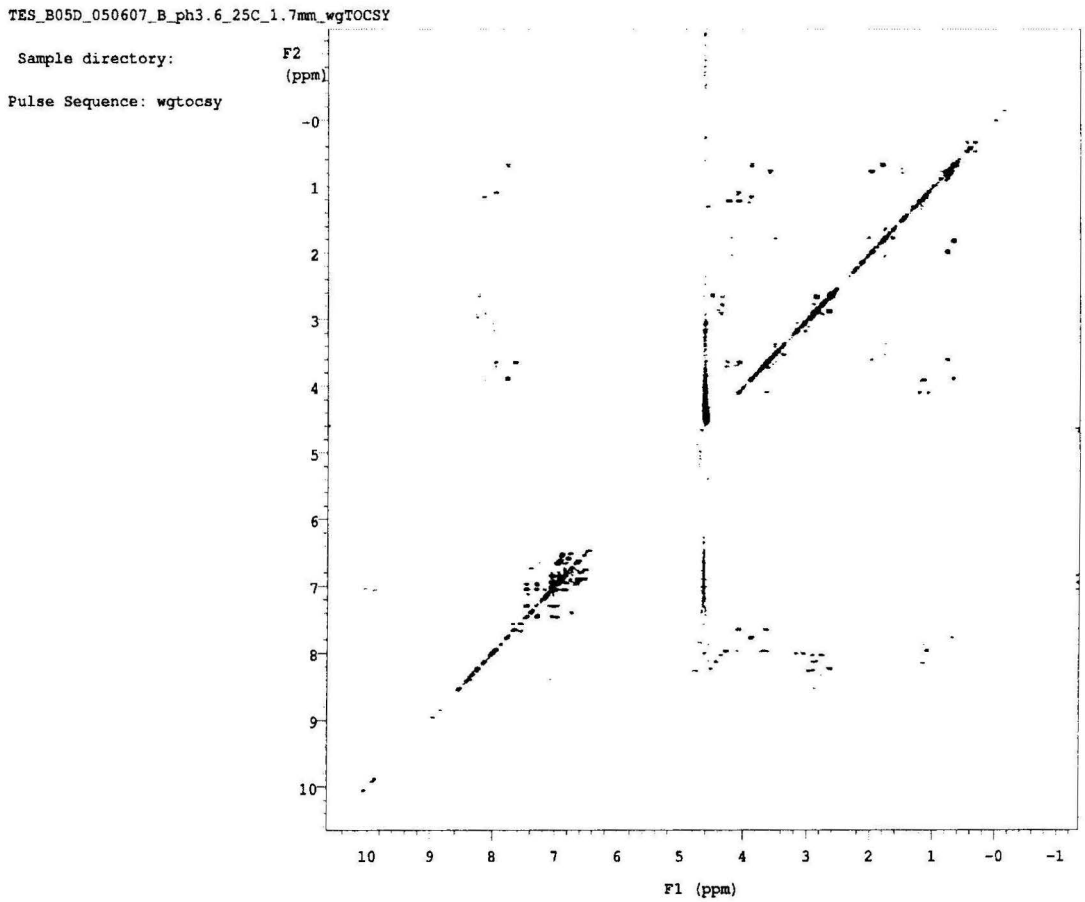
We acquired a 1-D NMR spectrum and a TOCSY spectrum for this sample.

Figure 26 – 1-D NMR Spectrum of Tes_B 05d050607b



The 1-D spectrum lacks a proper TSP peak; therefore no estimates on concentration can be accurately made, however the sample does appear concentrated enough to run 2-D experiments. The obvious residue seen in this spectrum is that of Trp around 10 ppm. There is also a double of doublets around 7 ppm that is characteristic of Tyr residues ring protons. The two His show peaks around 7 and 8 ppm for the ring protons, which slightly overlap with the Tyr. The two Ala show peaks around 1.39 ppm for the βH's. The Val peaks around 0.5 ppm are downshifted, as characteristic of conophans due to the presence of the neighboring Trp ring causing shielding.

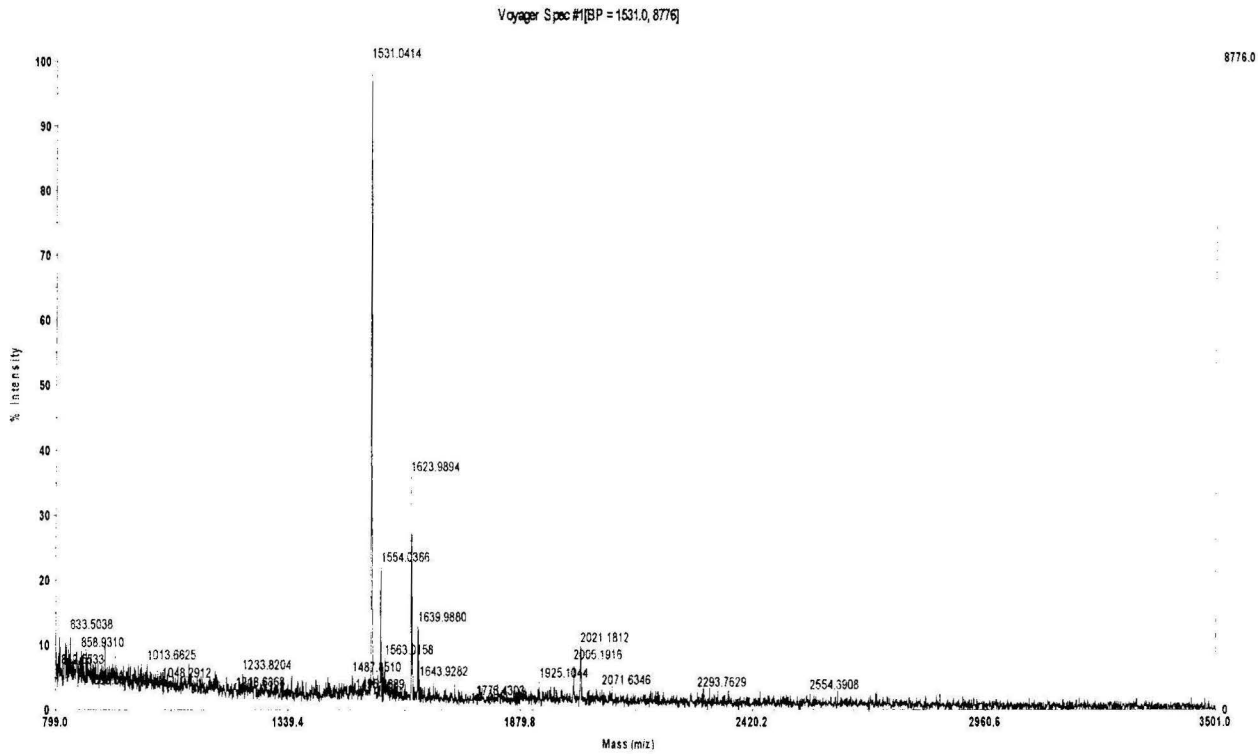
Figure 27 – TOCSY Spectrum of Tes_B 05d050607b



This spectrum confirms the presence of the Trp residue at 10 ppm. Otherwise, the spectrum is not intense enough to make accurate assignments. Additionally, without a NOESY spectrum, assignments cannot be confirmed.

About one third of the sample was reduced and alkylated yielding the following mass spectrum (figure 28):

Figure 28 - TOF MS of the Reduced and Alkylated Tes_B 05d050607b4 (MW= 1531.06 Da)



The reduced and alkylated mass spectrum revealed that there are no cysteines present in this peptide. This peptide is therefore a linear conopeptide. The sequence analysis of the peptide yielded the following results:

Sequence: VYHAHPYSNAVWS

The expected molecular weight of this peptide as calculated by Protein Prospector MS-Product software is 1530.71 Da, yielding a 0.38 Da difference in MW as calculated by the mass spectrum. This conopeptide is a conophan that closely resembles a conophan that was recently discovered in *Conus gladiator*, gld-V. The sequence homology is quite striking:

Tes_B 05d050607b: VYHAHPYSN-A-VWS

gld-V: AOAN-S-VWS

The gld-V peptide exhibits a D-Val in a homology site of the Tes_B 05d050607b peptide raising the possibility that the tessulatus peptide may have a D-Val as well. The homology also extends to the Pro and Hyp that are in adjacent sites of the two peptides. The homology is convincing evidence for the argument that this peptide may indeed be a conophan.

The following peptide peak to be analyzed was Tes_B 08 from Superdex 30. The peak was dissolved in 500 μL of 0.1 M NH_4HCO_3 and ran through the Superdex Peptide column for 68 minutes at range 2 in wavelengths 220 nm, 280 nm, and 250 nm. Figure 29 shows the elution profile for this run.

Figure 29 – Elution Profile of Tes_B 08 in the Superdex Peptide Column

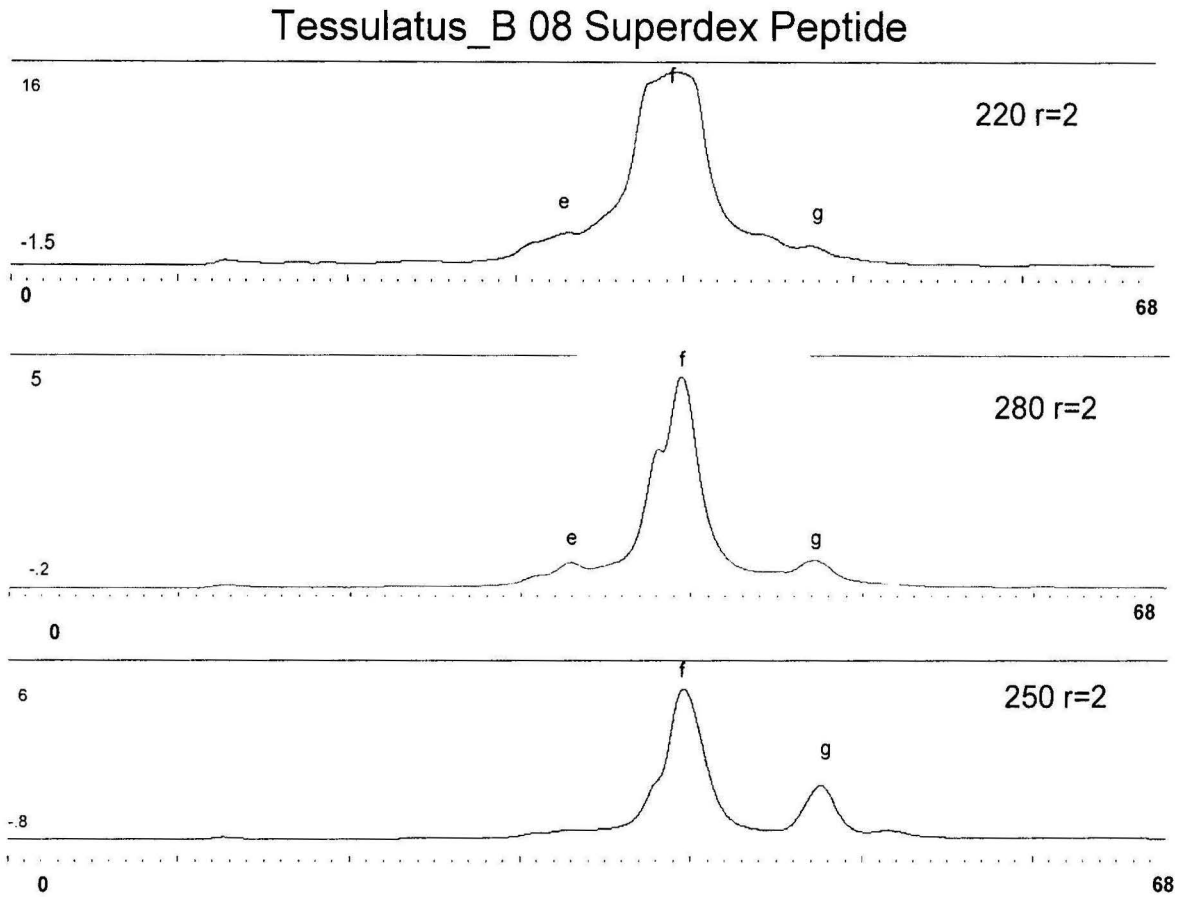


Table 8 – Elution Times of Tes_B 08 in the Superdex Peptide Column

Peak #	Elution Time (minutes)
a	12.77
b	16.66
c	18.86
d	23.70
e	33.10
f	39.58
g	47.45

From this separation, the major peak, peak f, was chosen for further separation in the semi-preparative column. The sample was dissolved in 1000 μL of 0.1% TFA and

yielded the following chromatogram at range 1 and wavelengths 220 nm, 280 nm (figure 30):

Figure 30 - Elution Profile of Tes_B 08f in the Semi-preparative Column

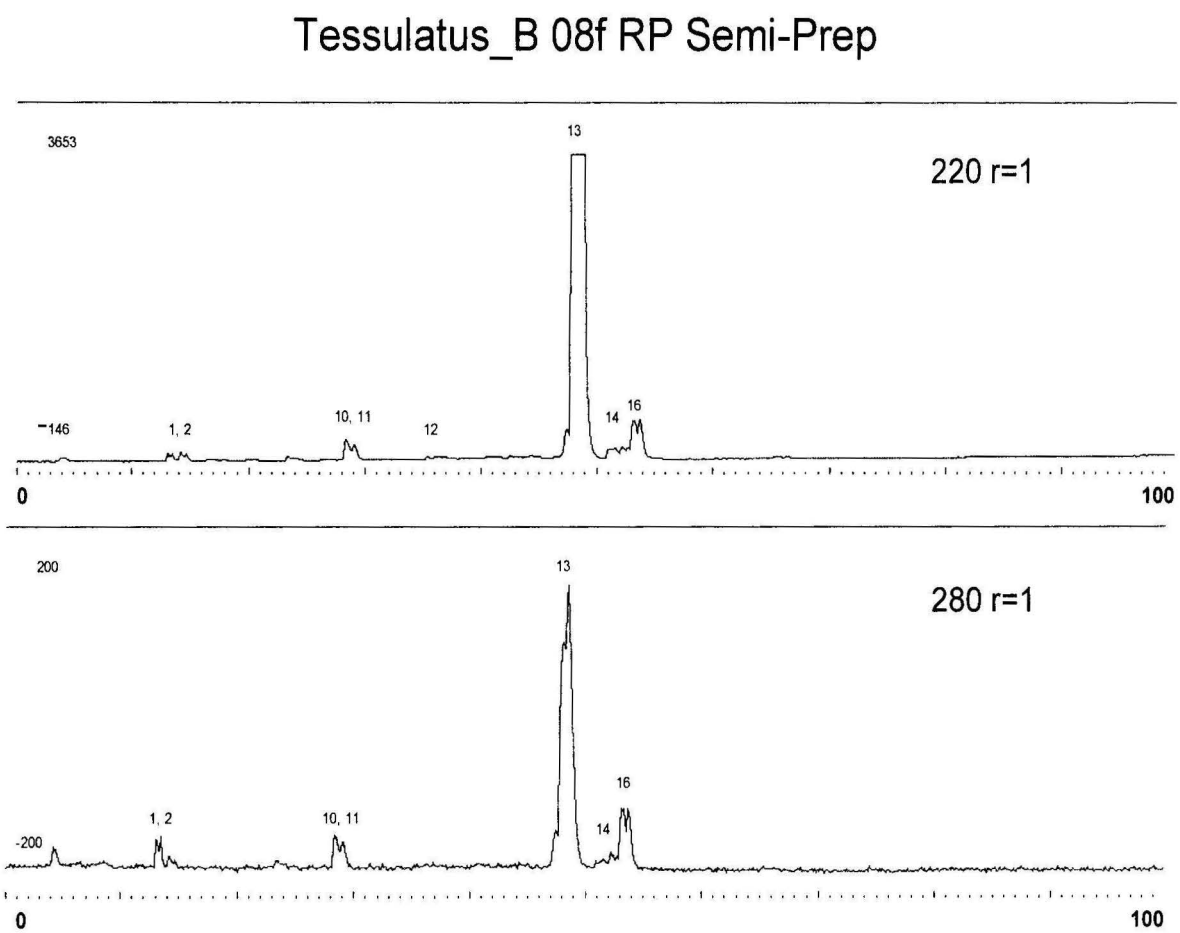


Table 9 – Elution Times of Tes_B 08f in the Semi-preparative Column

Peak #	Elution Time (minutes)
1	12.70
2	13.15
3	14.13
4	16.80
5	18.17
6	20.30
7	23.38
8	24.21
9	26.66
10	28.37
11	29.10
12	35.49
13	47.40
13(b)	48.41
14	51.18
15	52.22
16	53.18

Mass spectra were acquired for all these peaks. Figure 31- 33 show the spectra for the peaks that were later used in other experiments.

Figure 31 – TOF MS of Tes_B 08f12 (MW = 1806.63 Da)

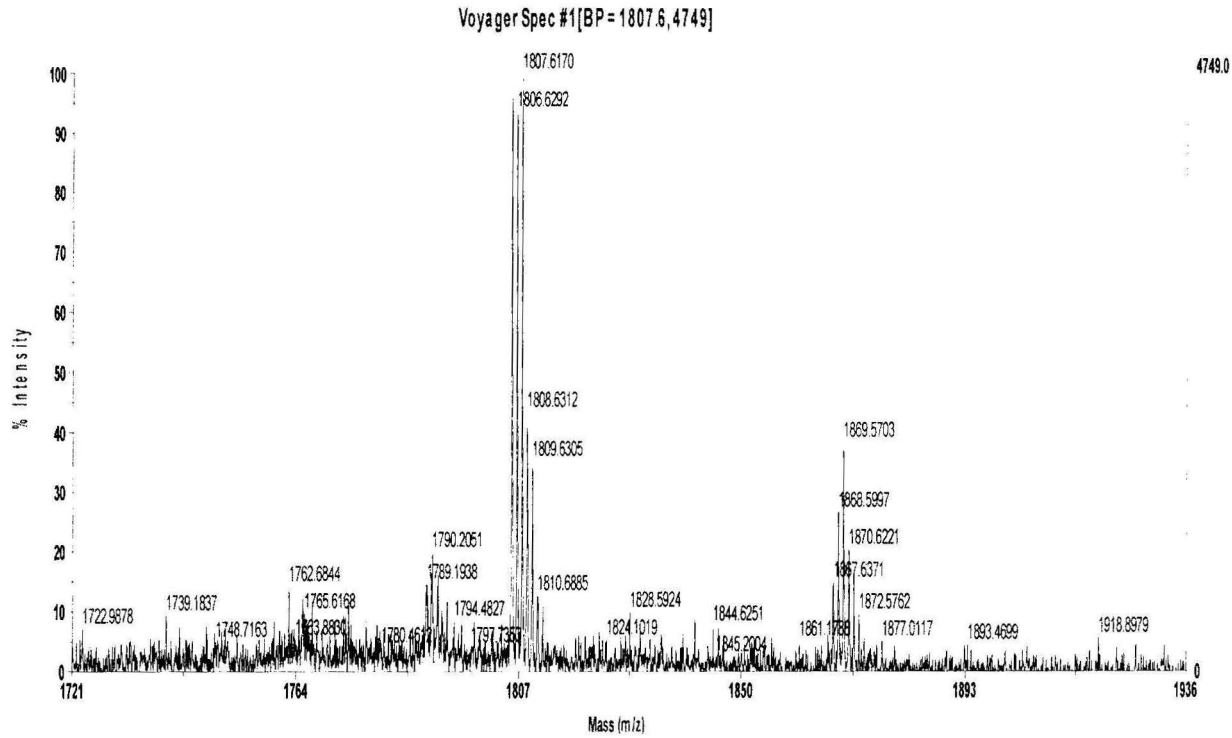


Figure 32 - TOF MS of Tes_B 08f13 (MW = 1751.65 Da)

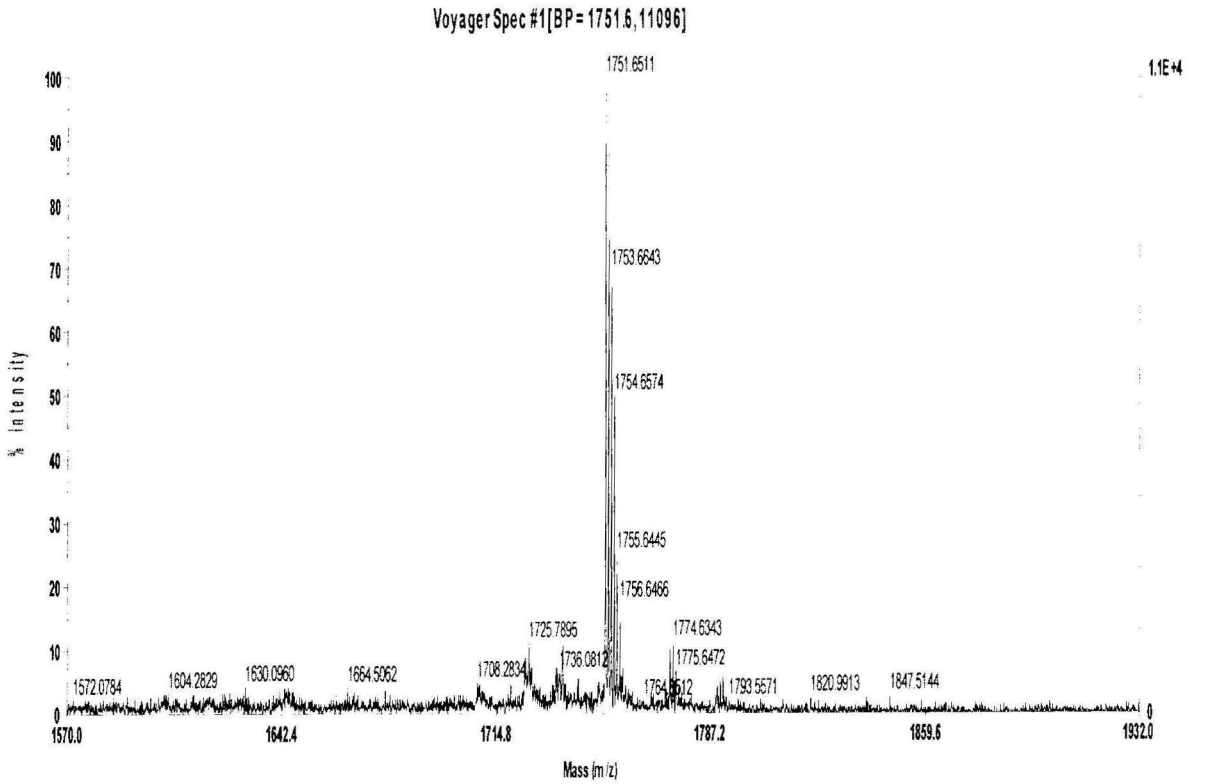
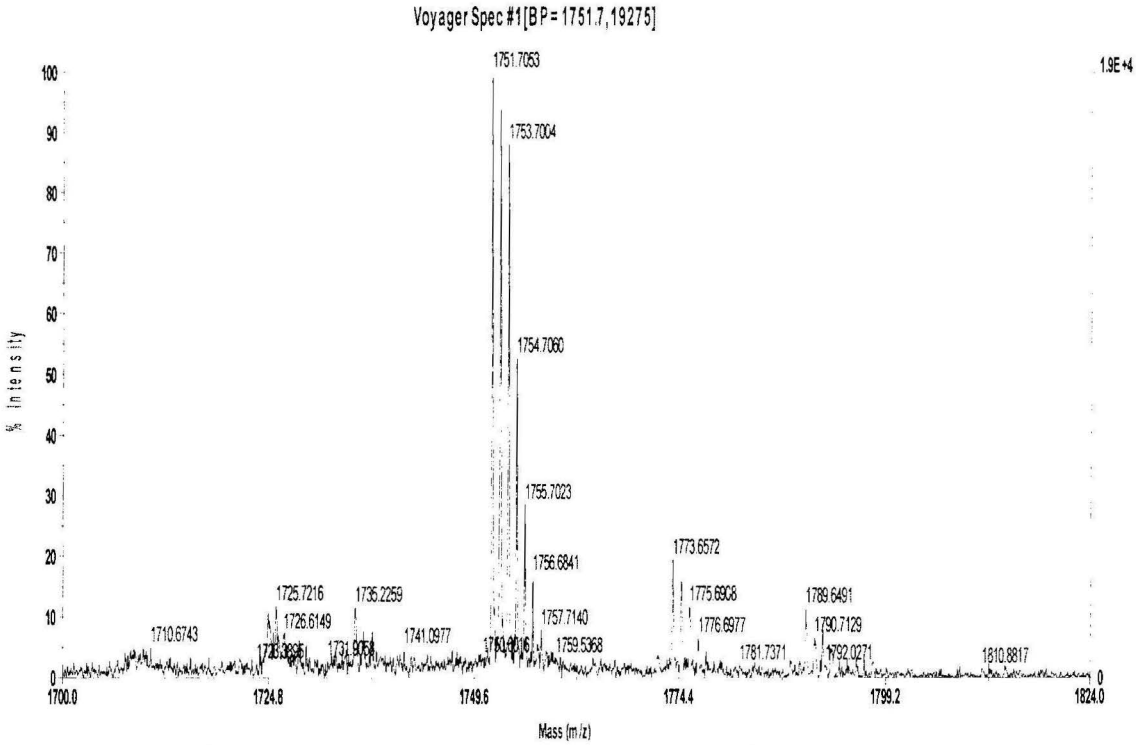


Figure 33 – TOF MS of Tes_ B 08f13(b) (MW = 1751.71 Da)



Peak Tes_B 08f12 was one of the peaks that were chosen for further analysis. The sample was dissolved in 500 µL of 0.1% TFA and ran in the analytical column for 47 minutes at range 1 in wavelengths 280 nm and 250 nm, and 220 nm. Figure 34 shows this chromatogram:

Figure 34 - Elution Profile of Tes_B 08f12 in the Analytical Column

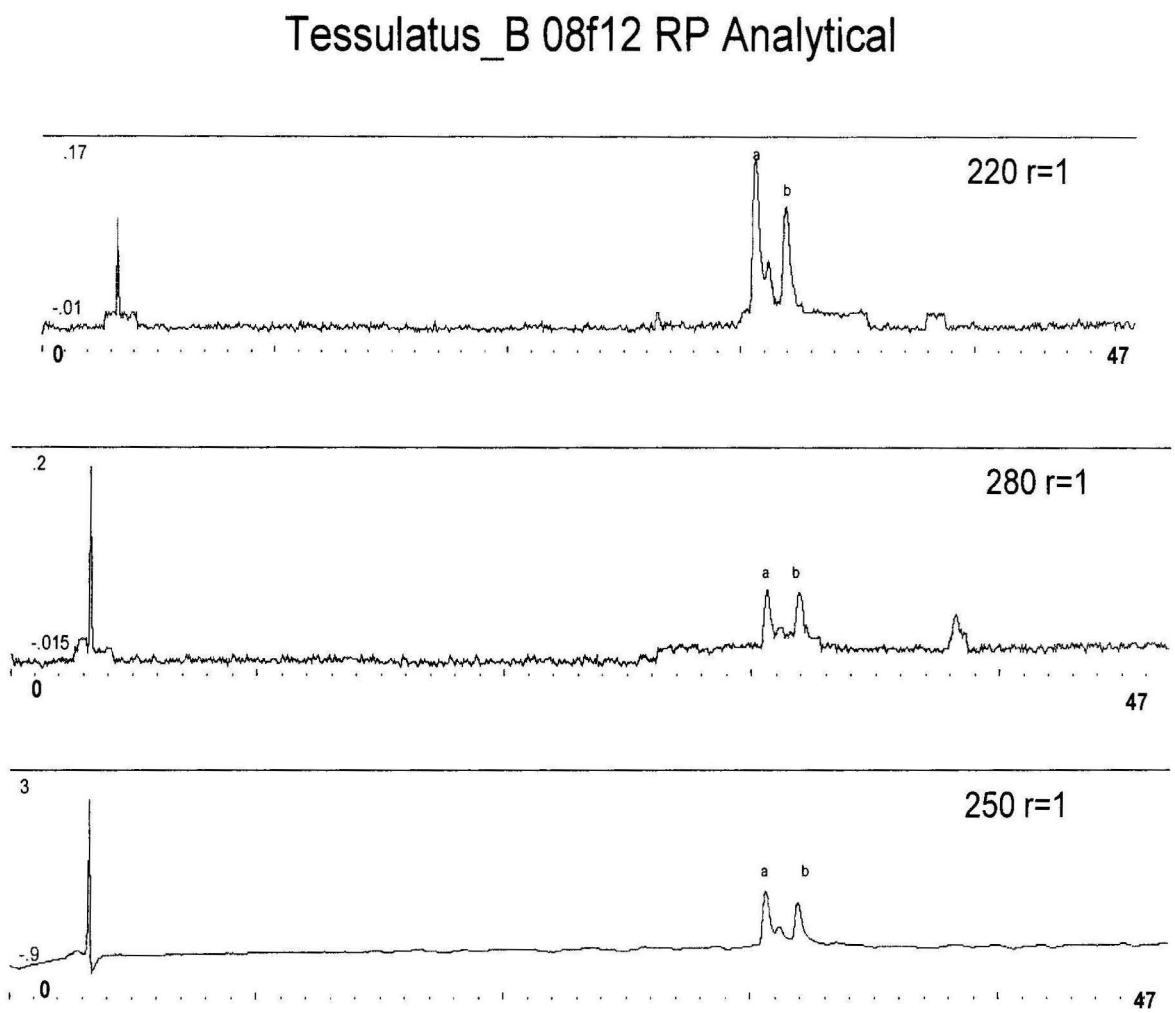
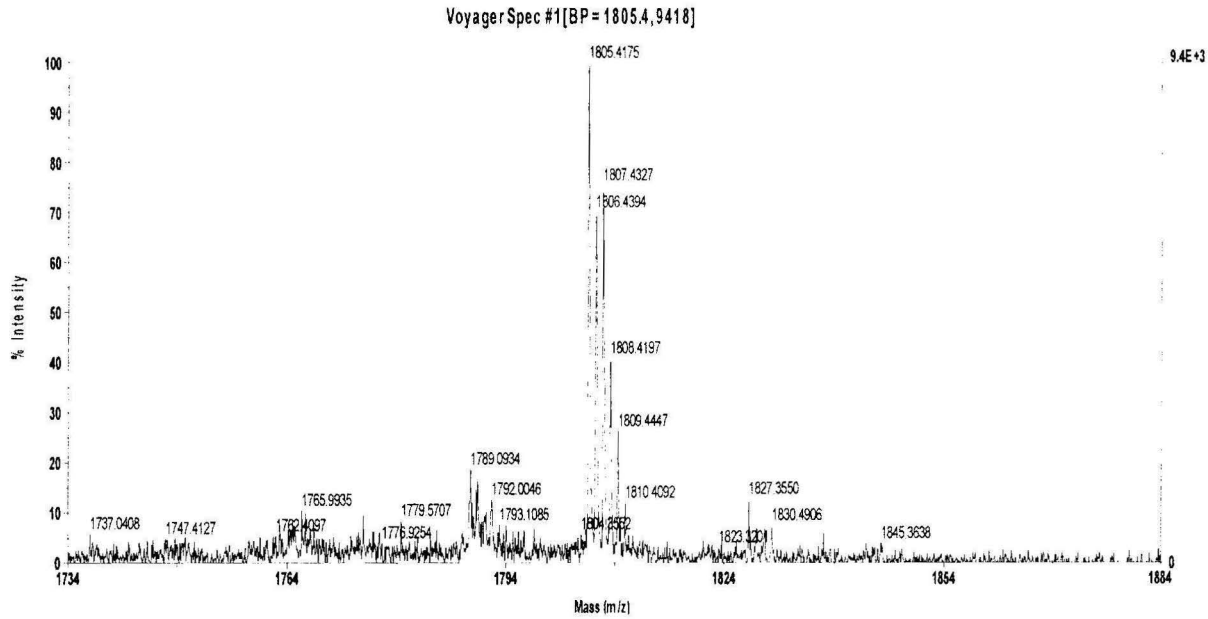


Table 10 – Elution Times for Tes_B 08f12 in the Analytical Column

Peak #	Elution Time (minutes)
a	30.55
b	31.90

From this separation, peak a was chosen to be further analyzed as it was the more pure peak. A mass spectrum was taken of this peak (Figure 35):

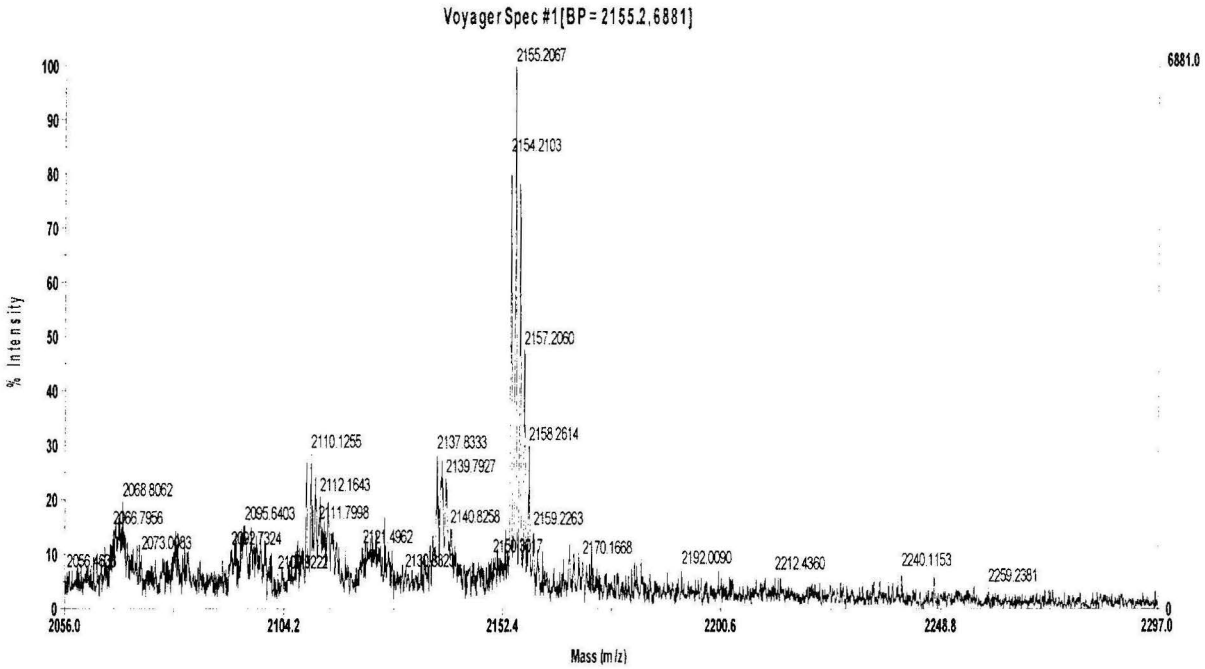
Figure 35 – TOF MS of Tes_B 08f12b (MW = 1805.41 Da)



The sample was then subject to 1-D NMR experiments that did not work due to insufficient quantities.

About one half of the sample was then reduced and alkylated revealing the following spectrum (Figure 36):

Figure 36 – TOF MS of Reduced and Alkylated Tes_B 08f12b (MW = 2155.20 Da)



This data revealed the presence of six cysteine residues (2155.20 – 1805.41 = 349.67. 349.67/58 = 6.03). The sample was then subject to sequencing revealing the following data:

Sequence: CCSRYCWRCTOCCPN

This peptide appears to be a mini-M as well.

The Tes_B 08f13 (combined with Tes_B 08f13 (b)) peak was the next to be further analyzed since it was the major peak of the run. The sample was dissolved in 1000 µL of 0.1% TFA and ran in the analytical column for 65 minutes at range 1 in wavelengths 280 nm and 250 nm, ant at range 1.5 at 220 nm. Figure 37 shows this chromatogram:

Figure 37-- Elution Profile of Tes_B 08f13 in the Analytical Column

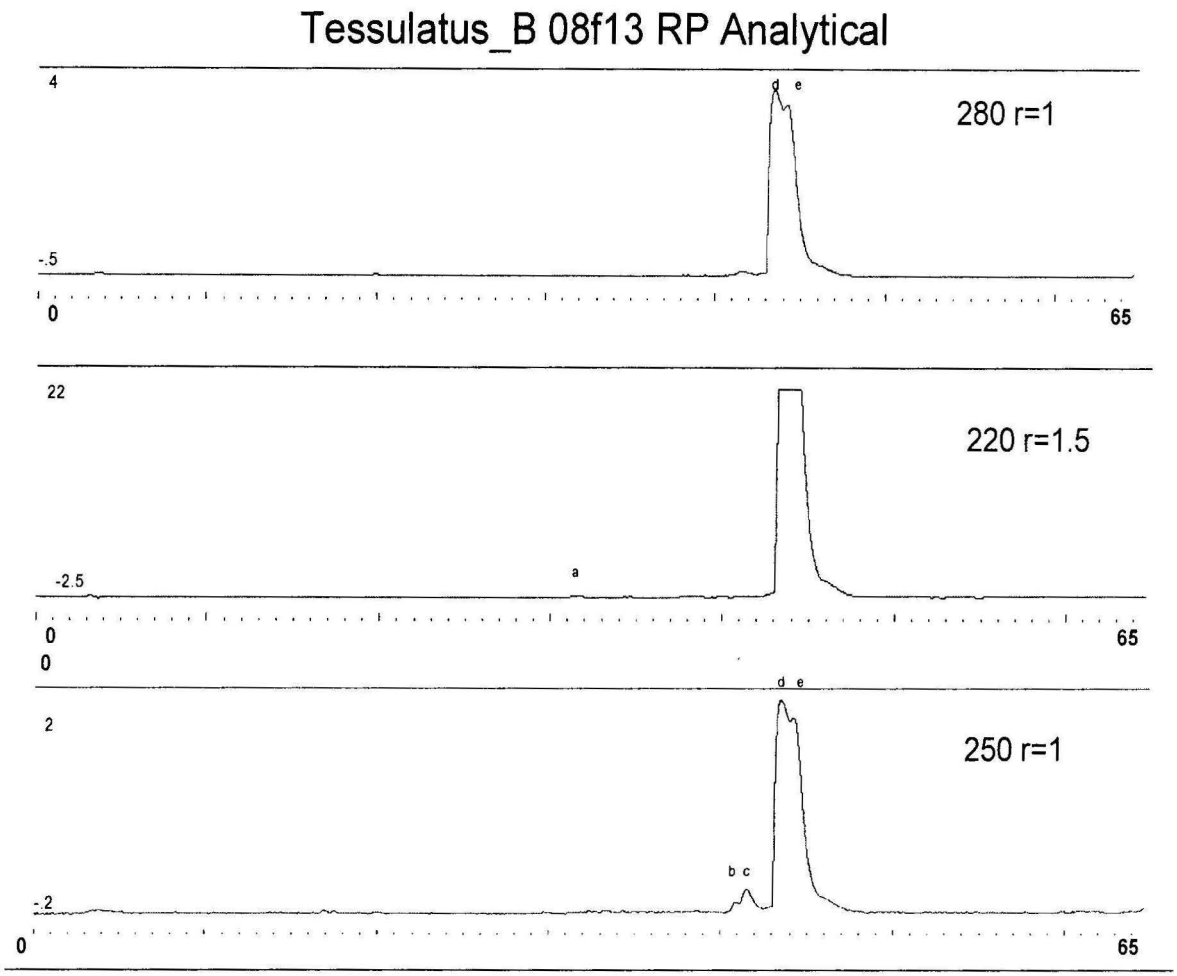


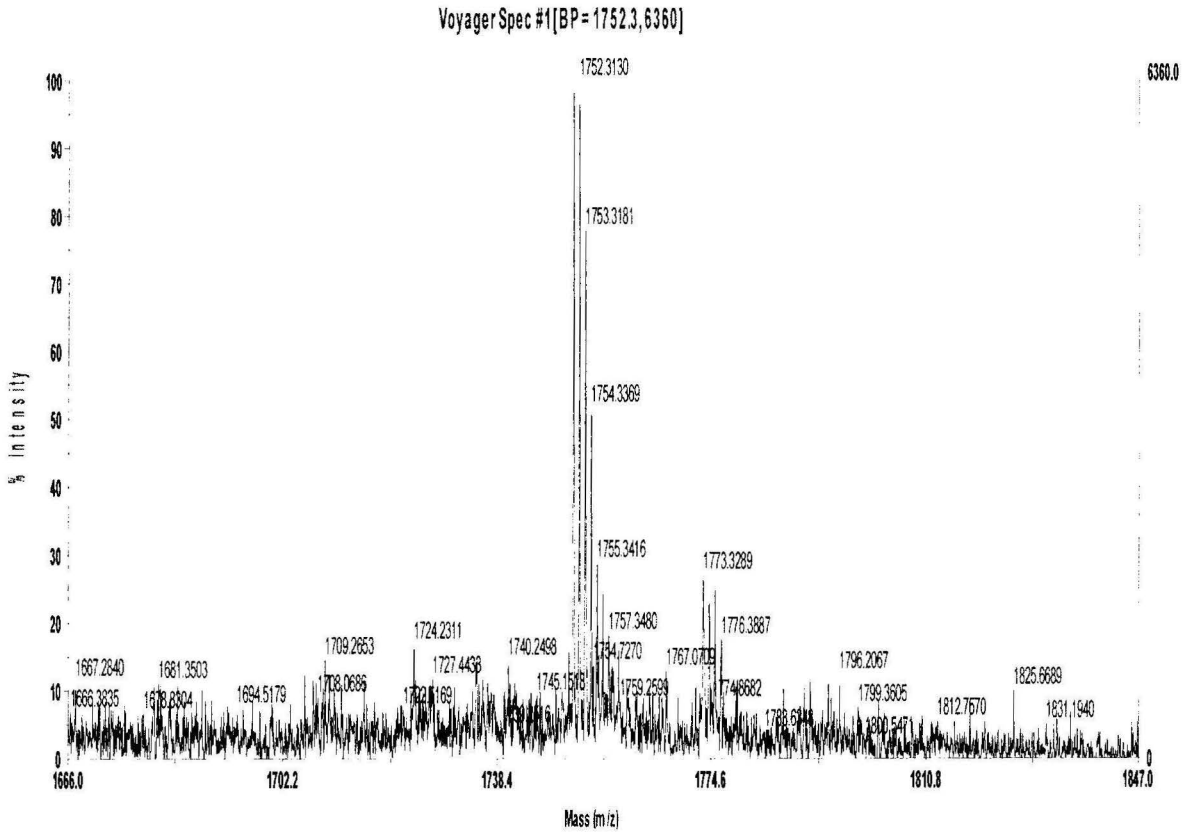
Table 11 – Elution Times for Tes_B 08f13 in the Analytical Column

Peak #	Elution Time (minutes)
a	31.20
b	41.00
c	41.37
d	43.52
e	44.20

From this separation peak d was chosen for further analysis since it was the major peak.

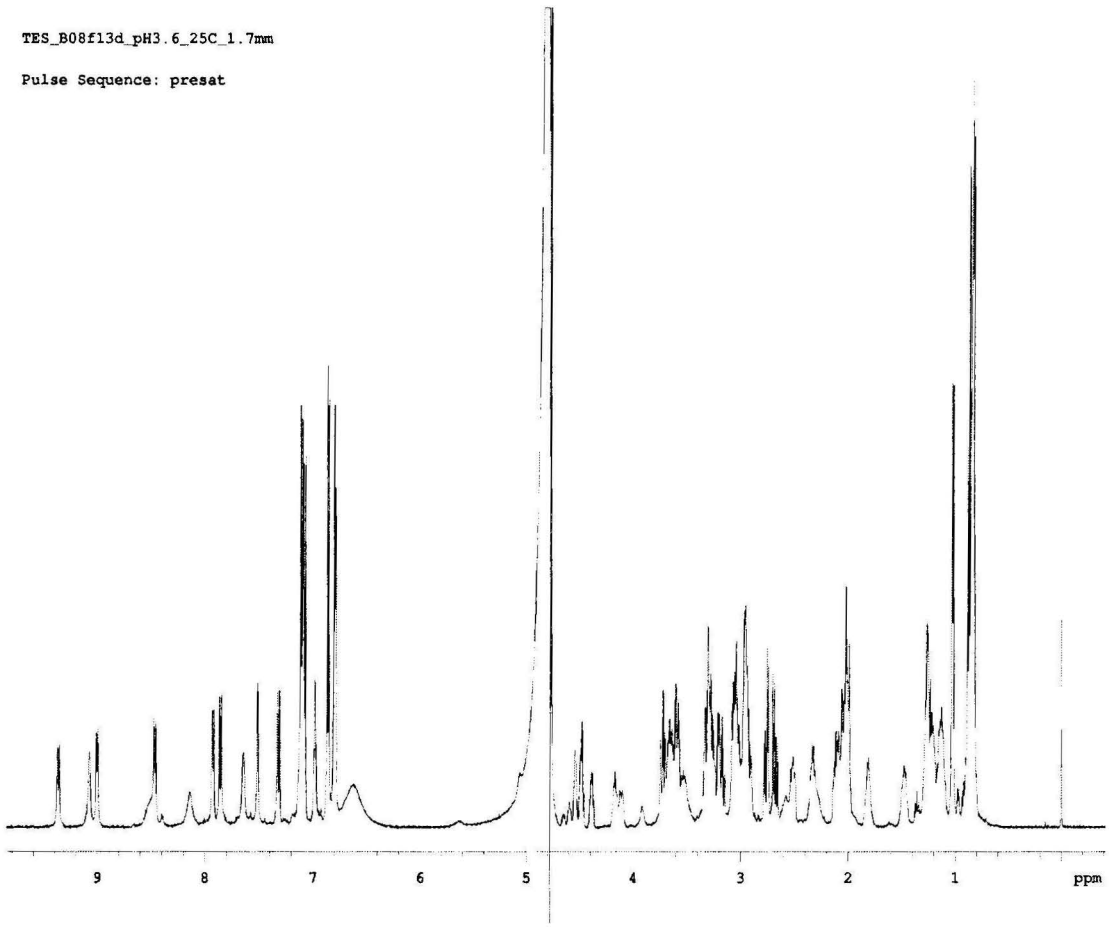
A mass spectrum of the sample was obtained (Figure 38):

Figure 38 – TOF MS of Tes_B 08f13d (MW = 1752.31 Da)



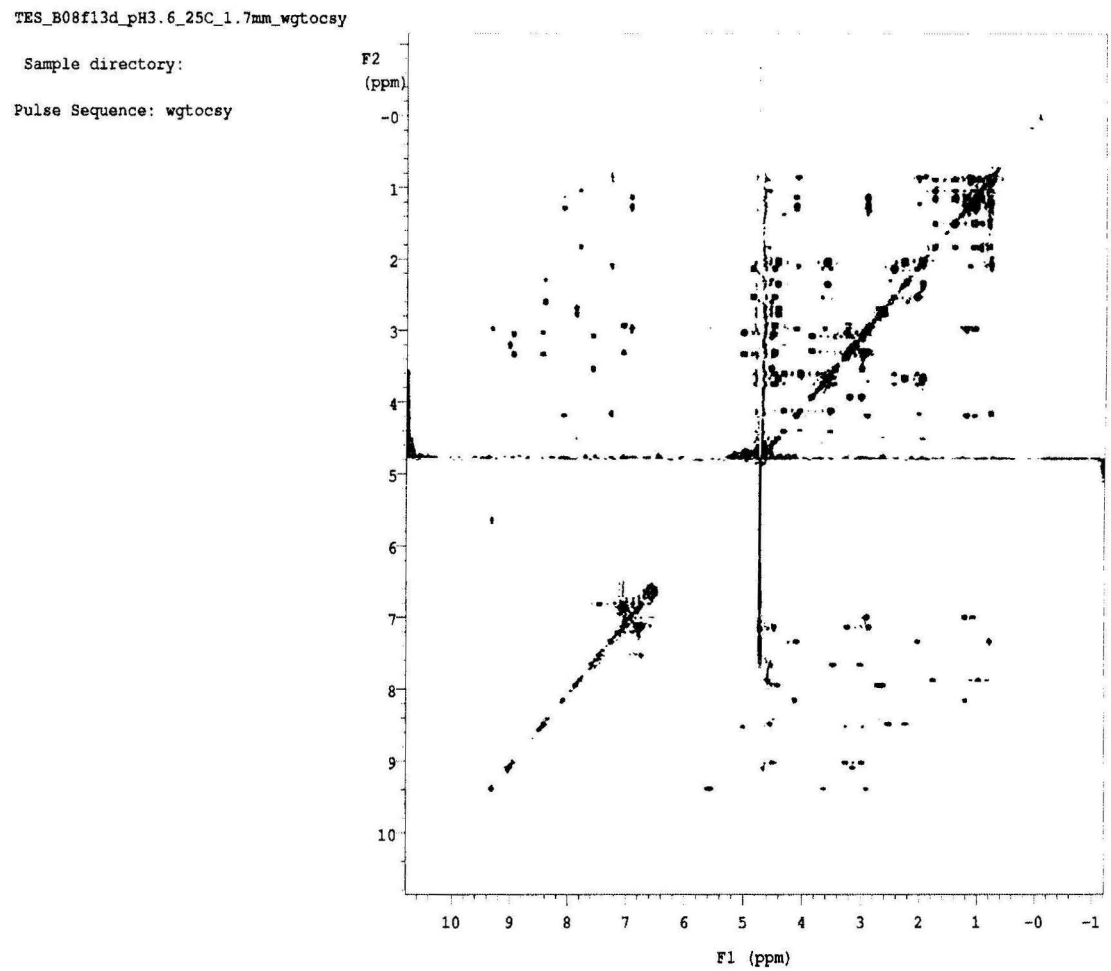
The sample was subsequently subject to 1-D NMR experiments that revealed great concentrations (Figure 39):

Figure 39 – 1-D NMR Spectrum of Tes_B 08f13d



Tyr resonates at 7.15 and at 6.86 ppm with the ring protons, as seen in this spectrum. Ile shows peaks at 1.48 and 1.19 ppm for the γH's, at 1.90 ppm for the βH, at 8.19 for the NH, and at 0.89 for the δH's. An Arg peak is seen around 6.6 characteristic of that NH. Ile peaks are shown below 1 ppm for the methyl groups. The sample also has slight impurities. This experiment revealed enough quantities, in comparison to the TSP peak, to run 2-D NMR experiments as well, so a TOCSY spectrum was taken of this compound (Figure 40).

Figure 40 – TOCSY Spectrum of Tes_B 08f13d



Unfortunately, upon close examination of this data, we determined that the sample was not pure since the chromatography peak was so broad. Therefore we decided to run it through the analytical column one more time. The sample was lyophilized and dissolved in 500 µL of 0.1% TFA and ran isocraticaly at 33% of the 60% ACN in the analytical column for 35 minutes at range 2 at 220 nm and range 1 at 280 nm and 250 nm. Figure 41 shows the elution profile for this run.

Figure 41 – Elution Profile for Tes_B 08f13d in the Analytical Column

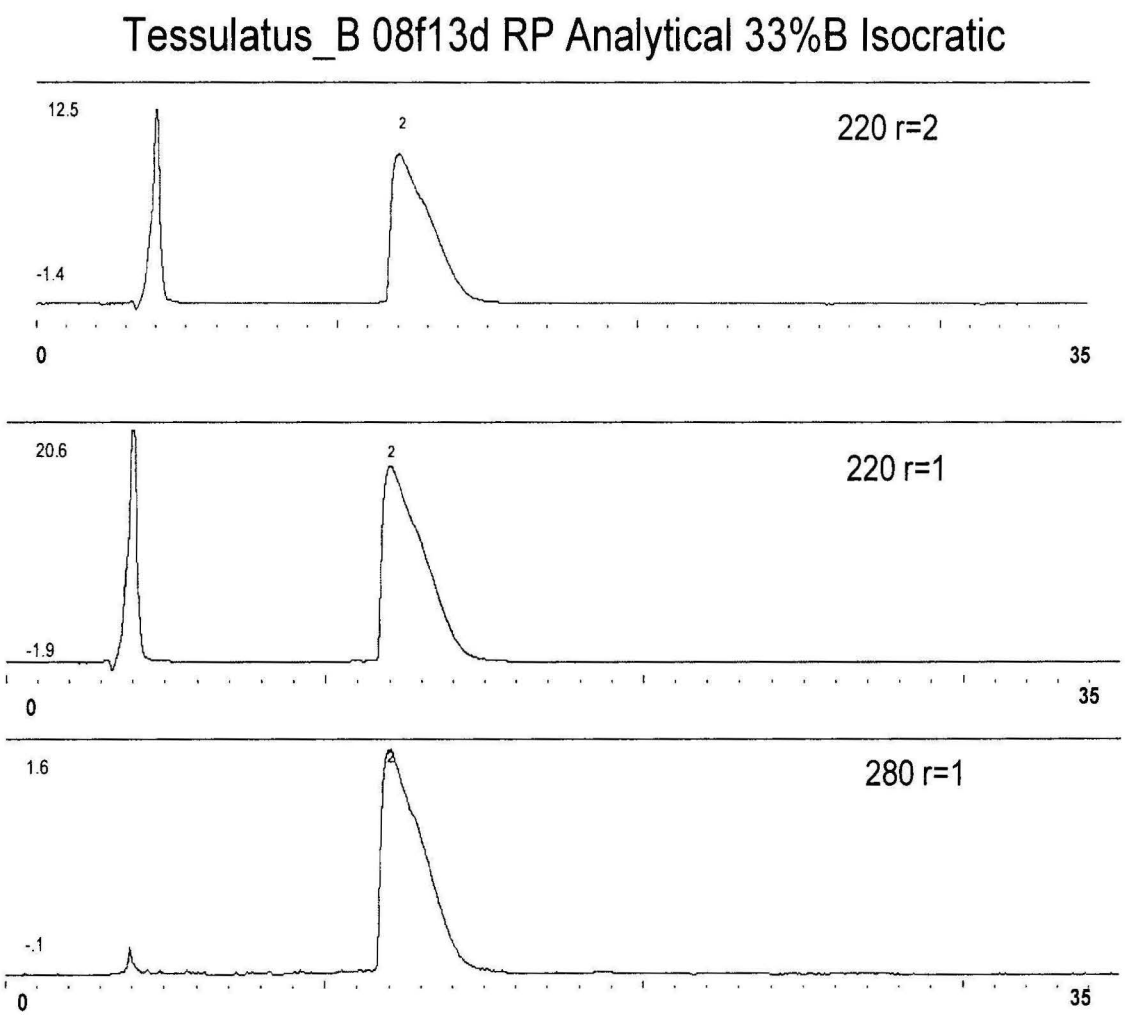


Table 12 – Elution Times of Tes_B 08f13d in the Analytical Column

Peak #	Elution Time (minutes)
1	4.22
2	12.19

Several fractions were taken from peak two of this run in an attempt to separate components that elute very closely together. Seven 30 second fractions were taken altogether. The broadness of the peak indicates that more than one peptide is present in

this peak. Mass spectra and sequences were taken for both fractions 1 and 7, as they were the furthest apart (Figure 42 and 43).

Figure 42 – TOF MS of Tes_B 08f13d2(1) (MW = 1751.47 Da)

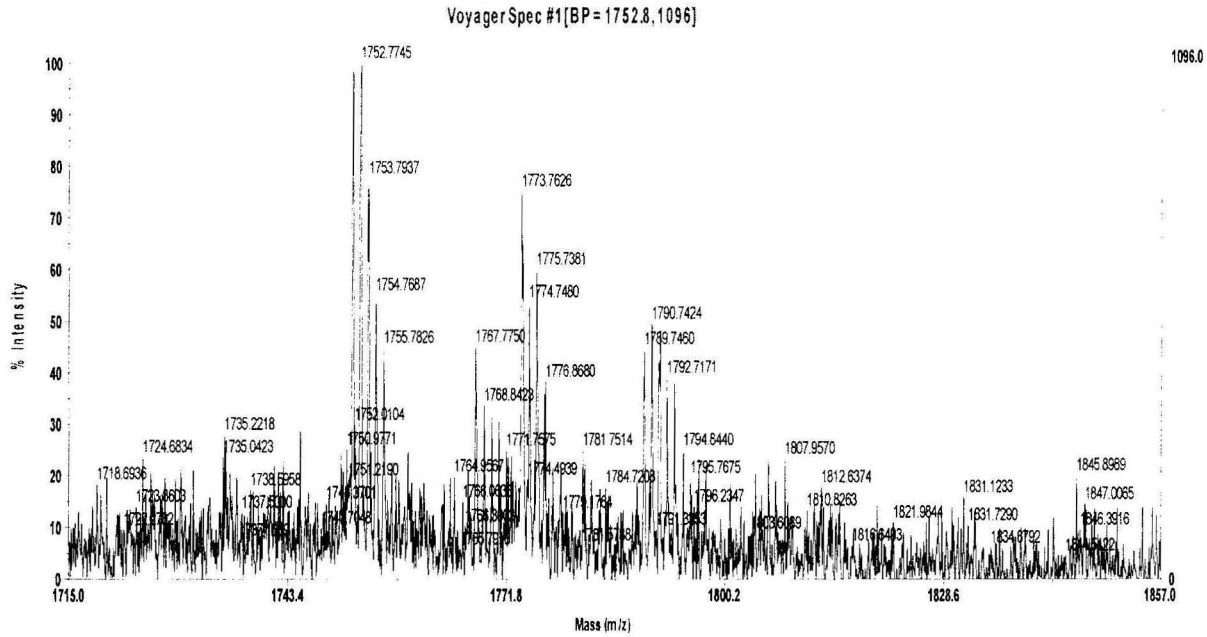
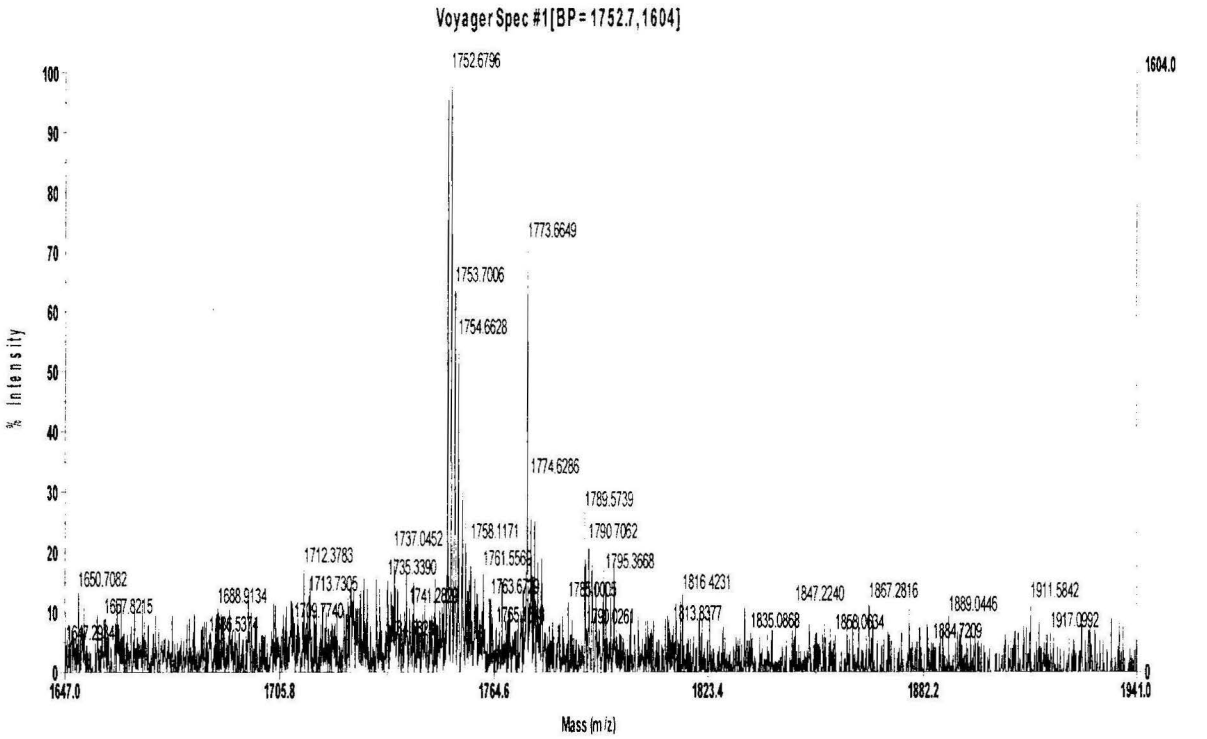


Figure 43 – TOF MS of Tes_B 08f13d2(7) (MW = 1751.69 Da)



Both samples were reduced and alkylated yielding the following mass spectra (Figure 44 and 45):

Figure 44 - TOF MS of Reduced and Alkylated Tes_B 08f13d2(1) (MW = 2101.98 Da)

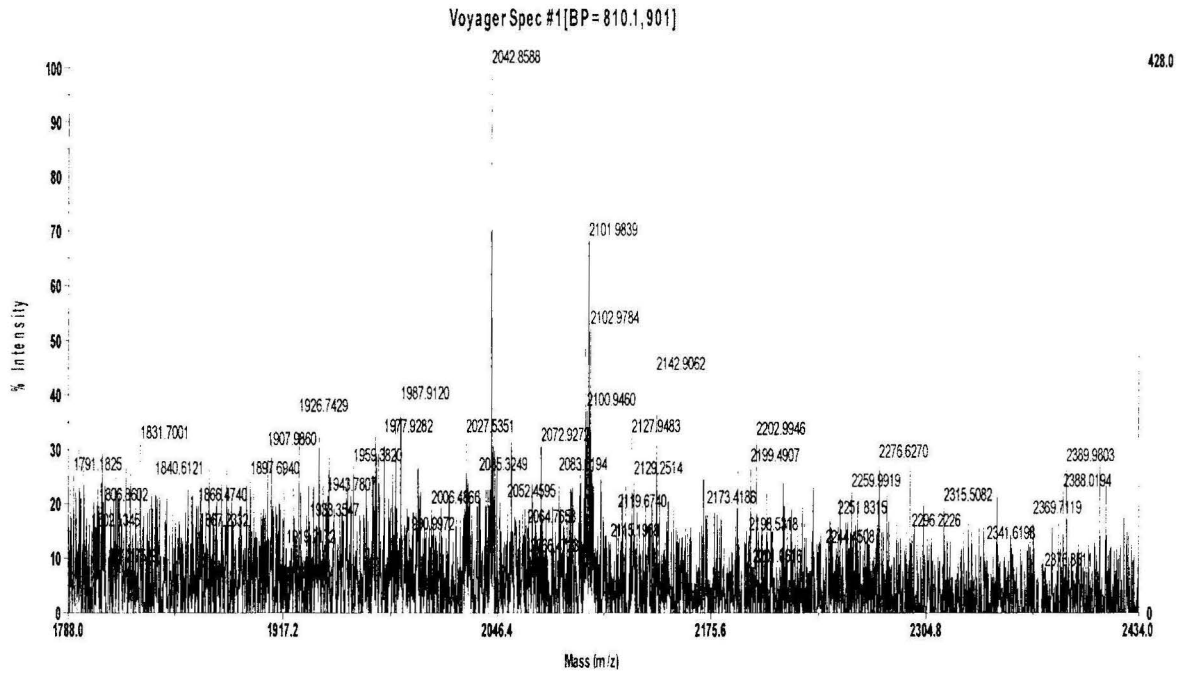
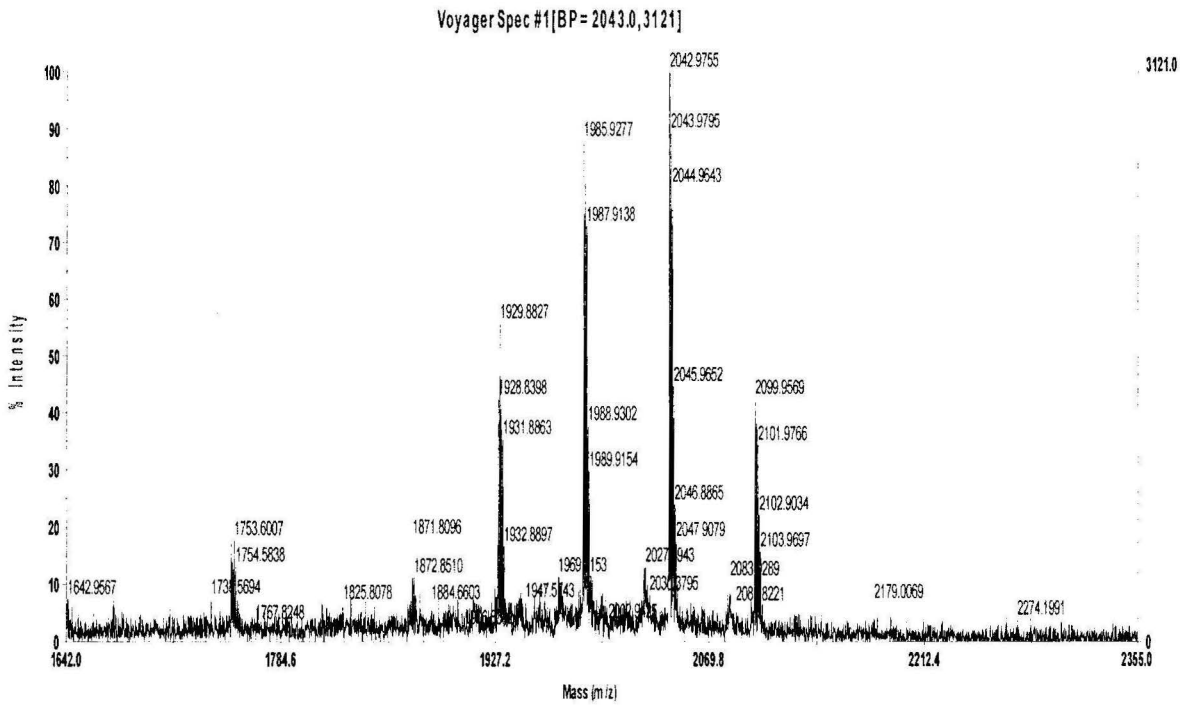


Figure 45 – TOF MS of Reduced and Alkylated Tes_B 08f13d2(7) (MW = 2101.98 Da)



Each peptide had a mass increase of about 349 Da indicating the presence of six cysteine residues (2101.98 – 1752.97 = 349.62. 349.62/58 = 6.02).

They were individually sequenced yielding the following results:

Sequence: CCSRYCYICIOCCPN

Sequence: CCSRYCYICIOCCPN

Since both sequences were identical, and both of their calculated masses closely matched the expected masses, one conclusion that can be drawn from the fact that they did not co-elute is that an amino acid in one of the peptides may be an epimerized D-amino acid. This would explain the identical sequences but different elution times. However, further work is required before this conclusion could be drawn. Synthesis of the peptide may aid in elucidating the reason behind this discrepancy.

This peptide also appears to be a mini-M.

Another peak that was analyzed was the Tes_B 08f14 peak from the same semi-preparative run as the previous peptide. The mass spectrum of this peak and the chromatography profile of this run in the analytical column are shown in figure 46 and 47.

Figure 46 – TOF MS of Tes_B 08f14 (MW = 1735.65 Da)

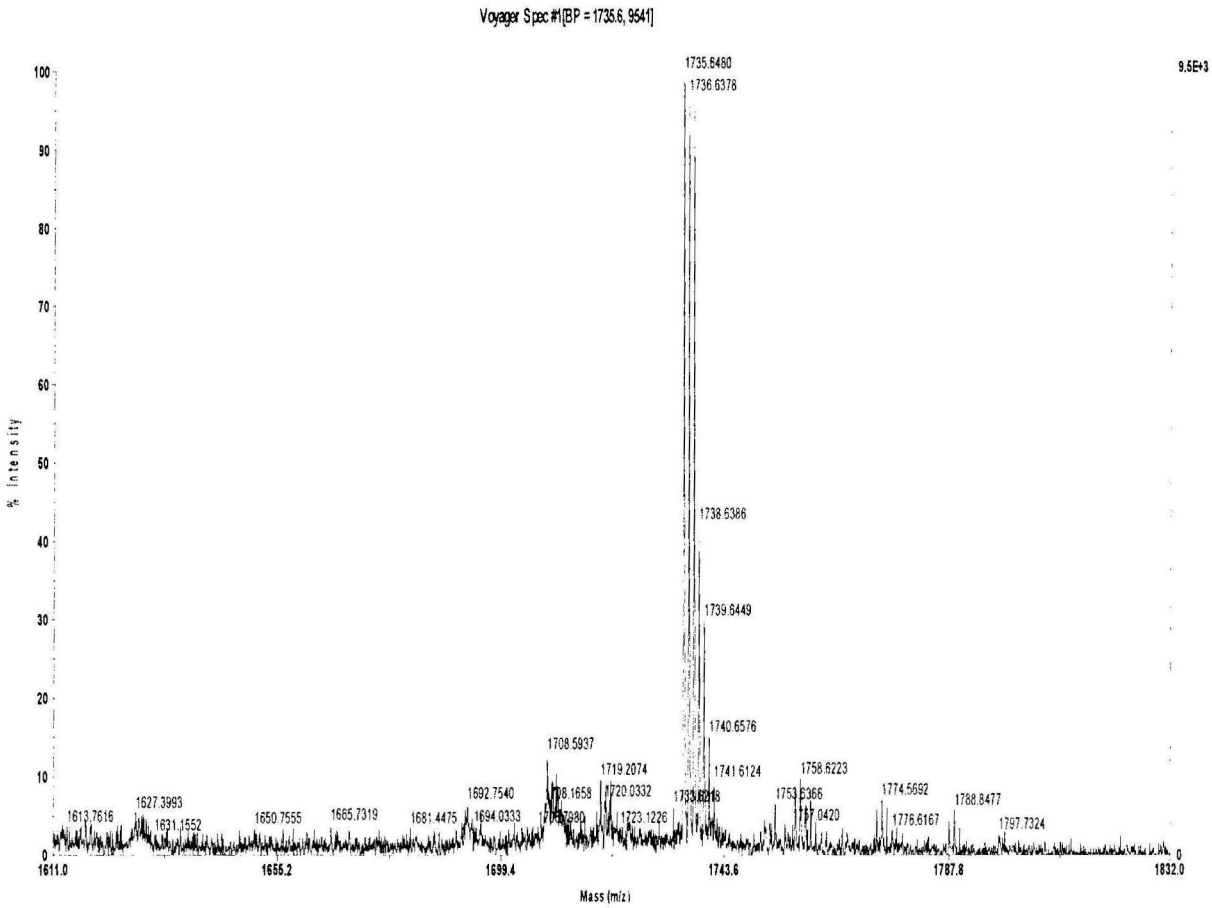


Figure 47 – Elution Profile of Tes_B 08f14 in the Analytical Column

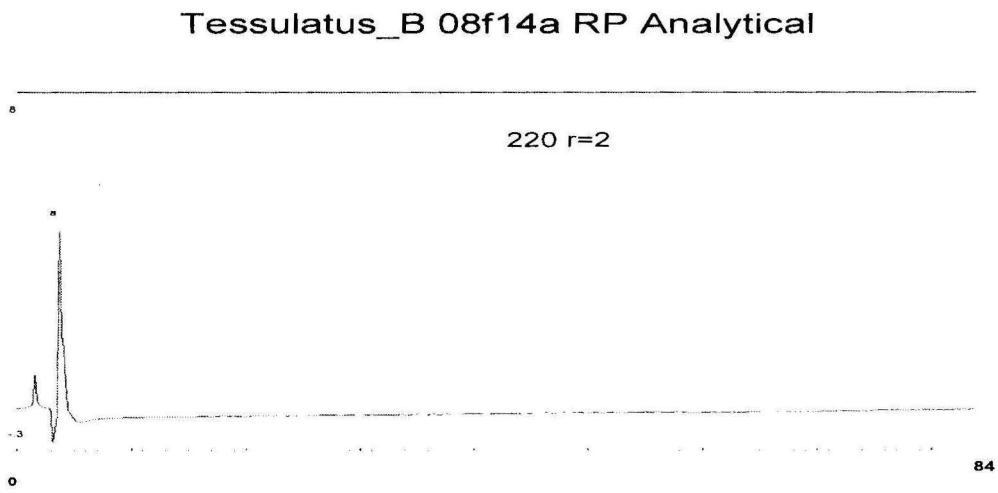
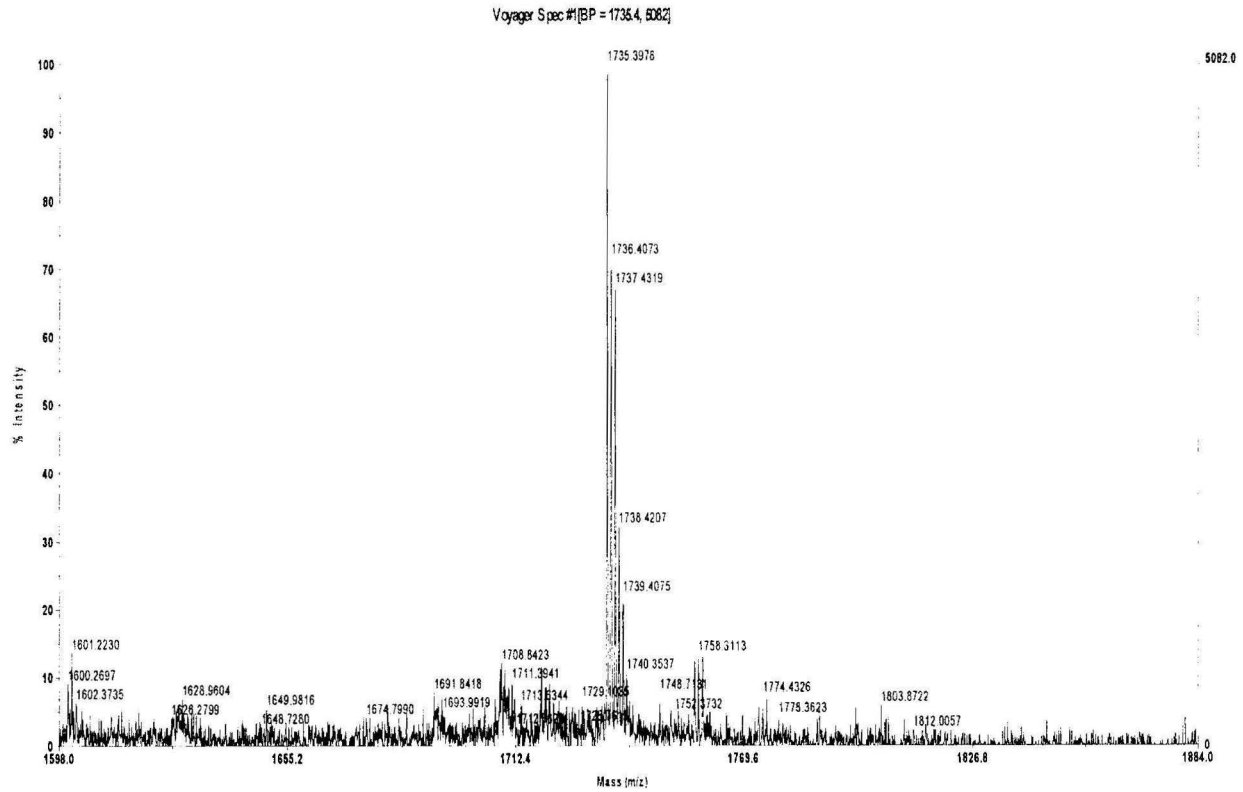


Table 13 – Elution Times of Tes_B 08f14 in the Analytical Column

Peak #	Elution Time (minutes)
a	4.20

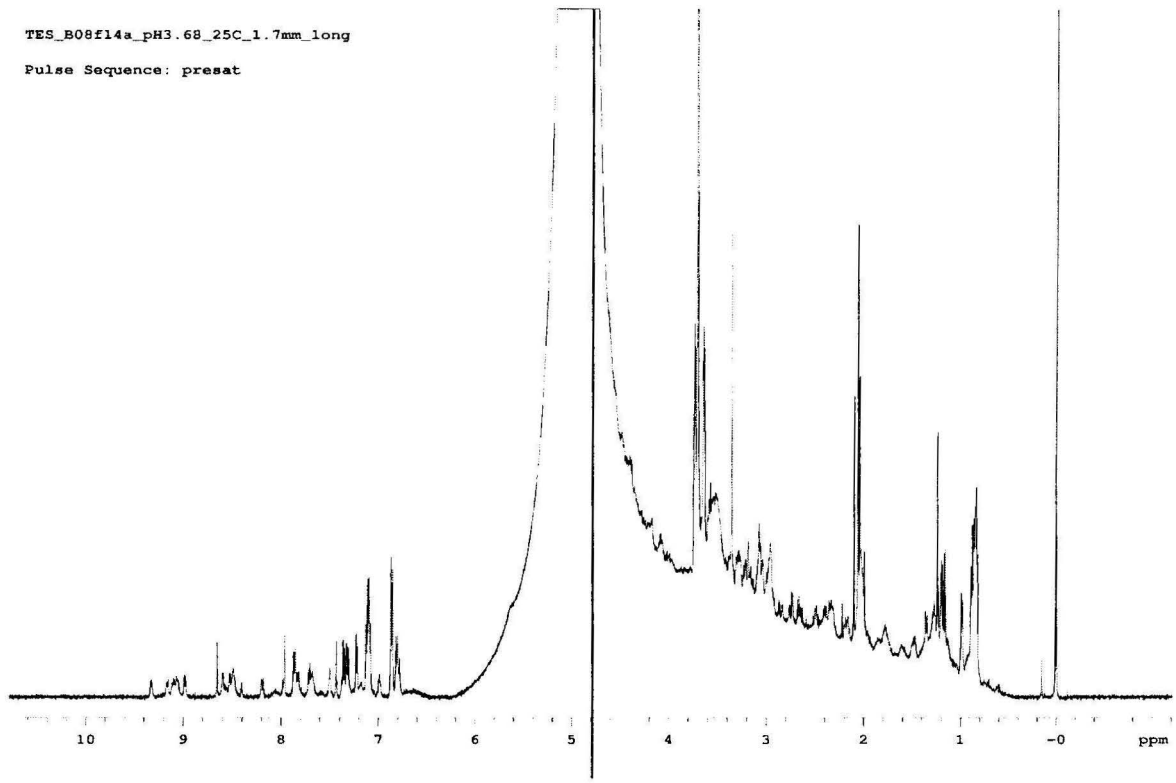
The mass spectrum of this peak is shown in figure.

Figure 48 – Mass Spectrum of Tes_B 08f14a (MW = 1735.40 Da)



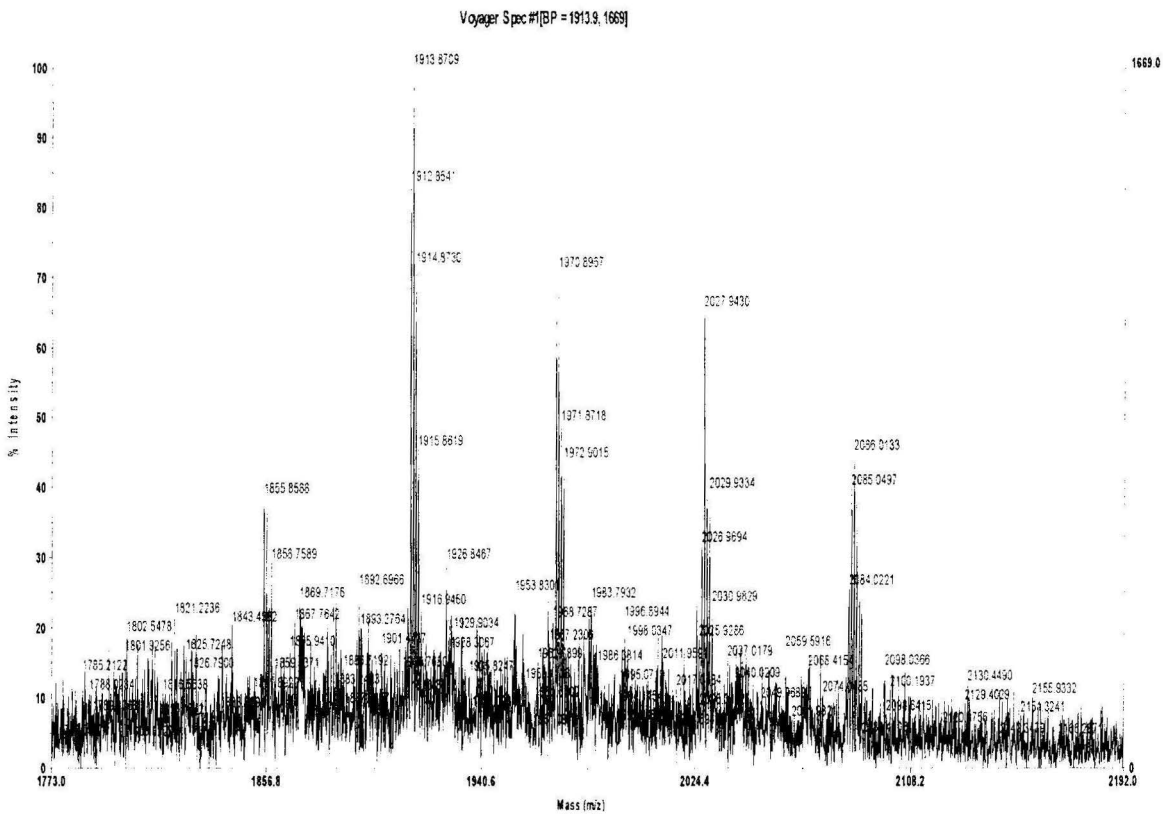
The NMR spectrum is shown in figure 49.

Figure 49– 1-D NMR Spectrum of Tes_B 08f14a



The sample was then reduced and alkylated yielding the following mass spectrum (Figure 50):

Figure 50 – TOF MS of the Reduced and Alkylated Tes_B 08f14a (MW = 2086.01 Da)



The sequence for this peptide was not properly obtained. Further work is still needed in order to confirm the sequence. However, as evident from the mass spectrum, there are probably six cysteine residues present in the peptide (2086.01-1735.40 = 350.61. 350.61/58 = 6.04)

The next peak to be analyzed was Tes_B 09 from Superdex 30. This peak was dissolved in 500 μL of 0.1 M NH_4HCO_3 and ran through the Superdex Peptide column for 70 minutes at range 2 in 220 nm and 280 nm, and range 4 at 220 nm. The following chromatogram was obtained:

Figure 51– Elution Profile of Tes_B 09 in the Superdex Peptide Column

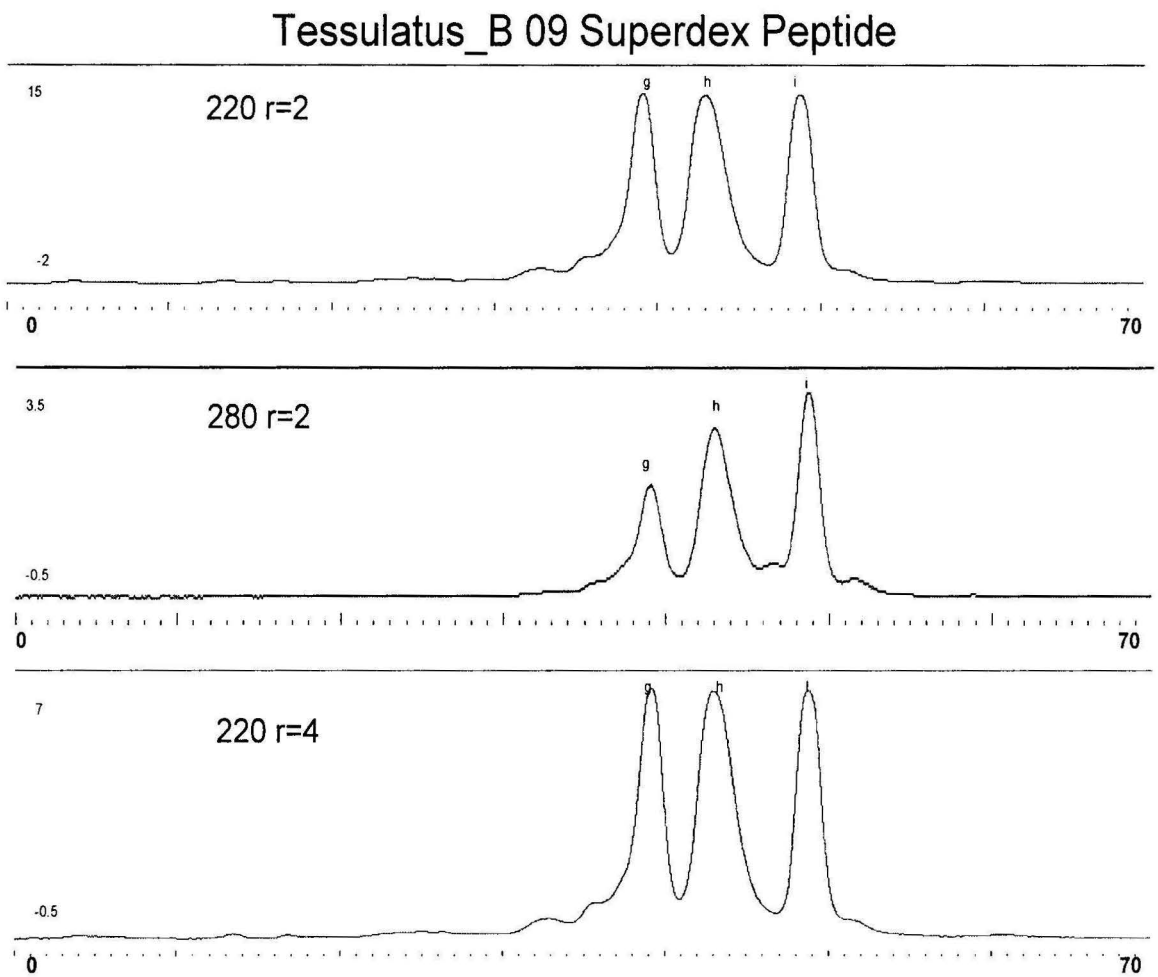


Table 14 – Elution Times for Tes_B 09 Superdex Peptide Run

Peak #	Elution Time (minutes)
a	4.20
b	13.44
c	16.77
d	23.60
e	24.97
f	32.85
g	39.00
h	42.90
i	48.75

Peak Tes_B 09g was then chosen for further separation in the semi-preparative reverse phase column. This run lasted for 100 minutes at range 1 in 220 nm and 280 nm.

Figure 52 – Elution Profile of Tes_B 09g in the Semi-preparative Column

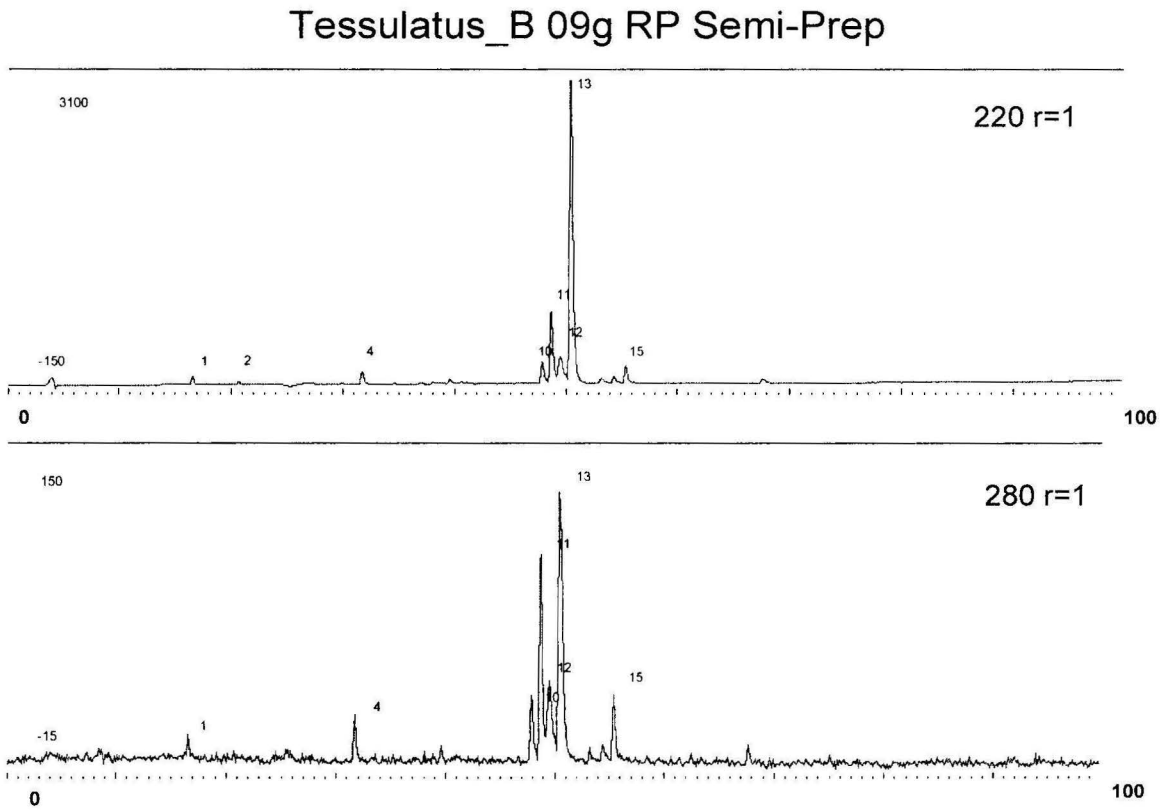
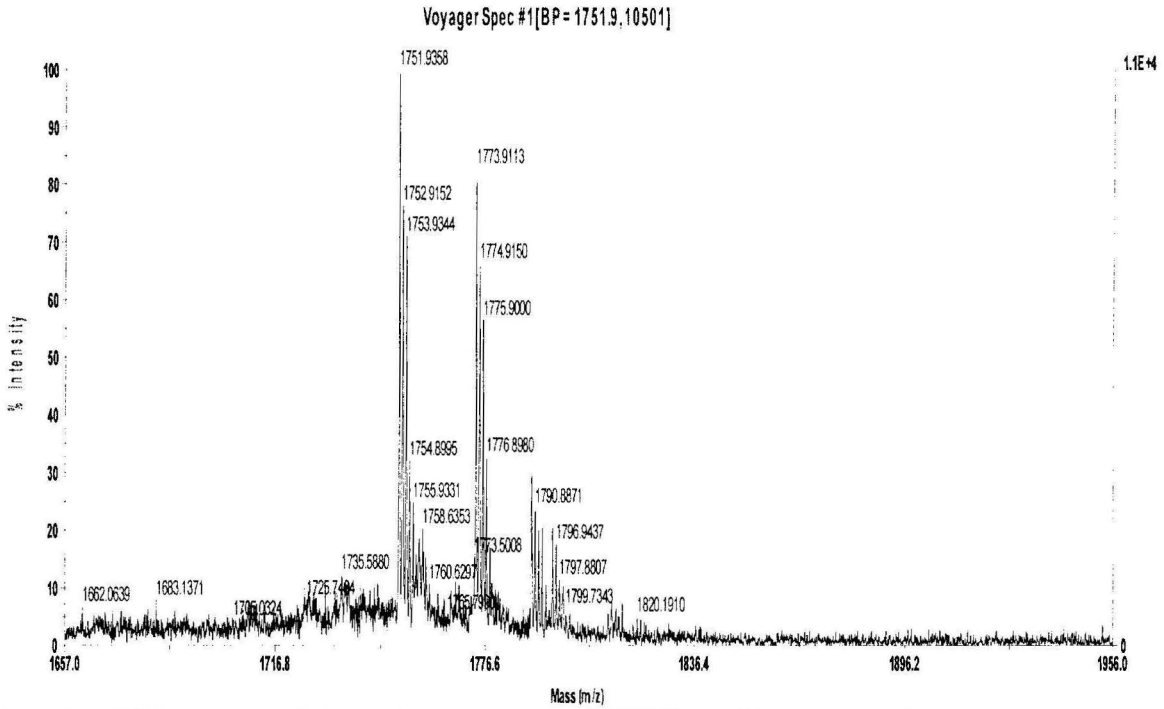


Table 15 – Elution Times for Tes_B 09g in the Semi-preparative Column

Peak #	Elution Time (minutes)
1	16.60
2	20.76
3	29.75
4	31.60
5	34.50
6	36.85
7	38.08
8	39.50
9	41.06
10	47.76
11	48.63
12	49.43
13	50.42
14	54.33
15	55.32
16	67.65

Peak 13 was then chosen for further analysis as it was the major peak. A mass spectrum was taken for this compound (Figure 53):

Figure 53 – TOF MS of Tes_B 09g13 (MW = 1751.93 Da)



This peak was then furthered purified in the analytical column. This run lasted for 56 minutes at range 1 in 220 nm and 280 nm (Figure 54):

Figure 54 – Elution Profile of Tes_B 09g13 in the Analytical Column

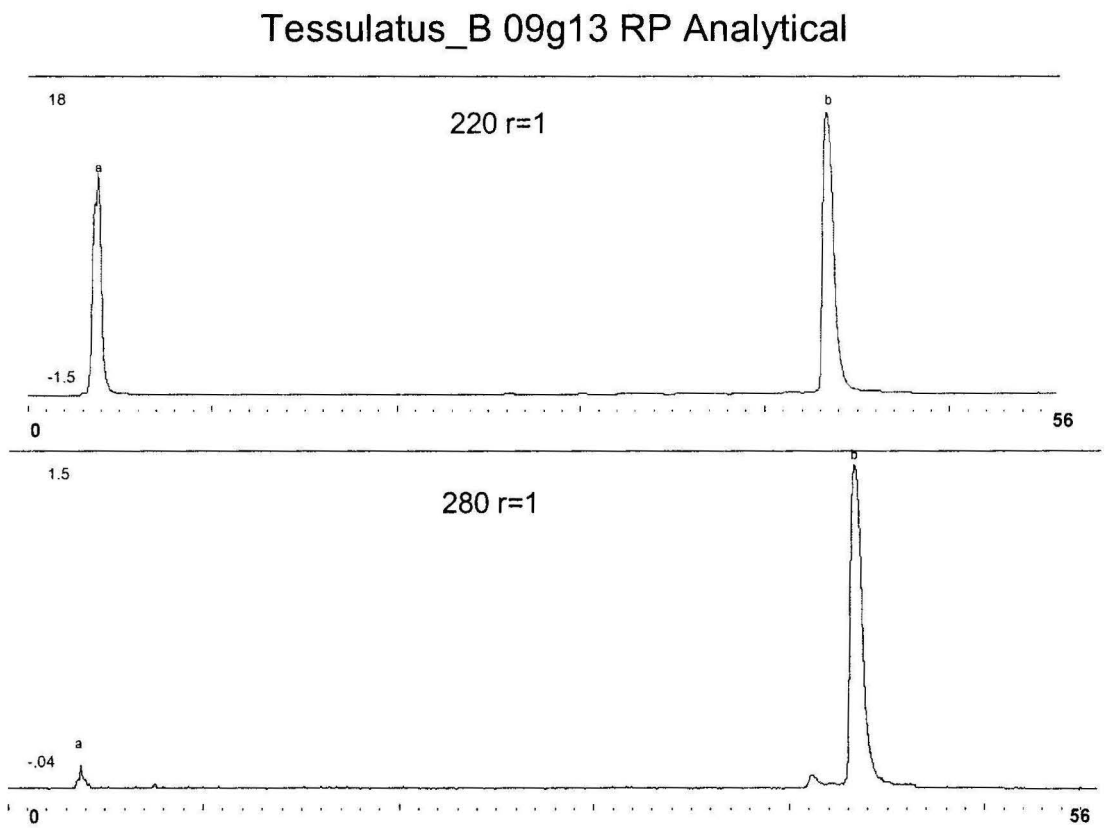
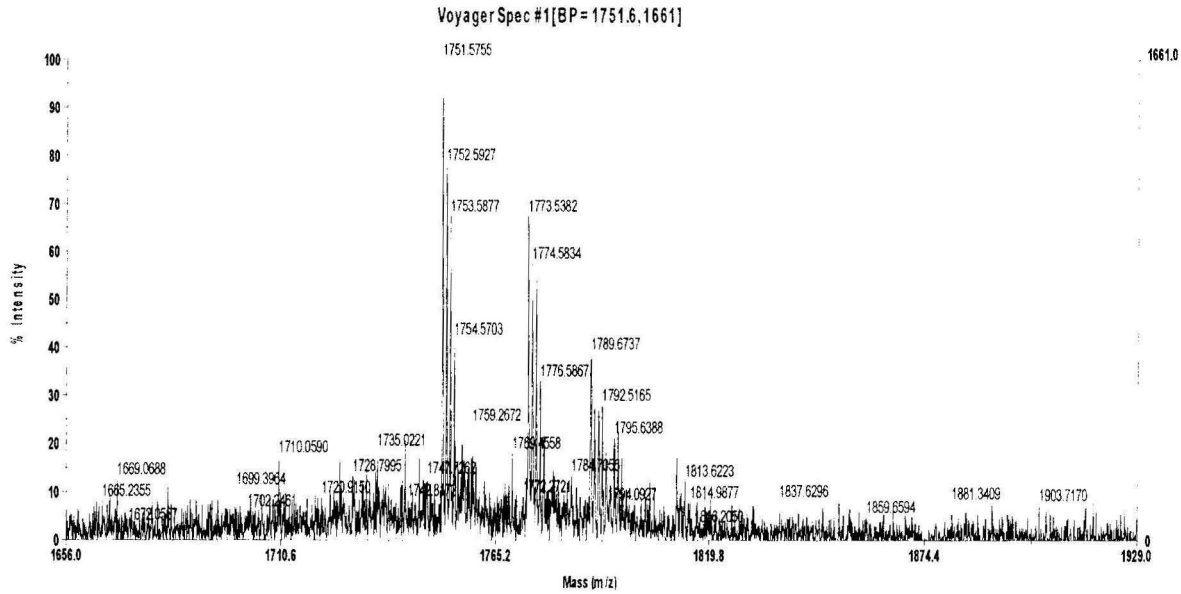


Table 16 – Elution Times for Tes_B 09g13 in the Analytical Column

Peak #	Elution Time (minutes)
a	4.10
b	43.27

A mass spectrum was obtained from peak b from this run (Figure 55):

Figure 55 – TOF MS of Tes_B 09g13b (MW = 1751.58 Da)



The sample was then subject to a 1-D NMR experiment that yielded the following spectra (Figure 56-57):

Figure 56 – 25°C 1-D NMR Spectrum of Tes_B 09g13b

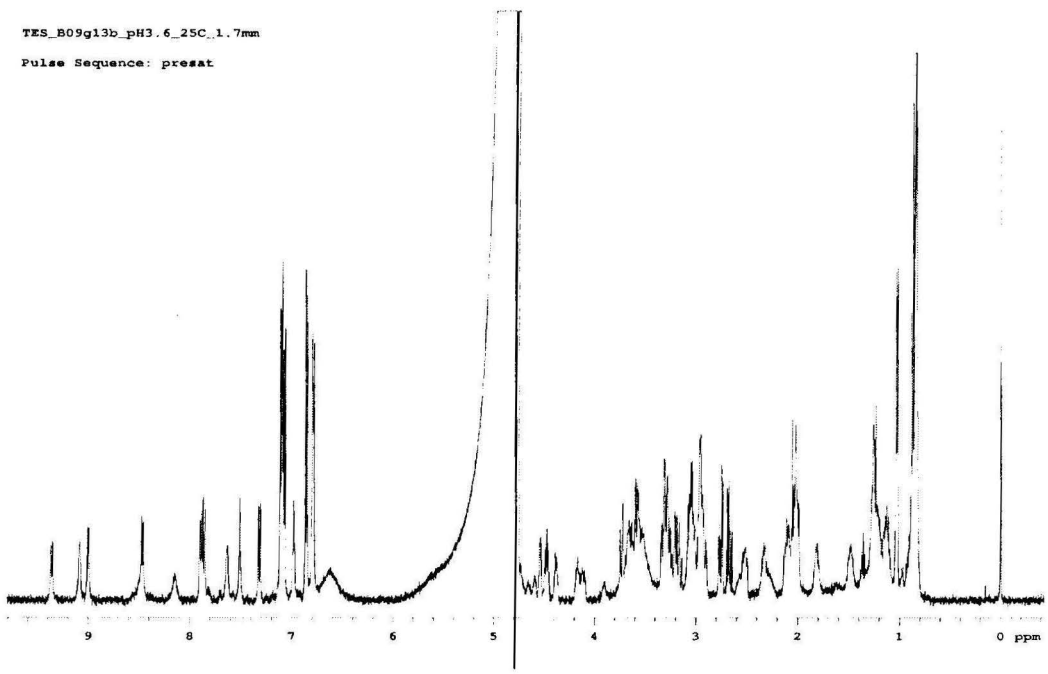
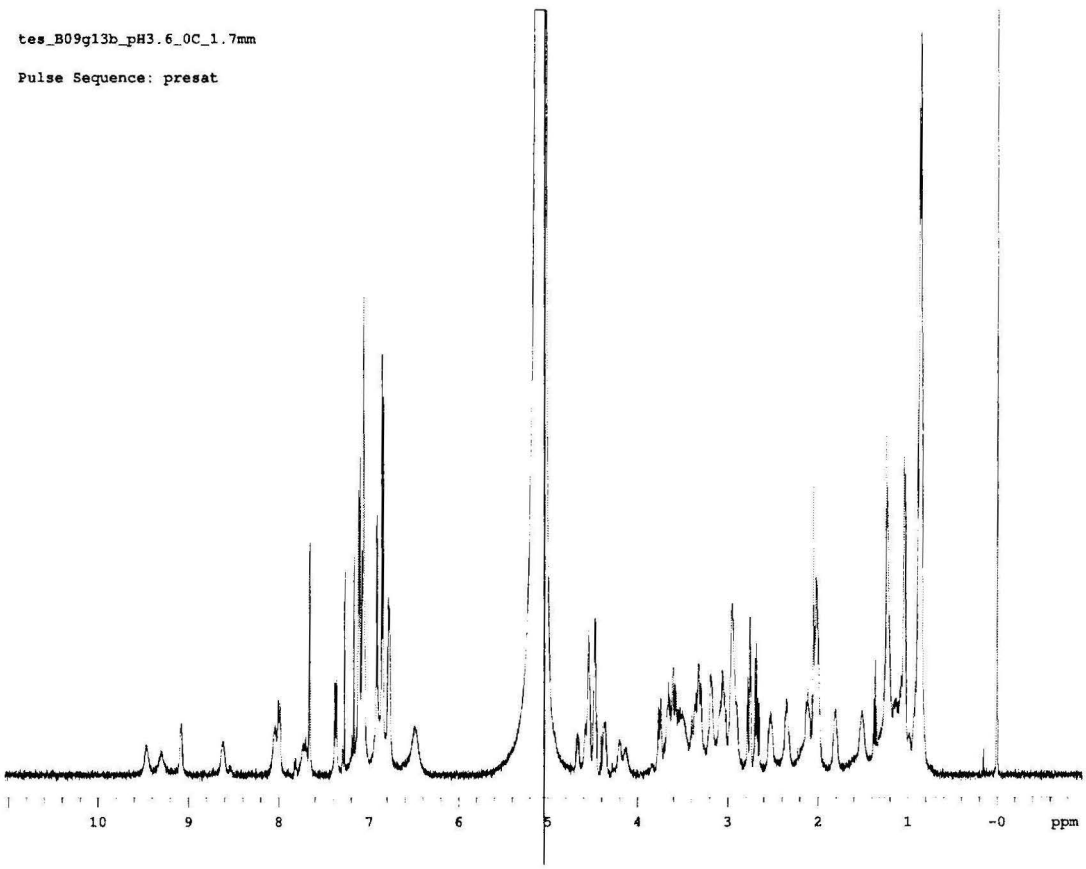
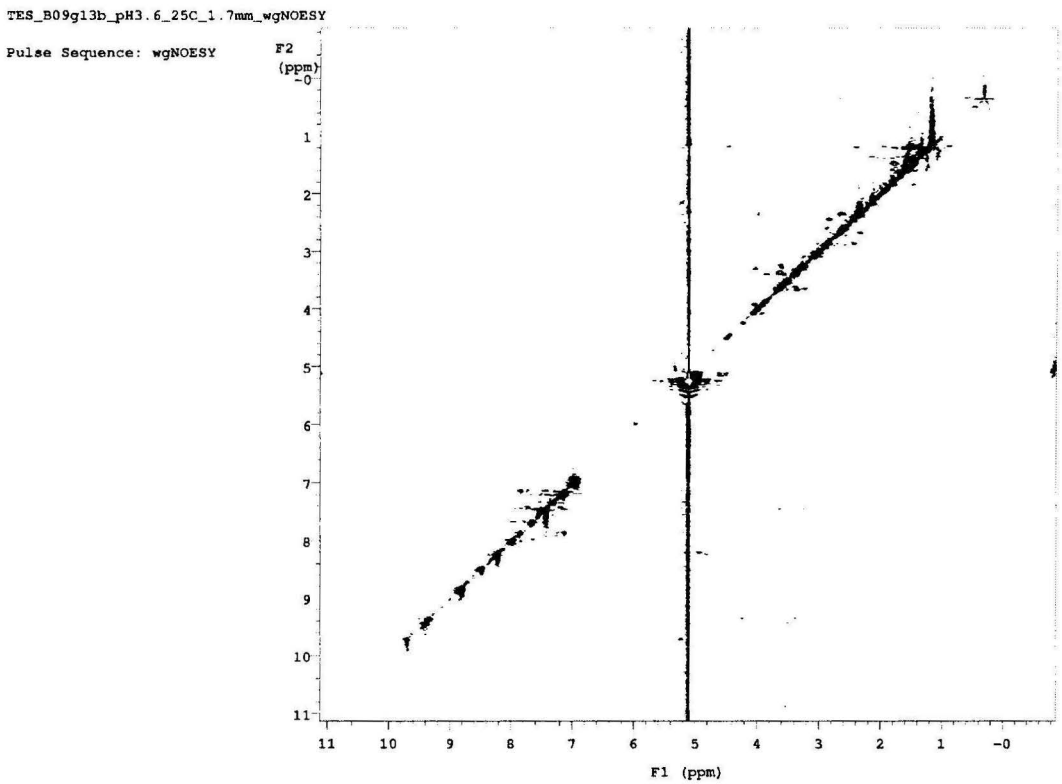


Figure 57 – 0°C 1-D NMR Spectrum of Tes_B 09g13b



This NMR spectrum closely resembles the spectrum of Tes_B 08f13d. There are no notable differences other than peak intensities. The two peptides have the same sequence. The only difference that may result in the difference in elution times is an L to D-epimerization that cannot be detected by NMR. The 1-D in 0°C is less structured that at 25°C but shows basically the same peaks, give or take a few. This experiment revealed enough quantities for 2-D NMR experiments as well, so both a NOESY and a TOCSY spectrum were taken.

Figure 58 – NOESY Spectrum of Tes_B 09g13b



The NOESY spectrum would have been a key factor in making sequence specific assignments in conjunction with the TOCSY data; however, the spectrum is very weak due to lack of sufficient concentration, and is therefore of no use. And the TOCSY spectra do not provide enough information for making sequence specific assignments.

Figure 59 – 25°C TOCSY Spectrum of Tes_B 09g13b

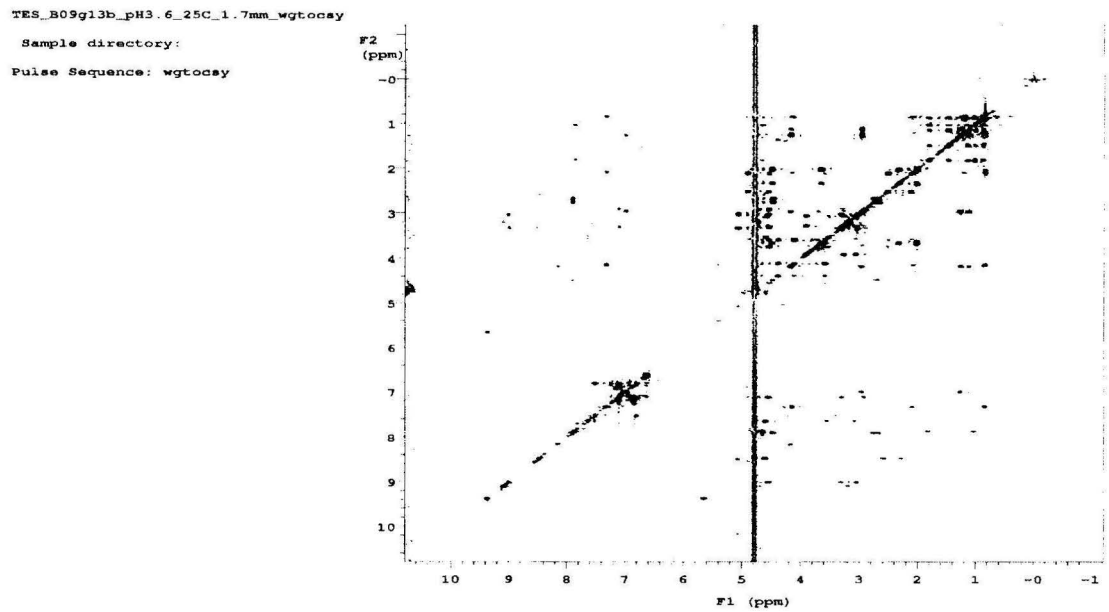
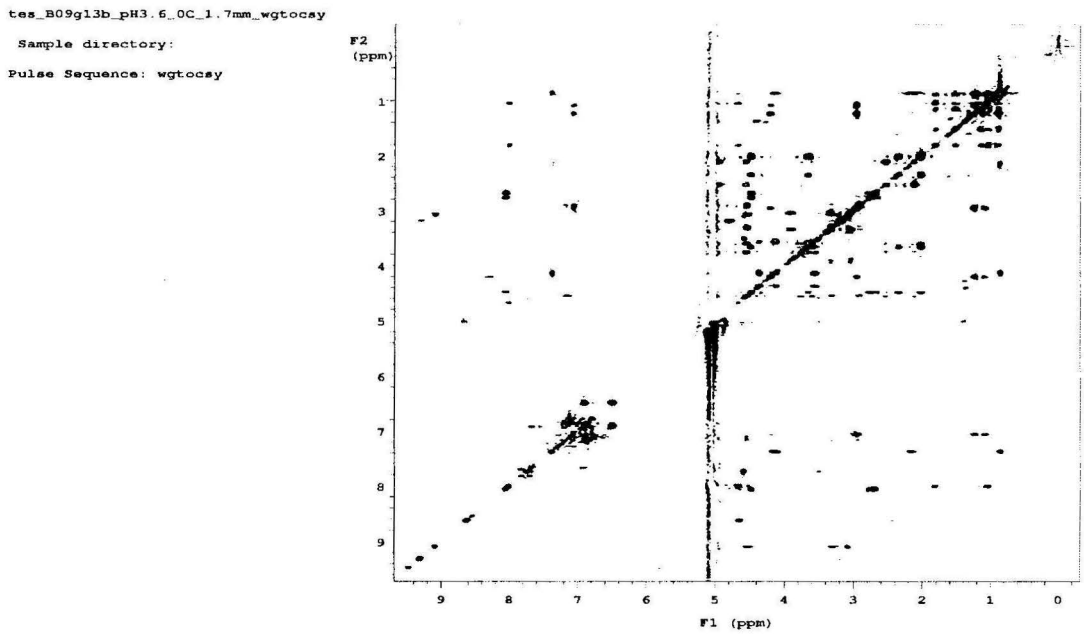
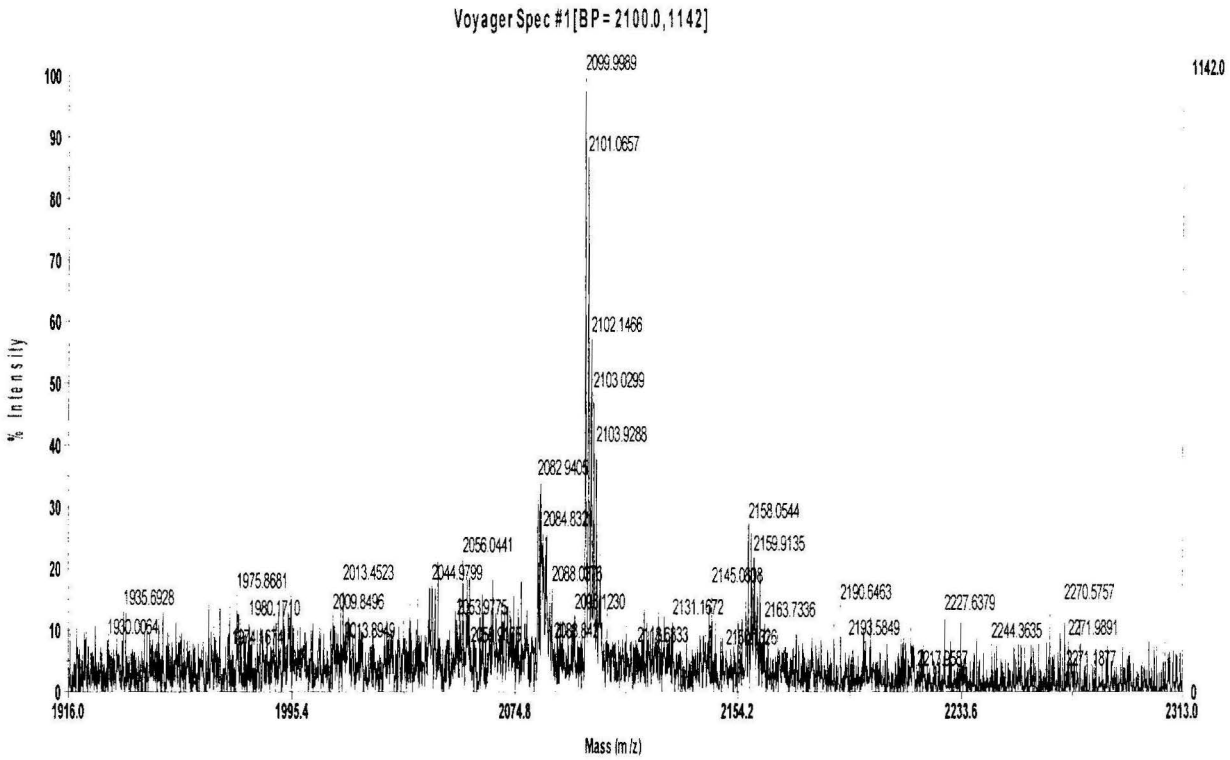


Figure 60 – 0°C TOCSY Spectrum of Tes_B 09g13b



Once the NMR experiments had been conducted, about one third of the sample was reduced and alkylated yielding the following mass spectrum (Figure 61):

Figure 61 - TOF MS of the Reduced and Alkylated Tes_B 09g13b (MW = 2101.07 Da)



This spectrum indicated the presence of six cysteine residues (2101.07 – 1751.58 = 349.49. 349.49/58 = 6.03). The sample was then sequenced yielding the following data:

Sequence: CCSRYCYICIOCCPN

This sequence is the same as that of Tes_B 08fd(1) and Tes_B 08fd(7). This occurrence further supports the theory that this peptide may have a D-amino acid and therefore is not co-eluting. Again, this peptide is a mini-M.

Peak 11 was then analyzed from the Tes_B 09g. Figure 62 show the elution profile of this peak in the analytical column at wavelengths 220 and 280 nm at range 1, and at 220 nm and range 0.5:

Figure 62 – Elution Profile of Tes_B 09g11 in the Analytical Column

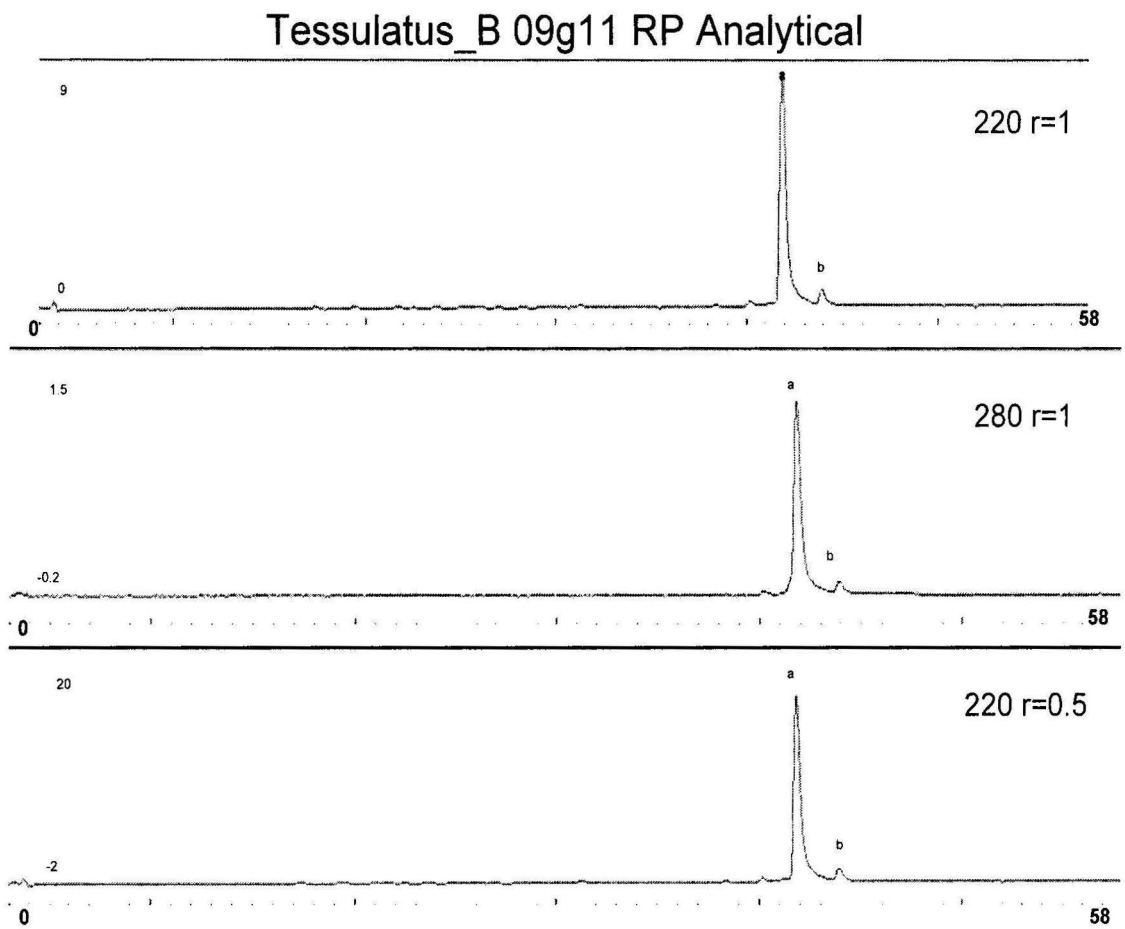


Table 17 – Elution Times of Tes_B 09g11 in the Analytical Column

Peak #	Elution Time (minutes)
a	42.03
b	44.02

The mass spectrum of this sample indicated that the peak was still somewhat contaminated, but an NMR spectrum was acquired nonetheless (Figure 63 and 64).

Figure 63 – TOF MS of Tes_B 09g11a (MW = 1774.69 Da)

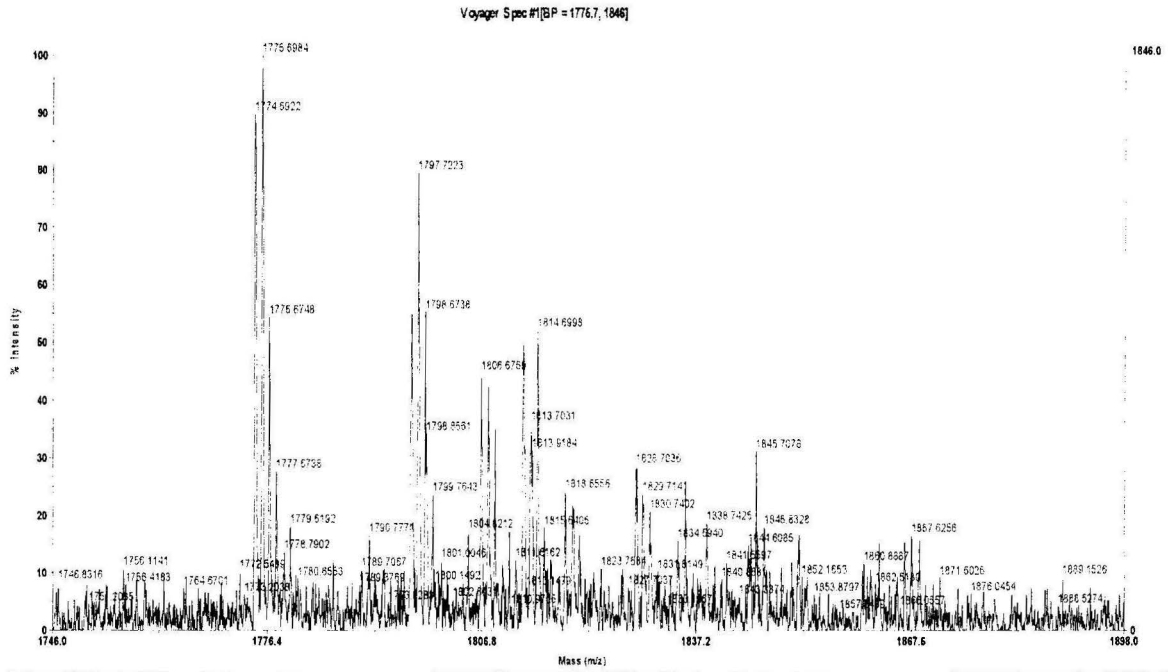
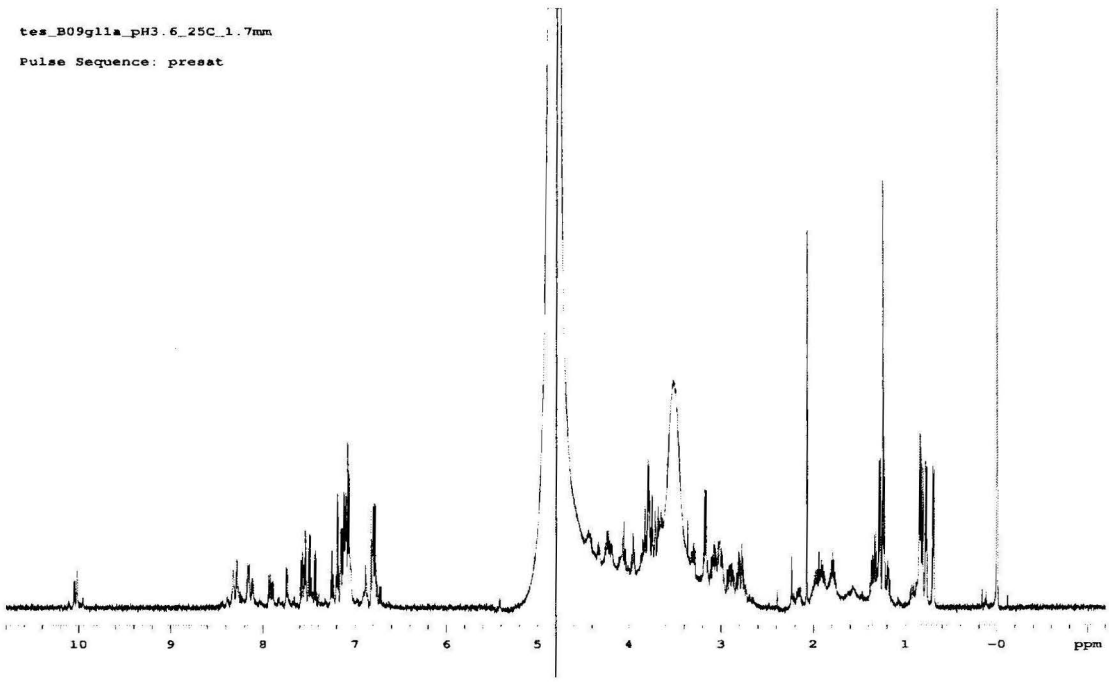
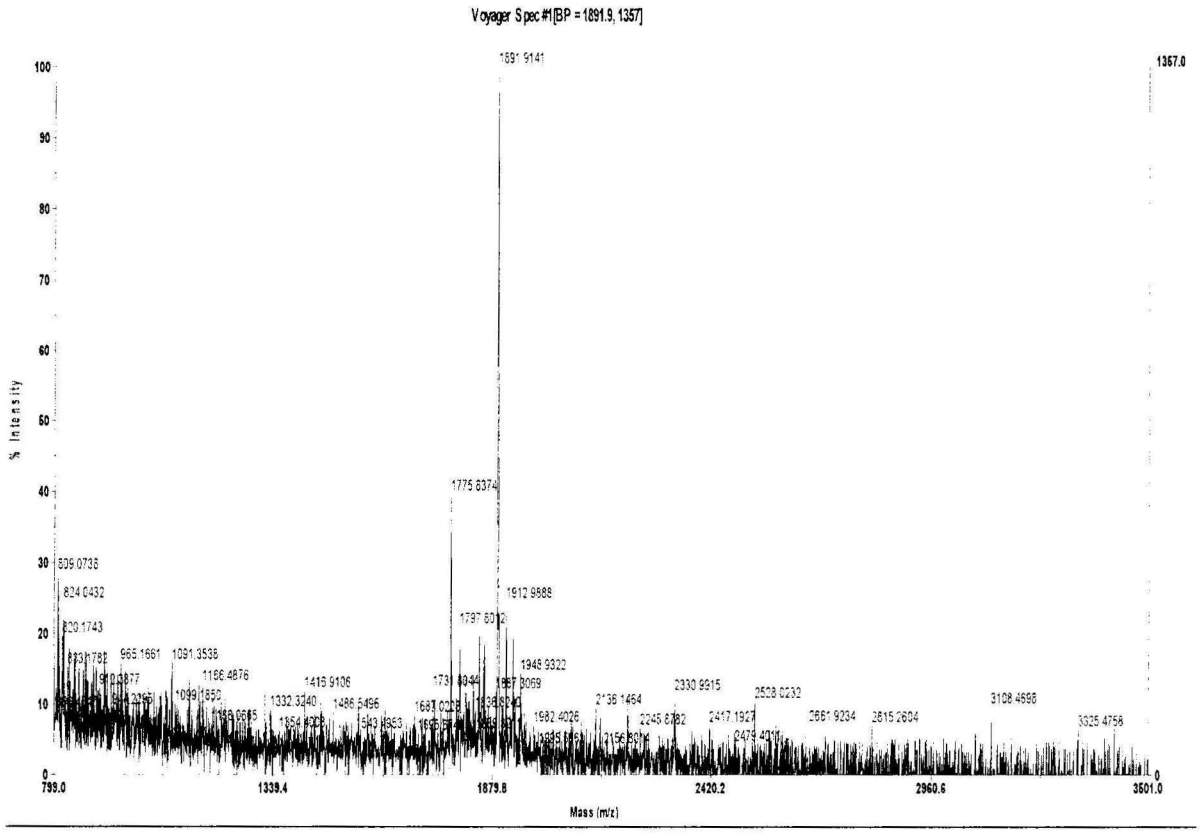


Figure 64 – 1-D NMR Spectrum of Tes_B 09g11a



This spectrum clearly shows the presence of a Trp at 10 ppm, as well as the presence of aliphatic residues below 1 ppm. The doublet of doublets is also observed for Tyr around 7 ppm. The TSP peak is much grater that the amino acid peaks, signaling a very dilute sample. Unfortunately there was insufficient concentration to run 2-D NMR experiments, so half the sample was thus reduced and alkylated, yielding the following spectrum (Figure 65):

Figure 65 – TOF MS Spectrum of the Reduced and Alkylated Tes_B 09g11a (MW =1891.91 Da)



The reduction and alkylation data suggests that there are two cysteines in this peptide (1891.91 – 1774.69 = 117.22. 117.22/58 = 2.02). Regrettably, the sequence data is not clear, presumably due to the impurities in the sample, and no sequence has yet been obtained for this conopeptide.

Conclusions

Several combinatory analytical techniques were employed in this study in order to elucidate structural data of some of the peptides present in the venom of the cone snail *Conus tessulatus*. These techniques included HPLC, mass spectrometry, NMR and peptide sequencing. By these techniques, five novel compounds were discovered and characterized. The literature does not indicate any reported sequences form *Conus tessulatus*. Therefore, these are the first conotoxins reported form this species. Among the reported peptides are three M-conotoxins, one α-conotoxin and one conophan. The sequences of these peptides are as follows:

Table 18 – Conus tessulatus Partial Peptide Library

Name and Sequence	**Mass Spec MW (Da)**	**Calculated MW (Da)**	**Difference in MW**	**Framework**	**Superfamily**
1. Tes_B05D1011F GGCCSYOPCIANNPFCG	1736.48	1714.65	21.83 = Na	CC-C-C	α
2. Tes_B05D050607B4 VYHAHPYSNAVWS	1531.08	1530.71	0.38	Linear	Conophan
3. Tes_B05D02 CCSQDCRVCIOCCPY	1703.37	1702.61	0.76	CC-C-C-CC	M
4. Tes_B08F12B CCSRYCWRCTOCCPN	1805.42	1804.65	0.77	CC-C-C-CC	M
5. Tes_B08F13D(2)1 CCSRYCYICIOCCPN	1751.47	1750.65	0.82	CC-C-C-CC	M
6. Tes_B08F13D(2)7 CCSRYCYICIOCCPN	1751.69	1750.65	1.04	CC-C-C-CC	M

These findings support and show the extreme diversity of the venom components of *Conus tessulatus*. This work is the first step in determining the functionality and uses of these incredibly promising peptides. This work will continue in an effort to determine the molecular targets and mode of actions of these compounds. Once these goals have been established, the great therapeutic value of these peptides will be readily available for many uses in neuroscience.

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