ISOLATION AND CHARACTERIZATION OF NOVEL CONOPEPTIDES FROM CONUS DALLI

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Isolation and Characterization of Novel Conopeptides from *Conus dalli*

By

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To the one who gave me the strength and lit my path

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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 his 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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ABSTRACT

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Conus dalli is a cone snail species that preys upon mollusks (molluscivorous) and it belongs to the same clade as the better studied *Conus textile*. They have different biogeographical distribution: *C. dalli* is restricted to the Panamic area; whereas *C. textile* is a widespread species found from the Red Sea to Hawaii. The venom of *C. textile* is an extremely complex mixture of conopeptides characterized for their very high content of modified amino acids; particularly, for their high content of γ-carboxy glutamate (Gla). Therefore, it is expected that the venom of *C. dalli* is equally complex and it might provide us with a library of novel conopeptides. We have collected 6 specimens of *C. dalli* from the Pacific coast of Panama. Their venom ducts were dissected and 40 mg of crude venom were extracted.

Venom was separated using SE-HPLC and RP-HPLC and several single-component fractions with unique molecular weights have been found. 1D and 2D NMR methods in conjunction with mass spectrometry techniques have been applied to the main

components of the venom. Three novel conopeptides have been isolated and characterized; dal_C1011h, dal_C0910, and dal_C0805g. dal_C1011h is a 27-residue hydrophobic conotoxin that belongs to O-superfamily, dal_C0910 is a 16-residue conotoxin that belongs to M-superfamily, and dal_C0805g is a 12-residue linear conopeptide the belong to the Conorfamide family. The details on the characterization of these conopeptides along with a comparison with previous data obtained from *C. textile* are presented.

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1. Introduction:

Marine snails of the genus *Conus* (cone snails) are venomous predatory gastropods found on or near coral reefs in tropical waters throughout the world. The families *Conidae*, *Turridae*, and *Terebridae* constitute the superfamily *Conoidea*, members of which are characterized by the possession of a venom apparatus. The most deadly are the *Conidae* family that includes approximately 1000 species of the genus *Conus* (11). Approximately 250 species have been found in the Atlantic Ocean, including the West African species and approximately. 50 species have been found in the Panamic Region (tropical west coast of the Americas) (2).

Cone snails inject venom into their prey by way of a disposable hollow tooth. All of the approximately 1000 members of the genus *Conus* are venomous hunters of fish (piscivorous), worms (vermivorous) or mollusk (molluscivorous) (1). Cone snail venom is typically composed of 40 to 200 small peptide components, each of which range in size from 6 to 50 amino acids (2). The great diversity and specificity of *Conus* toxins and conopeptides has been attributed to intense evolutionary pressure.

Conopeptides can be divided into two major groups: (1) disulfide rich which contain multiple disulfide bonds (2 or more), referred to as conotoxins, and (2) non-disulfide rich (those with a single disulfide linkage or none at all).

It is the diversity, specificity and simplicity of conopeptides that contribute usefulness as receptor blocker. Conopeptides have been isolated and shown to inhibit or

induce muscle contraction in mice, block sodium, potassium, or calcium channels, and induce acetylcholine receptor (nAChR) (3).

Cone snails have been able to deal with evolutionary pressure by undergoing constant change. The arsenal of peptides used to immobilize prey is generated by what is often referred to as combinatorial strategy. An immature toxin consists of a highly conserved prepropeptide, which is made up of 60 to 90 amino acids at the N-terminus and 10 to 30 amino acids at the very cysteine rich C-terminus (4). The N-terminal part of the precursor peptide changes only slightly from toxin to toxin within a given *Conus* species, but the C-terminal end is extremely variable. This variability is attributed to high propensity for mutation to genes responsible for encoding the amino acids that constitute the C-terminus. The mature toxin, which was once the C-terminus, is held together tightly by rigid network of disulfide bridges. The variability in the family of conotoxins is observed in the size of the loop regions between the cysteine residues (5).

The venom peptide needs to be sufficiently stable to survive chemical degradation in solution at ambient temperature and enzymatic degradation by processing proteases present in the venom itself (6), as well as those in the tissues of prey species. This stability is often achieved naturally through the use of post-translational modification (PTM) and/or disulphide bonds that fold the peptide into a stabilized structure. Typical PTMs include amidation (C-terminal), sulphation (Tyr), bromination (Trp), glycosylation (Thr), γ-carboxylation (Glu), hydroxylation (Pro), pyroglutamation, N-C-cyclization and isomerization to D-amino acids.

The pattern of disulfide cross-link is a decisive determinant of peptide structure. Each *Conus* venom peptide gene superfamily has one or two characteristic patterns of

disulfide cross-link. In most cases, the disulfide connectivity can be deduced from the arrangement of Cys residues (Table 1.1). Diversity within families of conotoxins arises from the many different combinations of amino acids occupying the loop regions between the cysteine residues.[1] The conotoxin family has yielded unique scaffolds, which are optimal for presenting critical residues within a pharmacophore (7). Approximately 80% of all known conotoxin peptides contain a disulfide bond pattern that can be described as either a 2-loop, 3-loop, or 4-loop framework (5). The 2-loop framework is common to the α-conotoxins of the A-superfamily. In general, α-conotoxins are known to have very potent and specific binding affinities for acetylcholine receptors (nAChR). The 3-loop framework which known as M-superfamily consist of three main families of conotoxins, μ-, κM-, and ψ-conotoxins, which are all active toward voltage gated sodium channels and nicotinic acetylcholine receptors. The neurological target of Mini-M is not yet known, but it did induce scratching behavior in mice (48). The 4-loop framework is common to the ω-, κ-, δ- and μO-conotoxins of the O-superfamily. The ω-conotoxins have shown unprecedented activity which inhibits ion flow through calcium voltage-gated channels, the κ-conotoxins block potassium voltage-gated channels, and the δ- and μO-conotoxins are specific for sodium channels (8-25).

Other *Conus* peptides that do not belong to the A-, O- or M-superfamilies include the conantokins, conopressins and conodipines. Table 1 lists examples of peptides from each of the known families of conotoxins.

[1] To indicate that sulfur atom is part of disulfide bond, cysteine residue written as cysteine.

Family	Peptide	Sequence	Ref(s)
A-superfamily	disulfide linkages	-CC---C----C (2-loop, 1-3, 2-4)	9,10
α-conotoxin	GI	ECCNPACGRHYSC	11
	disulfide linkages	-CC------C-C-C------C-	12
αA-conotoxin	PIVA	GCCGSYONAACHOCSCKDROSYCGQ-NH_2	12
O-superfamily	disulfide linkage	C------C------CC---C------C (4-loop, 1-3, 2-5, 3-6)	9,13
ω-conotoxin	GVIA	CKSOGSSCSOTSYN---CCRS–CNOYTKRCY	11
κ-conotoxin	PVIIA	CRIONQKCFQHLDD---CCSRK-CNRFNK-CV	15
δ-conotoxin	PVIA	EACYAOGTFCGIKOGL---CCSEF-CLPGV--CFG-NH_2	16
μO-conotoxin	MrVIA	ACRKKWEYCIVPIIGFIYCCPGLICGPFV--CV	17
M-superfamily	disulfide linkage	--CC-----C----C----CC- (3-loop, 1-6, 2-4, 3-6)	18
μ-conotoxin	GIIIA	RDCCTOOKKCKDRQ-CKOQRCCA	19,4
ψ-conotoxin	PIIIE	HOOCCLYGK-CRRYOGCSSASCCQR	20
	disulfide linkage	CC----C----C-CC (3-loop, 1-6, 2-4, 3-5)	4
κM-conotoxin	RIIIK	LOSCCSLNLRLCOVOACKRNOCCT	47
Mini-M-conotoxin	Mr3a	GCCGSFACRFGCVOCCV	48
Miscellaneous			
conantokins	conan-tokin-G	GEγγLQγNQγLIRγKSN	22
conopressins	Arg-cono-pressin-S	CIIRNCPRG	23
conodipines	conodi-pine-M	AATCTHWALIYFKTVQLFGWXHFNYQVDATYCP QFQPCMPXX	24

Table 1.1 List of the conotoxin superfamilies and representative families for each. One-letter amino acid codes are used except for O (*trans*-4-hydroxyproline), γ (γ-carboxyglutamate and X (unidentified amino acids).(4)

1.1 Mollusk hunting cone snails

The fossil recorded that *Conus* can be divided into 17 clades of species that originated from the second Eocene period (55.5 million–38 million years ago) radiation of *Conus* (25,26). Four of these clades (I to IV) comprise fish-hunting species; two (V, VI) comprise snail-hunting species and worm hunting clades (VII-XVII) (Table 1.2). *C. textile* and *C. gloriamaris,* belonging to clade V, are more closely related to each other than the other eight species (Table 1.3). The δ-conotoxins from snail hunting species *C. textile* and *C. gloriamaris* are divergent from each other.

Clades	*Conus* species example	prey
Fish-hunting		
I	*striatus*	Fish
II	*geographus*	Fish
III	*purpurascens*	Fish
IV	*radiatus*	Fish
Mollusc-hunting		
V	*textile*	Gastropods
VI	*marmoreus*	Gastropods
Worm-hunting		
VI	*lividus*	Hemichordates;
polychaetes		
VIIII	*glans*	Errant polychaetes
IX	*planorbis*	Errant polychaetes
X	*betulinus*	Sedentary polychaetes
XI	*ebraeus*	Errant polychaetes
XII	*vexillum*	Errant polychaetes
XIII	*virgo*	Sedentary polychaetes
XIV	*arenatus*	Sedentary polychaetes
XV	*sponsalis*	Errant polychaetes
XVI	*tessulatus*	Errant polychaetes
XVII	*imperialis*	Errant polychaetes

Table 1.2: *Conus* clades (25)

Mollusk-hunting Clades
Clade V A. *Conus ammiralis, Conus textile, Conus glariamaris, Conus aureus, Conus legatus, Conus retifer, Conus bengalensis, Conus telatus, Conus victoriae.* B. *Conus aulicus, Conus episcopatus, Conus auratinus.* Probable other species in Clade V: *Conus omaria, Conus pennaceus, Conus magnificus.*
Clade VI *Conus marmoreus, Conus bandanus, Conus arenosus, Conus nocturnus.*

Table 1.3: Mollusk-hunting Clades (26)

1.1.1 *Conus textile*:

C. textile is a typical mollusk-hunting cone, which distributed in entire Indo-Pacific area. The venom of *Conus textile* is rich in peptides and has been studied by several laboratories using different methodological approaches. Peptides that have been described from this venom are summarized in Table 2. Behavioral analysis of the effects of the venom when injected into lobsters revealed a characterization posturing, or "King Kong" behavior, which was elicited by a single isolated peptide, the "King Kong" peptide (27). This peptide has a characteristic pattern of cysteine residues, -C-C-CC-C-C, where dashes represent intervening amino acid sequences of different lengths. This pattern is characteristic of the O-superfamily of conotoxins (Table 1.1). In contrast to the ω-peptides, which contain a number of amino acids with positively charged and small side chains, the King Kong peptide has relatively acidic side chains and contains a high number of hydrophobic residues.

Independent studies have shown that the King Kong peptide, as well as several peptides of similar sequence (TxVIA, TxVIB), produces paralysis in mollusks by

prolonging the duration of the sodium component of the action potential (28,29). Interestingly, while these "delta" peptides appear to bind to mammalian sodium channels, they have no physical effect on mammalian sodium currents (30).

Additional studies have revealed two peptides in *C. textile* venom with effects on mollusk calcium current TxVII (31), and synaptic transmission ε-TxIX (32). In these two instances, effects are observed in only micromolar concentration ranges. The disulfide scaffold of TxVII is C-C-CC-C-C, which is the same as sodium channel agonists of the *C. textile* venom. The disulfide scaffold of ε-TxIX is -CC-CC-, which is distinct from the aforementioned *C. textile* peptides. In addition, the peptide has a significant amount of post-translational modification including γ-carboxylation of glutamate, bromination of tryptophan, and glycosylation of threonine (32, 33). Another peptide with the -CC-CC- cysteine pattern was predicted from the sequence of a *C. textile* cDNA clone (33).

The structure of the cDNA for the precursor of the King Kong peptide, along with two similar peptides KK-1 and KK-2, has been determined (Table 1.4). The precursor encodes a signal sequence of 18 residues and a 33 residue propeptide sequence, which contains a paired basic sequence that is presumably the recognition site for a processing enzyme. Significant variation occurs in those residues; the overall predominance of hydrophobic and acidic amino acid residues is maintained. Some variations observed in the propeptide sequence are largely conserved (34).

Similar results were obtained in an independent venom duct cDNA cloning study (35). Again, propeptide sequences and cysteine pattern were largely conserved. Amino acid sequences varied between cysteine residues, but negative charge and hydrophobicity were maintained for peptides with the C-C-CC-C-C cysteine framework (Tables 1 and 2).

This study also obtained cDNA clones for two peptides with the CC-C-C framework. C.t.86/86 is similar to the King Kong peptide, containing hydrophobic and acidic amino acids and having the C-C-CC-C-C cysteine framework. Comparison of the result of Edman degradation with the cDNA structure shows that the tryptophan adjacent to the fifth cysteine residue is modified. Several other peptides of the *C. textile* venom (*C.t.*27/49; (31,32) *C.t.* 37/34; Table 1.4) have the same modified residue near the fifth cysteine residue in the C-C-CC-C-C cysteine motif and are hydrophobic and acidic. The next most abundant peptide of the venom, *C.t.* 75/82, was not completely sequenced but does not have a pattern of cysteine residues similar to those of other peptides reported in this venom (Table 2).

Based on the aforementioned studies, several observations can be drawn as to the make-up of the *C. textile* venom. The venom is not only predominantly composed of peptides, but these peptides are largely similar in character. The most abundant peptides of the *C. textile* venom are of the O-superfamily (C-C-CC-C-C cysteine frame work). There is a number of these peptides (at least 15 have been identified), which are all neutral or acidic, and tend to have a high proportion of hydrophobic residues. Among this group of peptides, several, notably the King Kong peptide and peptide 86/86 (TX04), are expressed in high abundance in the venom, while the others are present in smaller amounts.

Thus, the *C. textile* venom is composed largely of members of a structurally restricted class of peptides, primarily δ or δ-like peptides, several of which are present in high abundance. In addition to the elaboration of the peptide similar to the δ-peptide, there several other classes of peptides present in the venom. These are members of

CC-C-C and CC-CC- families. In other venoms, the members of these families function as acetylcholine receptor antagonists, but the molecular targets for the C. textile homologs of either family remain to be determined.

Table 1.4: Peptides in Conus textile venom

Name	Isolation/characterization/assay	Sequence	activity	Reference
King Kong	HPLC/behavior in lobster	WCKQSGEMCNLLDQNCCDGYCIVLVCT	Posturing in lobsters Prolongs molluscan Sodium-channel	27,29
TxIb	HPLC/sodium agonist	WCKQSGEMCNVLDQNCCDGYCIVFVLT	Prolongs molluscan Sodium-channel inactivation	28,29
TxIIa		WGGYSTYCγVDSγCCSDNCVRSYCT		28
TxVII	HPLC/calcium antagonist	CKQADEPCDVFSLDCCTGICLGVCMW	Molluscanc calcium Antagonist(μM)	31
ε-TxIX	HPLC/calcium antagonist	γCCγDGW^{Br}CCT^{GLY}AAO	inhibits molluscan synaptic	32,33
Tx5.2	mouse behavior, cDNA		transmission(μM)	32,33
Tx5.1	cDNA	CCQTFYWCCVQGK		33
KK-1	cDNA cloning	CIEQFDPCEMIRHTCCVGVCFLMACI		34
KK-2		CAPFLHPCTFFFPNCCNSYCVQFICL		34
Txα1	cDNA cloning	PECCSDPRCNSSHPELCG-NH_2		35
Txα2		PECCSHPACNVDHPEICR		35
TxO1		CLDAGEVCDIFFPTCCGYCILLFCA		35
TxO2		CYDSGTSCNTGNQCCSGWCIFVCL		35
TxO3		CYDGGTSCDSGIQCCSGWCIFVCF		35
TxO5		CVPYEGPCNWLTQNCCDATCVVFWCL		35
TxO6		NYCQEKWDYCPVPFLGSRYCCDGLFCTLFFCA		35
C.t. 86/86	HPLC/mass abundance	YDCEPPGNFCGMIKIGPPCCSGW*CFFAACA		35,36
TXO4	cDNA cloning			35,36
C.t. 27/49	HPLC/mass abundance	SCSDDγQYC?SXTDCCSW*DC DVVC?		36
C.t. 31/32	HPLC/mass abundance	LCXDYTγXCSHAHγCCSW*NC YNGHCT		36
C.t. 37/43	HPLC/mass abundance	DCRGYDAPCSSGAPCCD?W* TC?ARTN?		36
C.t. 75/82	HPLC/mass abundance	YTPNDAESSVCYFLCLMGIDLDE?N?P?		36

1.1.2 Conus dalli

C. dalli is a mollusk-hunting cone, distributed strictly in the Panamic region. In contrast to *Conus textile*, which has been extensively researched, less research has been carried out with *Conus dalli* likely due to the restricted distribution of this species of cone snail. The information known for this cone snail species is contained in a patent by B.M. Olivera in the laboratories of the University of Utah, where only methods of cDNA cloning were used (Table 1.5) (38-40) due to the framework of cysteine residues found in *C. dalli* venom. Olivera's patent disclosed toxins Da6.1, Da6.2, Da6.3, Da6.5, Da6.6 and Da6.7 as members of the O-superfamily, and Da3.1, Da3.2 and Da3.3 as members of the M-superfamily. The neuropharmacology of these conotoxins has not been determined, since bioassays have not been obtained for this cone species.

Conus textile and *Conus dalli* belong to the same clade, "clade V," due to their similarity in shape and feeding habits. This is a good premise to compare the structure of crude venom of both snails, although each belongs to a different geographical area. Using cDNA cloning to determine the components of conotoxin compounds in C. *dalli* is not sufficient to reveal the complete structure of the conotoxin, since this method is unable to identify the post-translationally modified amino acids, which are considered to be one of the defining characteristics of *Conus textile.* However, comparing conotoxins isolated from the crude venom of *Conus textile* and *Conus dalli* can gives us an idea about the displacement of amino acids and their importance.

Table 1.5 *Conus dalli* conotoxin sequences determined by cDNA cloning (Patent WO02064740)

Number	Sequence	SEQ ID#
O-superfamily		
Da6.1	----**CY**DGGTG**C**D----SGNQ**CC**SG**W**-**C**--IFV-**C**L	(SEQ ID NO:202)
Da6.2	QVKP**C**RKEHQL**C**DLIFQN---**CC**RG**WYC**--LLRP**C**I	(SEQ ID NO:17)
Da6.3	-D--**C**QEK**WD**Y**C**PVPFLGSR**YCC**DGFI**C**--PSFF**C**A	(SEQ ID N0:223
Da6.5	----**C**AQSSEL**C**DALDSD---**CC**SGV-**C**--MVFF**C**L	(SEQ ID NO:72)
Da6.6	-VKP**C**SEEGQL**C**DPLSQN---**CC**RG**WHC**--VLVS**C**V	(SEQ ID NO:22)
Da6.7	-D--**C**QGEWEF**C**IVPVLGFV**YCC**PWLI**C**--GPFV**C**VDI	(SEQ ID NO:226)
M-superfamily		
Da3.1	---**CC**DDSE**C**DYS-**C**---WP**CC**ILS^	(SEQ ID NO.401)
Da3.2	--Q**CC**PPVA**C**NMG-**C**---EP**CC**#	(SEQ ID NO.402)
Da3.3	---**CC**NAGF**C**RFG-**C**---TP**CC**W^	(SEQ ID NO.403)

2. MATERIALS AND METHODS

2. *Cone Snail Descriptions and Collection*

The shell of *Conus dalli* has a spiraling pattern, which appears as alternating stripes of

a. Intricate white hills, outlined in brown, of varying sizes; they could also be described as scales (these "scales" are in a horizontal position when viewing the snail with the cone pointing east or west.)

b. Brown and black striped lines in a vertical position when viewing the snail with the cone pointing up.

The inside of the shell has a pinkish / purplish hue and the tip of the cone is pale pink. The tip is also taller and more pointed than that of *C. textile*, and the length of the cone has sides that are bowed out, giving the cone a slightly oval shape.

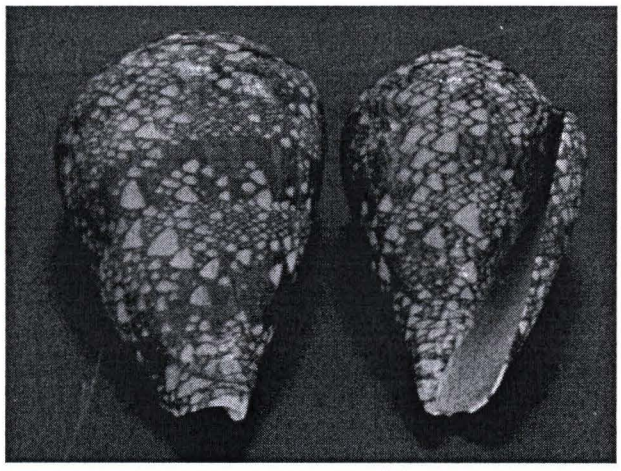

Figure 2.1 Photograph of the marine cone snail shell, *Conus dalli* used for this work.

Conus dalli generally measures up to 55 mm in length and up to 26 mm in diameter. This marine mollusk is commonly distributed in the Panamic area (from West Mexico to Panama), assuring an abundant supply of cone snails. The specimens used in this research were collected off the coast of Panama (Figure 2.2).

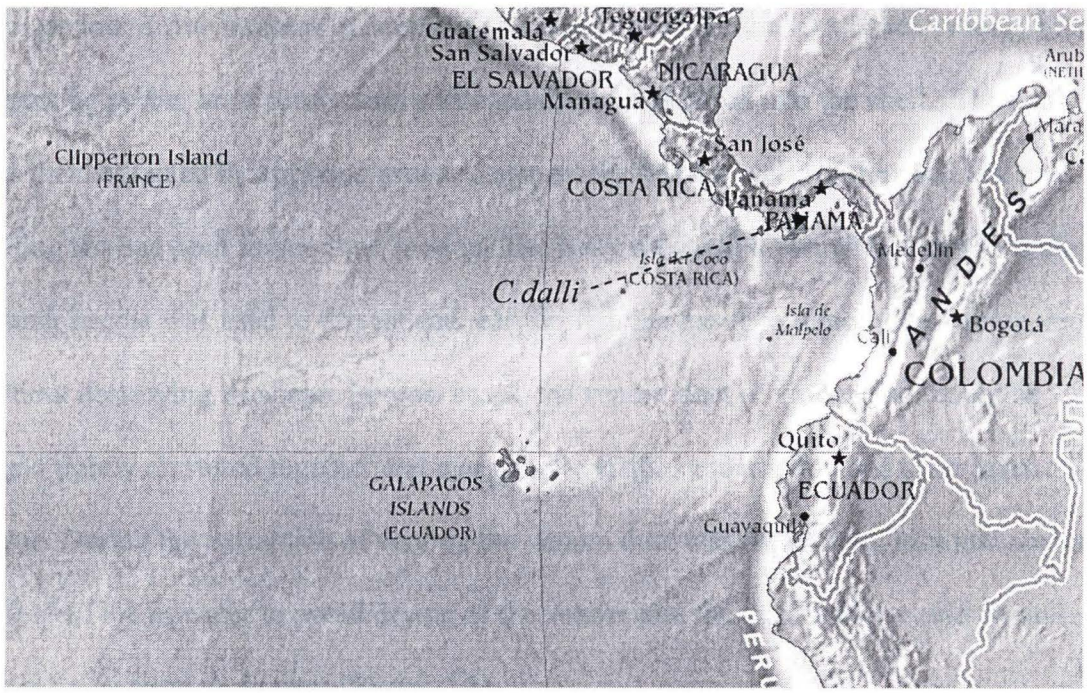

Figure 2.2 Map of Collection Sites for *Conus dalli.*

The specimens were transported alive to our laboratory and kept in aquaria. The majority of the cone snails collected were kept alive and then dissected at a later day, when the venom was finally extracted. Any cone snails that didn't survive the journey were frozen at –70 ˚C and studied separately.

2.2 Crude Venom Extraction

The first part of the venom extraction consisted of taking the animal out of the shell. At this stage, one can choose to break or not to break the shell; in either case, the snail will perish. Removal of the snail from each specimen without cracking the shell was the selected method. Since live specimens were used, each cone snail was placed on ice for a period of five minutes, to insure that the body would come out intact. A long, thin dissecting probe, or in some cases a fine needle, was inserted into the shell. The body was then extracted by applying pressure against the interior walls of the shell and then pulling the body out in one fluid motion. The body was held down with a needle, while a thinner needle was used to dissect and tear through the tissue to remove the venom duct, without destroying its shape. In most cases, the venom duct of *Conus dalli* could be found tightly clustered together and was usually visible under a thin and clear layer of tissue. During the extraction of venom, the venom duct was rinsed with minimal amounts of 0.1% TFA in water to avoid drying of the venom and the duct, which could complicate the entire extraction process (Figure 2.3).

Once the duct was isolated, it was placed on a flat or cylindrical surface so that it could be elongated and measured. During this process, the venom duct was stored in 0.1% TFA in water. The duct was then frozen and lyophilized. After lyophilization, around 5 mL of 0.1% TFA was added, the venom duct was crushed using a tissue grinder, the grinding process was repeated until the duct completely disappeared, the homogenous solution was centrifuged at 10,000 RPM for 20 minute to separate the "water soluble" crude venom from the other components, the supernatant fluid was separated from the solid, and the process repeated for the solid pellet at least three times

to insure all crude venom was completely extracted. The supernatant layers were then combined, frozen, and lyophilized, and the venom was obtained as light white/beige-colored powder. The venom at this stage was regarded as crude venom. The crude venom was weighed, labeled and placed at –70 ° C.

Two batches of *Conus dalli* crude venom were prepared; each batch of crude venom was given a different code. The amount of snails sacrificed and the amount of venom extracted varied in each batch preparation (Table 2.1).

Conus Dalli Sample Preparations	Number of Cone Snails Specimens	Total Crude Venom (extracted)
dal_A	2	10.0 mg
dal_C	4	25.3 mg

Table 2.1: List of Crude Venom Batches of *Conus dalli*

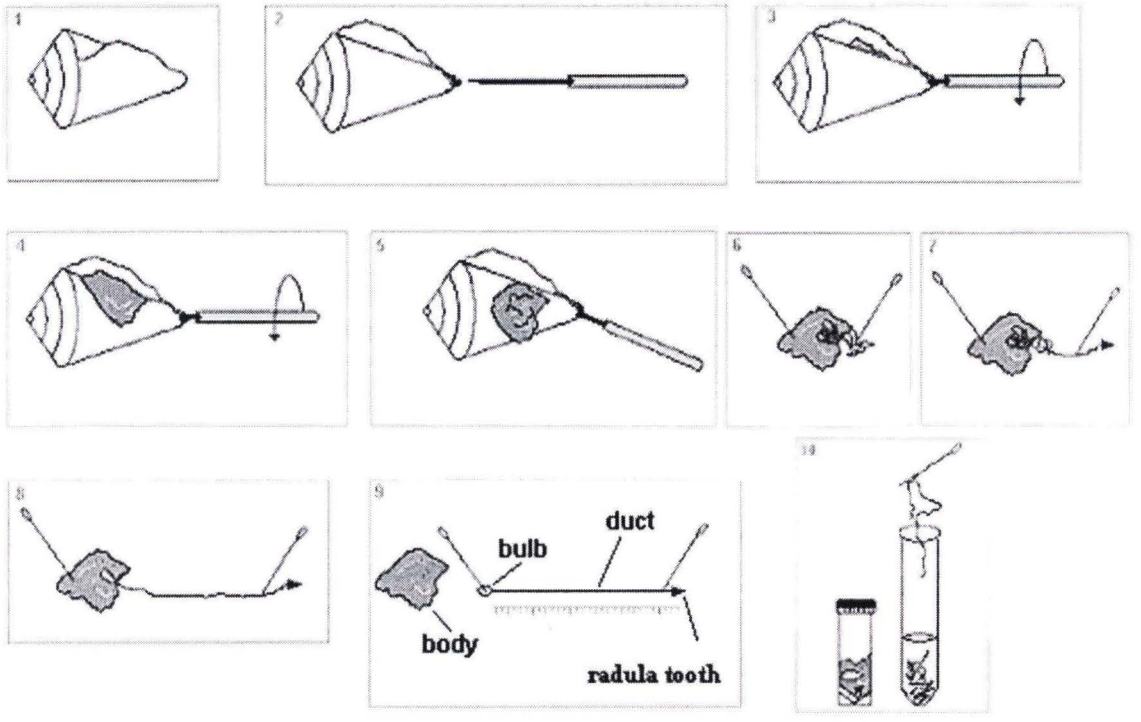

Figure 2.3: Schematic Drawing of the Dissection Procedure of a Cone Snail.

2.3 Peptide Isolation:

After obtaining the crude venom from the cone snails, two different methods involving combined chromatographic techniques were used to isolate the peptide (as shown in Figure 2.4). The first separation technique involved direct separation of the crude venom using semi-preparative RP-HPLC. At first, this technique was useful to obtain a chromatographic profile that outlined the complexity of the venom from *Conus dalli*. However, this chromatographic technique by itself did not meet the primary goal, which is to isolate pure peptides with low molecular weights in as few steps as possible, due to severe chromatographic overlap of the peptide components.

Therefore, isolation was improved by using the second method: Size exclusion HPLC followed by semi-preparative RP-HPLC and finally analytical RP-HPLC for further purification of selected fractions (when needed).

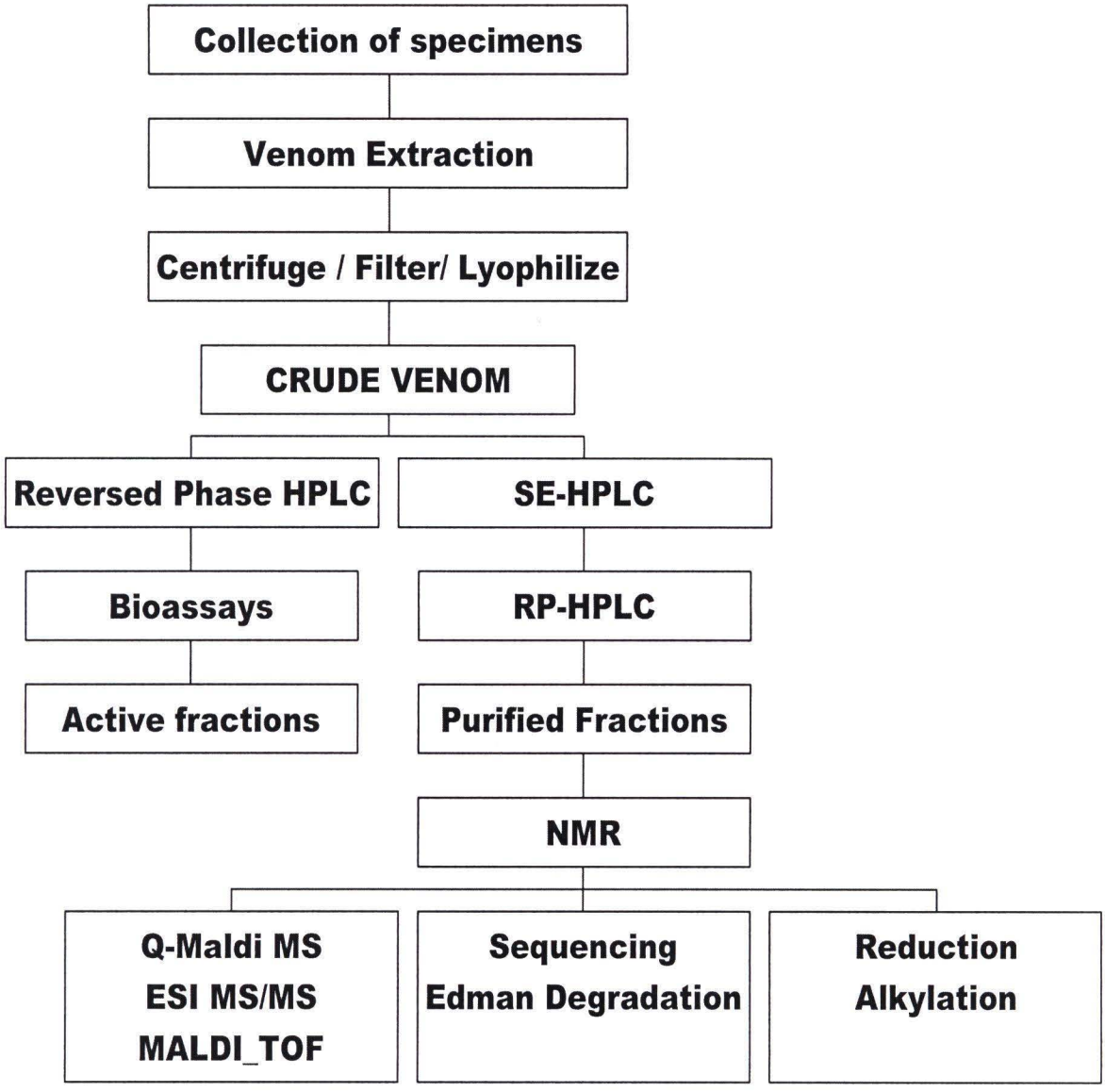

Figure 2.4: Protocol for the extraction and characterization of *Conus dalli* venom.

2.4 Reversed Phase HPLC

A 10.0 mg sample of *Conus dalli* crude venom (dal_A) dissolved in 0.1 % TFA/H_2O was injected into the Semi-preparative Reversed Phase HPLC (Figure 2.5). For this separation, a Vydac 5μm C-18 protein/peptide semi-preparative column (250 mm x 10 mm) and UV detector (Thermo Separation Products SM-4100) measuring the absorbance at λ = 220 nm and 280 nm were used. The solvent gradient (Thermo Separation Products CM-4100) established for the separation of conopeptides is a 100 min linear gradient starting at 100% of solution A and ending with 100% of Buffer B. The mobile phase used for this separation is the following:

Solution A: 0.1%TFA / H_2O

Solution B: 0.1%TFA / 60% ACN / H_2O

The flow rate used for all the Semi-preparative RP-HPLC separations in this work was 3.5 ml/min.

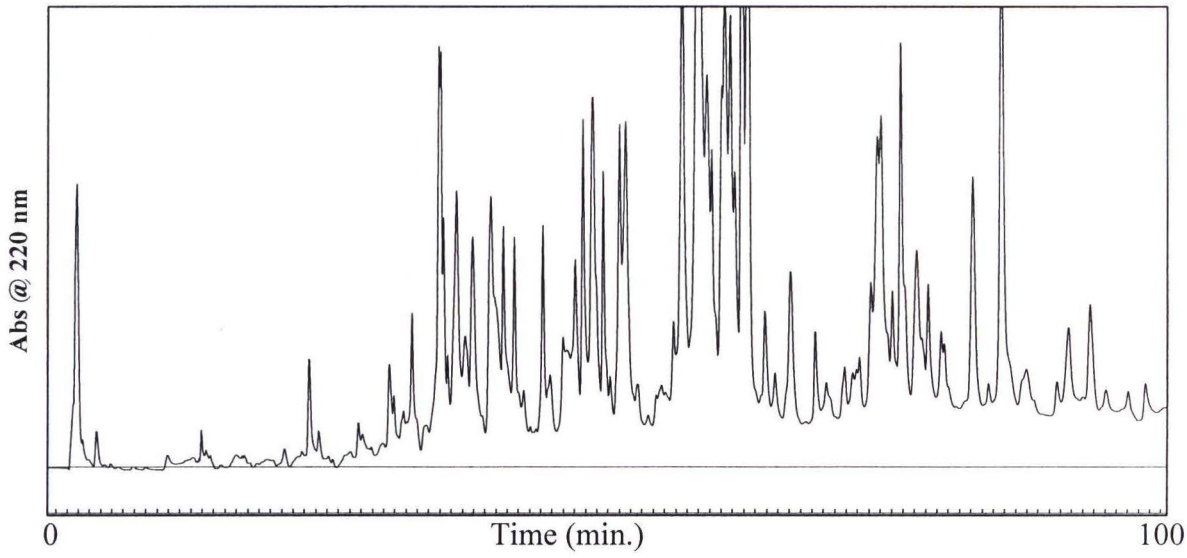

Figure 2.5: Semi-preparative Reversed Phase HPLC Chromatogram of *Conus dalli* crude venom (dal_A).

The chromatogram obtained for dal_A provided a general profile of the venom of *Conus dalli*. A total of 60 fractions were collected from this separation. Each fraction was subjected to fluorescence-based *in vitro* assays for screening the venom from *Conus dalli*. These assays allow the identification of venom fractions that possess biological activity at both ion channels and neurotransmitter receptors. These fluorescence-based functional bioassays where performed in murine cortical cell cultures, using Di-8-ANEPPS (4-[2-[6-(dioctylamino)-2-naphthalenyl] ethenyl]-1-(3-sulfopropyl)-pyridinium) to determine the membrane potential dye. The following dyes used for each ion channel are shown below (Table 2.2).

Fluorescent Indicator Dyes	**Voltage-gated Ion Channel**
SBFI dye (4,4'-[1,4,10-trioxa-7, 13- diazacyclopentadecane-7,13-diylbis(5- methoxy-6,2-benzofurandiyl)]bis-1,3-Benzenedicarboxylic acid)	Na^+ channel
PBFI dye 1,3-Benzenedicarboxylic acid, 4,4'-[1,4,10,13-tetraoxa-7,16- diazacyclooctadecane-7,16-diylbis(5- methoxy-6,2-benzofurandiyl)]bis-	K^+ channel
Fluo3 dye (N-[2-[[[[2-[bis(carboxymethyl)amino]-5- (2,7-dichloro-6-hydroxy-3-oxo-3H- xanthen-9-yl)phenoxy]methyl]methyl]oxy]- 4-methylphenyl]-N-(carboxymethyl) glycine)	Ca^+ channel

Table 2.2: Fluorescent Indicator Dyes.

In the case of sodium, an increase in fluorescence indicated a depolarization, meaning that it was a possible Na^+ channel opener. A decrease in fluorescence indicated a hyperpolarization and indicated the opposite, establishing a possible Na^+ channel blocker.

2.5 Size Exclusion Chromatography

Dal_C crude venom was separated using the second method, involving Size-Exclusion Chromatography (Superdex-30 column, Pharmacia Biotech, 560mm x 25.4mm) using an isocratic system. Each run was approximately 300-350 min long. The mobile phase used for these separations was 0.1 M ammonium bicarbonate (at a flow rate of 1.5 ml/min). A UV detector (TSP SM-5100 PDA Detector) was used to measure absorbance at the wavelengths 220 nm, 280 nm and 250 nm. Examples of the SE-HPLC chromatograms can be seen below (Figure 2.6 a and b).

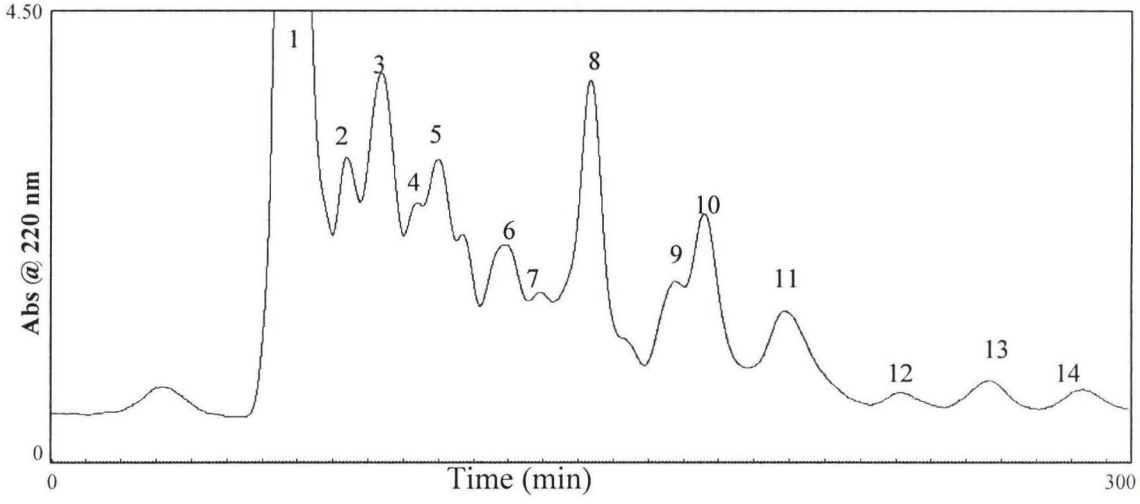

Figure 2.6a: Size Exclusion HPLC Chromatogram of dal_C. at λ=220 nm.

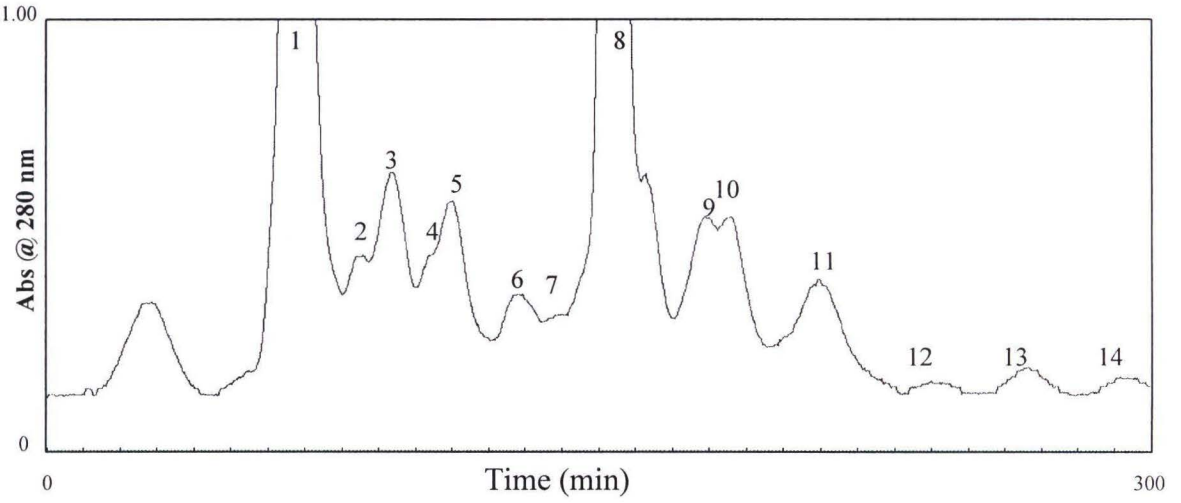

Figure 2.6b: Size Exclusion HPLC Chromatogram of dal_C. at λ=280 nm.

2.6 *Analytical Reversed Phase HPLC*

Selected fractions collected from the Semi-preparative RP-HPLC of each of the SEC fraction runs were subjected to further purification. For this separation, an analytical Phenomenex Jupiter 5μm C18 column (250 mm x 4.60 mm) and UV detector (TSP SM-4100) measuring the absorbance at λ = 220 nm and 280 nm were used. The quaternary pump (TSP CM-4100) produced a linear gradient starting at 100% of solution A and ending with 100% of solution B. The mobile phase used for these separations was the same as used for the Semi-Preparative HPLC runs (solution A: 0.1%TFA / H_2O, solution B: 0.1%TFA / 60% ACN / H_2O). The flow rate used for all the Analytical RP-HPLC separations in this research was 1.0 ml/min.

2.7 *Reduction/Alkylation*

Once a conopeptide was isolated and purified, the sample had to be reduced and alkylated before sequencing because of the possibility of disulfide bridges being present. The sample was reduced and alkylated prior to Edman sequencing using the following protocol:

1. The sample was lyophilized from 0.1%TFA / H_2O in 2 ml screw capped Eppendorf tubes.
2. The sample was reconstituted with 60 µl diionized water (d-H_2O).
3. The sample was lyophilized.
4. The lyophilized peptide was dissolved in 30 µl 10%ACN/10%NH_4HCO_3 (made fresh before use).
5. The pH was checked to ensure that it was in the range 7.5-8.5.
6. 5 µl of 45mM DTT (dithiothreitol) was added to the sample, and the sample was incubated at 37 °C for 20 min.
7. 5 µl 100 mM (iodoacetamide) was added to the sample, which was then incubated in the dark at room temperature for 30 min to 1 hour.
8. 5 µL of acetic acid was added to the sample to stop the reaction.

2.8 *Zip Tip clean up procedure*:

This procedure was used to clean conopeptides after reduction and alkylation or trypsin digestion as preparation for Edman degradation and mass spectrometer methods.

1. $ZipTip_{\mu\text{-}C18}$ pipette was pre-weted tips by aspirating 15 µL of 50% ACN/50% diionized water.
2. Sample was bounded to $ZipTip_{\mu\text{-}C18}$ pipette tips by aspirating and dispensing 10 µL of the sample five times.
3. $ZipTip_{\mu\text{-}C18}$ pipette tips was washed by aspirating 15 µL of 0.1% TFA. Repeat this step five times to insure Zip Tip is clean from all reagents except desired peptide.
4. Desired peptide was released from $ZipTip_{\mu\text{-}C18}$ resin by using 50% CAN/0.1% acetic acid.

2.9 *Molecular weight determinations*:

Four instruments were used to determine the molecular weights of desired peptides:

1. Matrix-Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) mass spectrometer (Hewlett Packard Model G2025A) using a DHB (dihydroxybenzoic acid) matrix and laser energy of 2.3-3.1µJ.
2. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry experiments are performed on an Applied Biosystems Voyager-STR mass spectrometer with delayed extraction. Samples were irradiated with a nitrogen laser (Laser Science Inc.) operated at 337nm and the laser beam attenuated by a variable attenuator and focused on the sample target. Ions produced in the ion source are accelerated with a deflection voltage of 30,000 V. The ions are then

differentiated according to their m/z using a time-of-flight mass analyzer or the time-of-flight reflectron mass analyzer using an α-cyano-4-hydroxy-cinnamic acid matrix.

3. LCQ Deca ion trap (Thermo Finnigan, San Jose CA). The electrospray ionization (ESI) tandem mass spectrometry experiments are performed on a Finnigan LCQ quadrupole ion trap mass spectrometer. Electrospray samples are typically introduced into the mass analyzer at a rate of 4.0 µl/min. The positive and negative ions, generated by charged droplet evaporation, enter the analyzer through a heated capillary plate. The emitter voltage is typically maintained at 4000V. Data acquisition was conducted using full scan mode to obtain the most intense peak as precursor ion, followed by high resolution zoom scan mode to determine the change state of the precursor ion and MS/MS scan mode to determine the structural fragments ion of the precursor ion.
4. Q-STAR XL MALDI MS/MS (Applied Biosystems, Foster City CA). The high performance quadrupole-quadrupole-time-of-flight hybrid mass spectrometer (QqTof-MS) is a compact, fully integrated system for peptide sequencing, protein characterization and metabolite identification. The mass spectrometer is a highly sensitive and very flexible tool for different types of offline analysis. The system enables accurate and sensitive mass determination with high resolution. This, together with different scan models (MS/MS fragmentation experiments), yield unequivocal molecular weights and structural information for both biomolecule and small molecule analysis.

2.10 *Amino Acid Sequencer:* Desired fractions were sequenced using a Procise™ 491A (Applied Biosystems Foster City, CA) amino acid sequencer.

The Procise Protein sequencing system is composed of four integrated modules: the Procise Protein Sequencer, the model 140C Microgradient Delivery System, the Model 785A UV Detector and a computer equipped with Procise control software and the Model 610A Data analysis software. N-terminal amino acids are cleaved sequentially from protein/peptide chains and the system then analyzes the resulting phenylthiohydantoin (PTH)-amino acid residues. The chemical process used is derived from the Edman degradation technique.

Edman degradation relies on both the high chemical reactivity of a protein's N-terminal amino acid and the ability to remove the selectively derived amino acid from the protein while leaving the rest of the peptide chain intact. Each cycle of the degradation occurs at the newly formed amino-terminal amino acid left by the preceding degradation. Repetitive cycles thus provide sequential removal of the amino acids from the primary structure of the peptide chain. A cycle is divided into three steps: coupling, cleavage, and conversion (Figure 2.7).

The sequencing process is not completed by Edman degradation alone. Once the amino acids are removed from the sample, they must be analyzed to determine their identity. The amino acid derived from each cycle is identified by comparison to the retention time on reverse phase high performance liquid chromatography (RP-HPLC) to the retention time of the PTH amino acid standards. Amino acids are separated on a C18 reverse phase column using acidic tetrahydrofuran with an increasing

acetonitrile/propanol gradient. Sensitivity of the HPLC system is the main component in determining the sensitivity of the sequencing system.

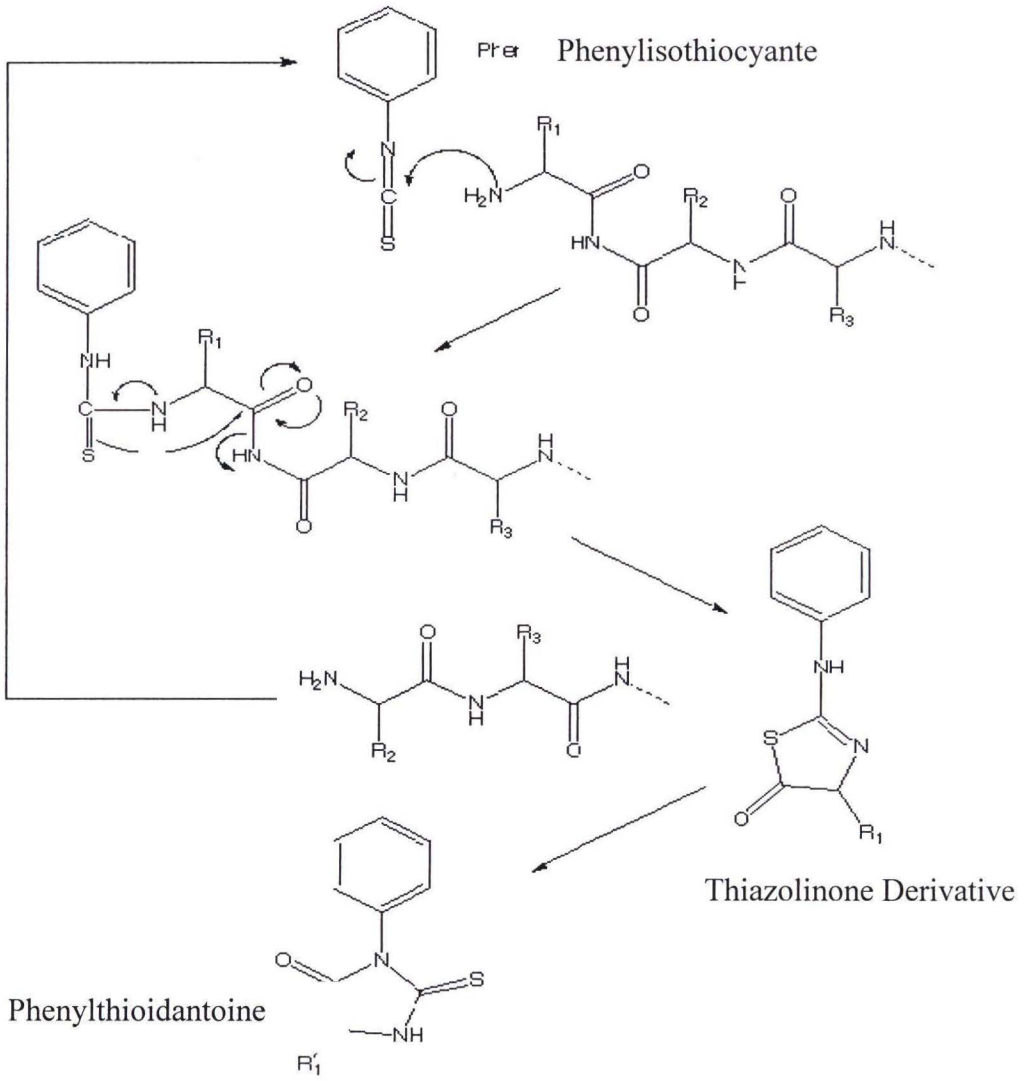

Figure: 2.7: Scheme of Edaman degradation procedures, which used for to determine protein sequence.

2.11 Nuclear Magnetic Resonance Spectroscopy

NMR spectra were acquired on a Varian Inova 500 MHz spectrometer equipped with three RF channels, pulse field gradients and waveform generators. Initially, 1D and 2D-TOCSY spectra were recorded using a using 3 mm sample tubes in 130 µl of NMR

solution in a 5 mm gHCX triple resonance probe. 1D spectra were acquired using 512 scans; whereas 2D spectra were acquired using 96 increments in t1 with 256 scans per increment in a phase sensitive mode. 2D spectra were processed using linear predictions in t1 to 1024 points and transformed to final size of 2k x 2k. The 1D spectra of picomolar amounts of the *C.dalli* conopeptides were acquired overnight using 3 mm sample tubes in 130 µl of NMR solution in a 5 mm gHCX triple resonance probe. All spectra were recorded at 25°C and 0°C in an NMR solution that consisted of 90% H_2O/10% D_2O using TSP as an internal standard. The pH for this solution was adjusted to 3.6 using 0.01 M solutions of HCl and NaOH and a Phoenix micro-pH probe. Water suppression was achieved using WATERGATE and Excitation Sculpting for the 2D experiments and WET and presaturation for the 1D ^1H spectra. The resonance assignments were carried out using standard biomolecular NMR procedures.The chemical shifts found in proteins fall into characteristic ranges, as shown in the Table 2.3. However, local influences such as hydrogen bonding, conformation, and neighboring residues (particularly aromatics) can have dramatic effects.

Type of proton	Chemical shift (ppm)
CH_3	0-1.5
β and other aliphatic protons	1-4
α; β in Ser, Met	3.5-5.4
Aromatic CH	6.5-7.7
NH (side chain)	6.6-7.6
NH (backbone)	8.1-8.8
NH (Trp)	~10

Table 2.3: Proton Chemical Shifts in Proteins.

3. RESULTS AND DISCUSSION

3.1 Chromatographic Analysis of Venom

When using the initial method of separation (direct Semi-preparative Reversed Phase HPLC of the crude venom) a total of 62 fractions were collected from the dal_A sample. From the chromatogram shown earlier, it is clear that the venom composition of *Conus dalli* is quite complex. Therefore, it was necessary to adopt an additional form of chromatography to enhance the separation of the components of the venom. The fractions collected from sample dal_A were separated into two parts. The first part was used for fluorescence-based functional bioassays. The information from the results of the bioassays confirmed that *Conus dalli* did indeed have biologically active components. A total of 27 fractions showed activity on the sodium, potassium, and calcium channels (Figure 3.1a). These fractions appeared to have more activity on the voltage-gated sodium and calcium channels, compared to the potassium channels as data shown in table's 3.1a, 3.1b, 3.1c, and 3.1d.

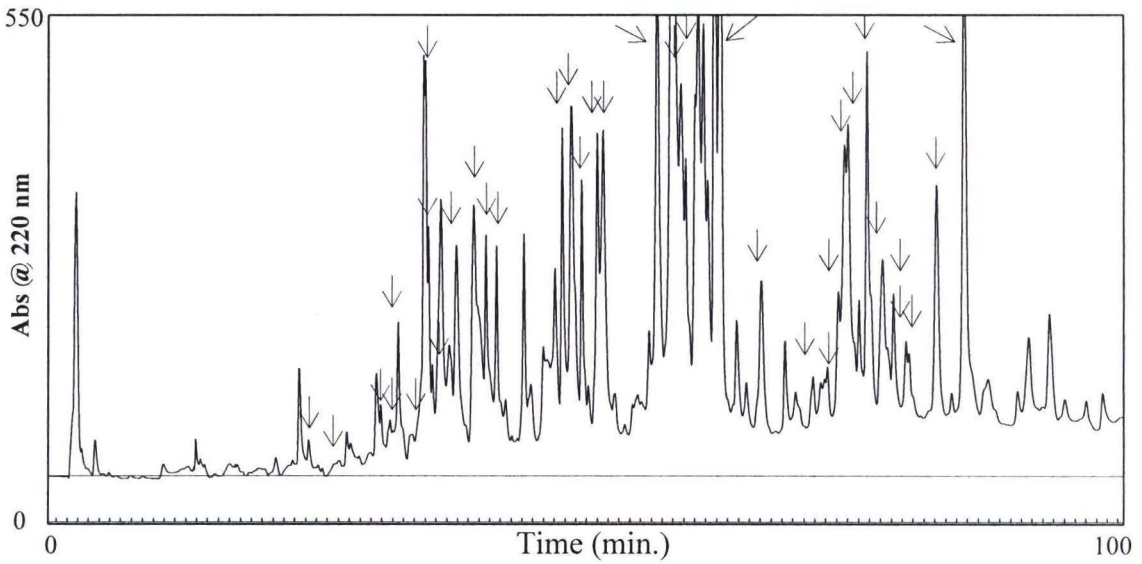

Figure 3.1a: Biologically Active Fractions of Sample dal_A.

The other portion of the sample was analyzed using low resolution MALDI-TOF mass spectrometry using the HP-2025A instrument to determine how many components were in each fraction. From the data, more than one fraction had several components with different molecular weights. The data of dal_A has been compiled and is shown in detail below.

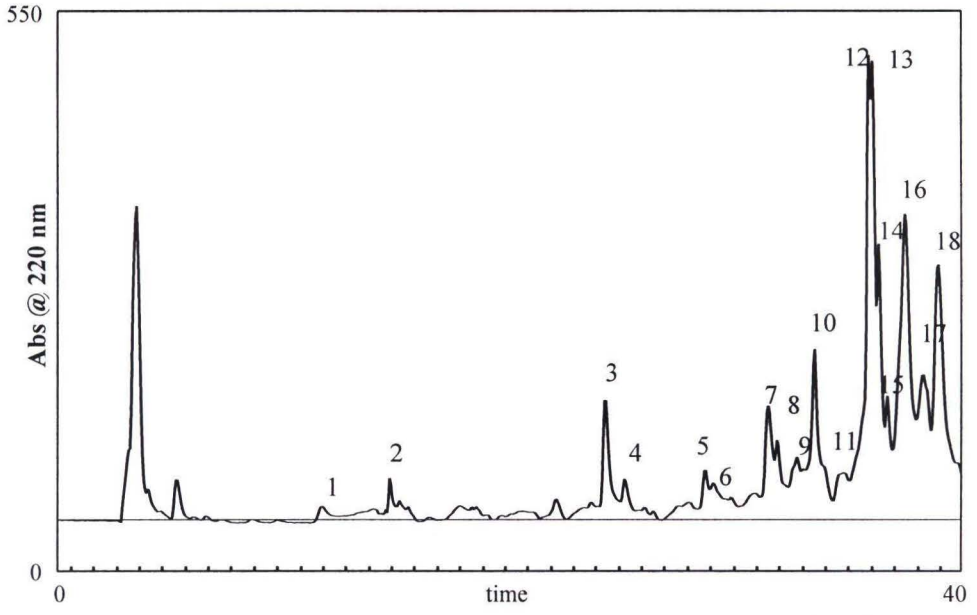

Figure 3.1b: Semi-preparative RP-HPLC of *Conus dalli* (dal_A) fractions 1-18.

Peak	Retention	MWt						
#	time	Da			# Cpds	Na	K	Ca - Fluo3
1	11.94							
2	14.87							
3	24.25	978.6	1279.1		2			
4	25.21					10.07	1.13	6.13
5	28.68	1319.2	1343.9		2			
6	29.12	1894.4			1			
7	31.35	1892.9			1			
8	32.10	1429.3	1874.3		2	9.43	2.07	5.77
9	32.55	1428.2	1874.1		2	7.13	0.97	5.17
10	33.24	1078.2	1118.0	2071.0	4	6.20	2.87	5.60
		3110.5						
11	34.50	1514.5	1953.5		2	8.93	3.40	5.27
12	35.51	1529.5	1636.2	1812.6	5			
		2899.5	4859.7					
13	36.05	1213.6	1235.0	1527.2	7.0	6.27	1.60	2.07
		1704.1	1811.1	2905.7				
		4888.5						
14	36.26	1230.7	2818.5	2881.6	4	9.63	2.93	6.20
		4870.7						
15	36.65	1211.7	1664.6	2800.4	5	4.68	1.27	6.07
		2867.0	4840.0					
16	37.05	1068.0	1118.5	1664.7	3			
17	38.15	1010.0	1666.0	1825.7	5	4.68	0.10	5.87
		2700.5	3008.0					
18	38.67	2614.8	3143.8		2	10.73	1.67	5.43

Table 3.1a: Retention times, Molecular weight (MALDI-TOF, Hewlett Packard), and Bioassay results for *Conus dalli* (dal_A) fractions 1-18.

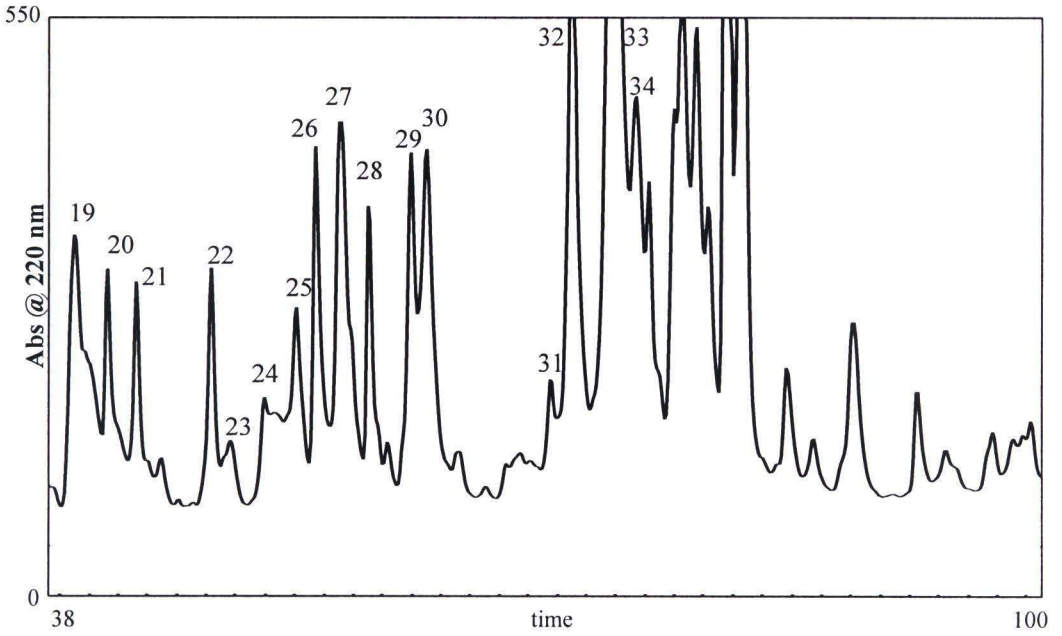

Figure 3.1c: Semi-preparative RP-HPLC of *Conus dalli* (dal_A) fractions 19-34.

Peak	Retention	MWt						
#	time	Da			# Cpds	Na	K	Ca - Fluo3
19	40.35	1175.3	1201.1	1445.9	8	5.43	5	4.23
		1743.3	2678.1	3166				
		3288.8	3744.2					
20	41.50	1429.3	1743.8	2666.5	4	13.00	-7.17	5.27
		3128.6						
21	42.45	3288.2				10.57	-6.93	5.03
22	45.00	1632.0	2186.3	3307.1	4	9.67	-5.3	5.43
		3823.1						
23	45.77	1305.8	1404.6	1803.7	4			
		3258.5						
24	46.85	1318.7	1999.1	2522.2	6			
		2903.5	3506.1	4008.0				
25	47.91	1343.8	2131.4	4016.4	3			
26	48.50	1343.7	2106.4	4026.9	5			
		7696.1	8589.9					
27	49.35	1787.3	8491.2		2	11.33	-7.00	6.57
28	50.39	1752.5	1861.2	8606.6	3	10.83	-3.70	4.80
29	51.67	1399.9	2050.9	7940.9	3	11.43	-6.10	5.33
30	52.26	1487.7	2081.0	2268.1	5	7.60	-6.40	6.87
		3323.0	7401.7					
31	56.81	2024.2	2439.3	2612.5	7			
		2939.8	4628.9	5345.1				
		9471.1						
32	57.39	3136.2	3412.5		2	3.27	-0.17	6.30
33	58.61	2934.4	3244.4	3644.9	4			
		4488.9						
34	58.98	2927.3	3284.9	3632.2	4	5.37	-7.53	6.23

Table 3.1b: Retention times, Molecular weight (MALDI-TOF, Hewlett Packard), and Bioassay results for *Conus dalli* (dal_A) fractions 19-34.

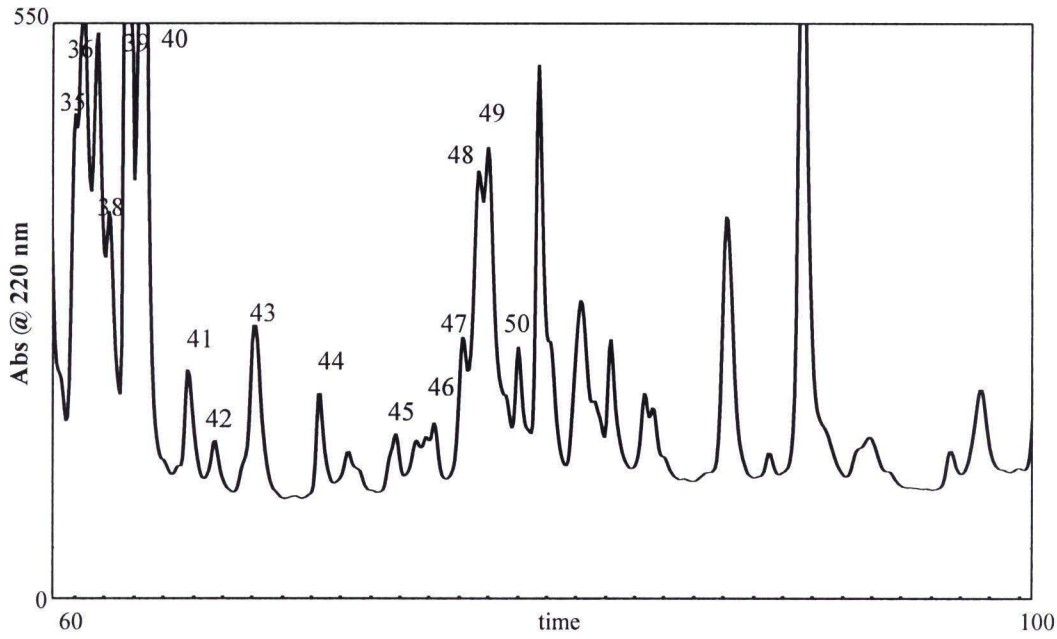

Figure 3.1d: Semi-preparative RP-HPLC of *Conus dalli* (dal_A) fractions 35-50.

Peak	Retention	MWt						
#	time	Da			# Cpds	Na	K	Ca - Fluo3
35	60.91	1480.9	1820	2659.4	6	6.1	-5.17	6.07
		3278.4	4489.6	5494.2				
36	61.21	1453.3	1793.6	3251.9	5	5	-3.43	3.17
		4471.7	5493.7					
37	61.7	3649.1	4479.8	5522.7	4			
		8652.8						
38	62.59	3187.1	6452.4	9655.1	4			
		12843						
39	63.1	3182.2	6517.7	9738.7	4	3.19	2.97	3.2
		12970.2						
40	64.71	2926.4				4.8	-4.27	3.67
41	65.24	2026.9	2767.5	3088.8	4			
		14207.9						
42	66.59	3050.7	3557.3	6224.5	3	6.83	1.57	3.8
43	69.12	3195.4	3882.4		2			
44	70.08	3123.8	3419.1	3859.8	4			
		4880.6						
45	71.6	2026.3	2751.4	3425.9	6	5.03	-1.07	2.97
		3718.6	4077.9	4973				
46	72.4	1703.1	2750.2	3400.5	6	5.93	-1.13	3.1
		3948.1	4918.6	5453.5				
47	73.92	2602.5	3501.3	3950.9	6		-8.13	3.13
		4854.1	4981.8	9919.8				
48	74.4	3493.2	4971.2		2	8.43	-3.1	3.6
49	74.9	1746.2	3214.3	3497.4	4	7.47	-1.3	3.83
		4846.9						
50	75.9	2558.3	2762.1	3057.7	7	7.27	-3.63	3.63
		3645	4037.1	4935.3				
		5133						

Table 3.1c: Retention times, Molecular weight (MALDI-TOF, Hewlett Packard), and Bioassay results for *Conus dalli* (dal_A) fractions 34-50.

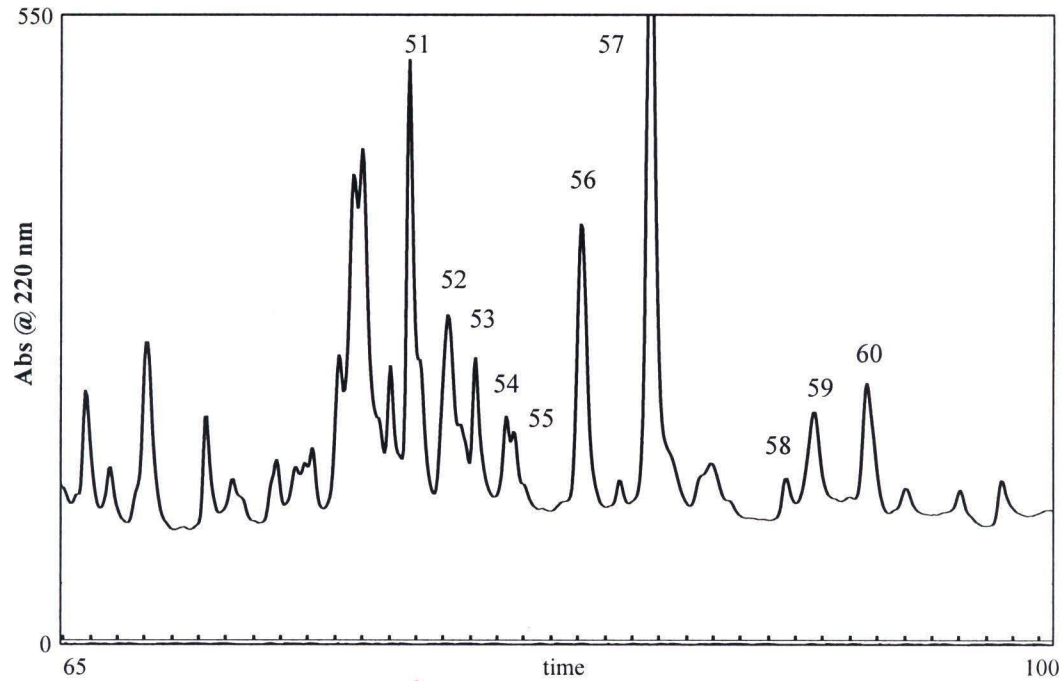

Figure 3.1e: Semi-preparative RP-HPLC of *Conus dalli* (dal_A) fractions 51-60.

Peak	Retention	MWt						
#	time	Da			# Cpds	Na	K	Ca - Fluo3
51	76.55	3016.9	4906.5		2	7.37	-1.47	2.33
52	77.84	727.1	2671.5	4742.7	3	6.3	-2.17	1.77
53	79.06	4957.4	9997.8		2	4.73	-8.7	3.77
54	80.19	2723.6			1	11.8	-8.33	5.1
55	80.65	2631.7	2663.3		2	7.77	-7.17	3.83
56	82.86	2750.1	3052.1	3649.8	3	8	-3.93	4.77
57	85.4	3062.2			1	10.3		
58	90.65	2957.2	3616.1		2			
59	91.32	2956.6	3644.1	4343.2	3			
60	93.34	2776.8	3638.1	4308.4	3			

Table 3.1d; Retention times, Molecular weight (MALDI-TOF, Hewlett Packard), and Bioassay results for *Conus dalli* (dal_A) fractions 48-63.

After completing the Semi-preparative RP-HPLC analysis of the venom of *Conus dalli,* the data obtained made it clear that further separation techniques would be required. The previous data showed that *Conus dalli* venom contained compounds of varying molecular weights (14208 kDa to 979 Da), which indicated that the venom of *Conus dalli* is complex. Many of the components present in the venom of *Conus dalli* have very high molecular weights, which are not suitable for this project.

Components with molecular weights below 3000 Da, with considerable concentration not lower than 30 pmol, are more suitable for this project since they are easier to characterize by different techniques like NMR, MALDI-TOF, and Q-TOF MALDI MS/MS. Therefore, Size Exclusion chromatography was adopted, because it separated components of the venom on the basis of the molecular weight and size. The sample was run through the Size Exclusion Chromatography Column at the following wavelengths: 220 nm (for presence of amino acids), 280 nm (for aromatic amino acids present in most conopeptides), and 250 nm (for the presence of cysteine bridges). The data for *Conus dalli* is shown below (Figure 3.2).

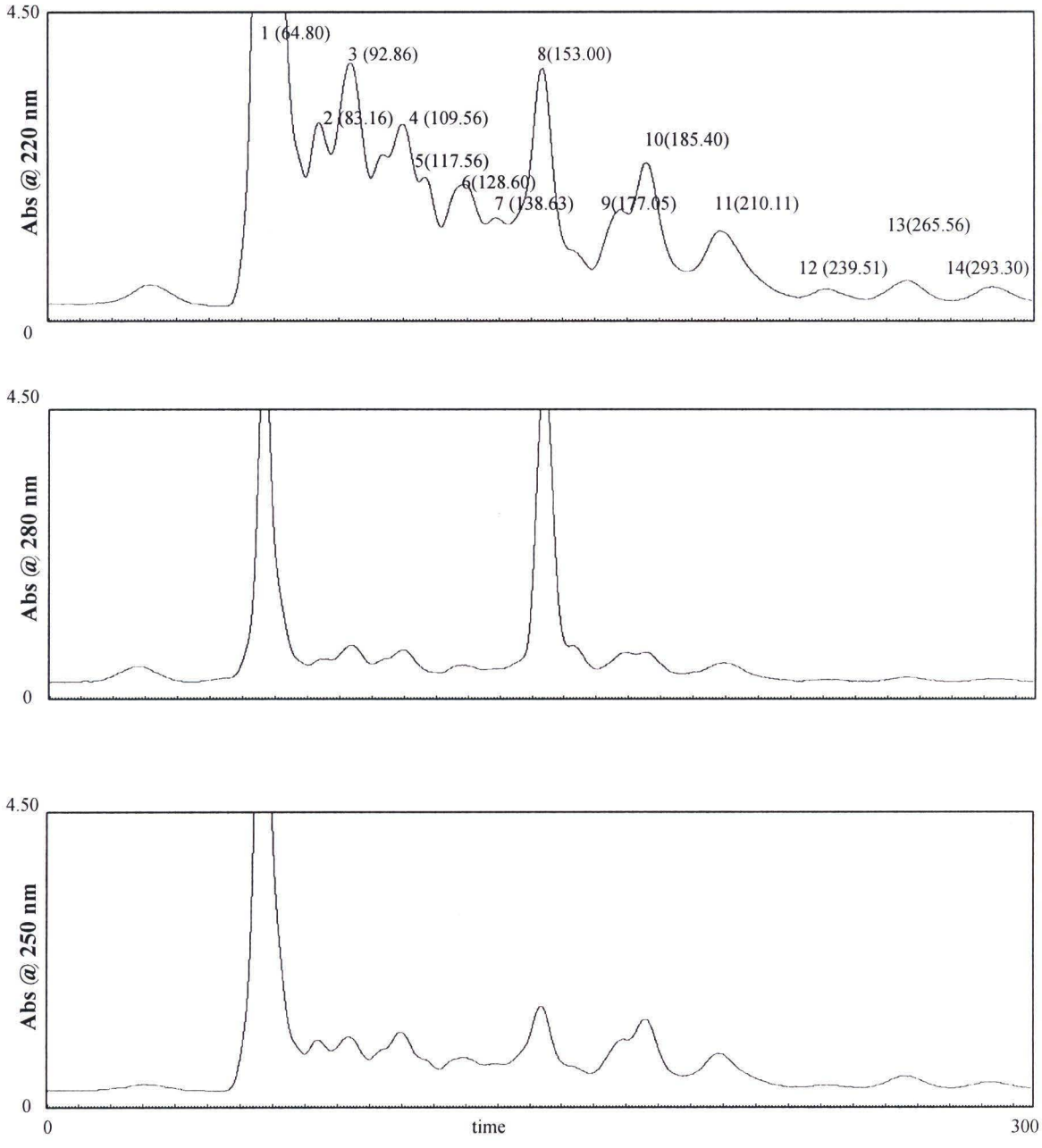

Figure 3.2: Size Exclusion chromatograms of dal_C at wavelengths 220nm, 280nm, and 250nm.

The size exclusion chromatogram of dal_C at λ = 280 nm was compared with a published Sephadex G-50 chromatogram of *Conus textile* at the same wave length (41,42). A height similarity was observed in the two chromatograms, which suggests that there is a resemblance between the two types of snail venom (Figure 3.3).

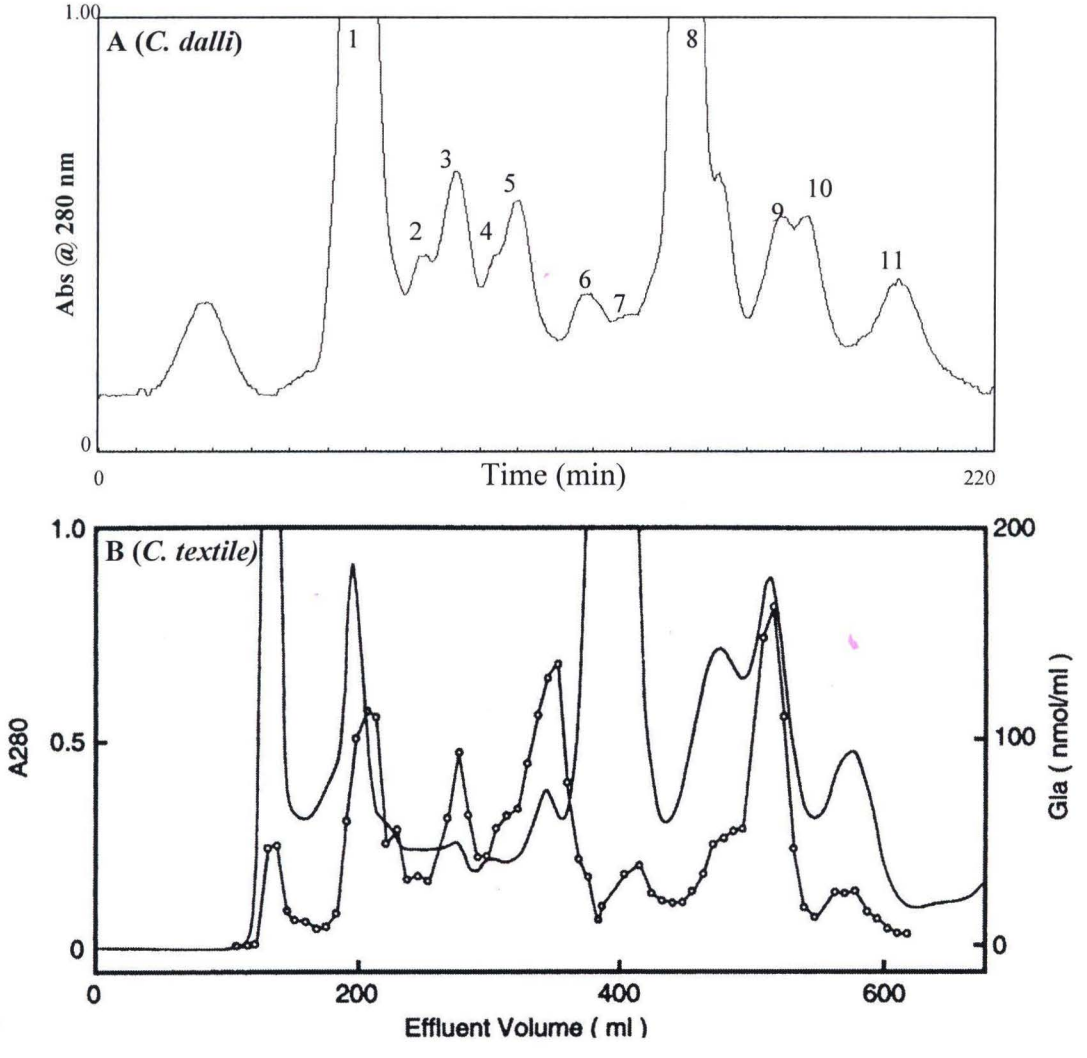

Figure 3.3: A) Size Exclusion Chromatograms of *Conus dalli* at λ = 280nm.

B) Profile of *Conus textile* venom extract Chromatographed on a Sephadex G-50 column. Where solid and circle line represent the absorbance of Gla-derivative (41, 42).

Each isolated fraction from the SEC-fractions was run on the Semi-Preparative Reversed Phase HPLC (RP-HPLC), using the linear gradient described earlier. Each fraction from the RP-HPLC was then subjected to MALDI-TOF mass spectrometry analysis. This method is considered a very useful tool for determining the molecular weight of each component and the purity of the fractions. If the RP-HPLC fractions needed further purification, the Analytical Reversed Phase HPLC was used for this purpose.

Each of the following figures will reflect the information below:

A: Separating crude venom using SEC-chromatography.

B: Semi-Preparative Reversed Phase HPLC for marked fraction from part a.

BI: Analytical Reversed Phase HPLC for fraction isolated from RP-HPLC for more purification if needed.

C: MALDI-TOF mass spectrometry analysis was done on each fraction from RP-HPLC (**B** or **BI**)

D: Q-TOF MALDI MS/MS was used for each isolated fraction to:

i. Determine the presence of disulfide bridges, which means the reduction alkylation process, must be applied before sequencing.

ii. Confirm the sequence obtained by other methods such as Edman degradation.

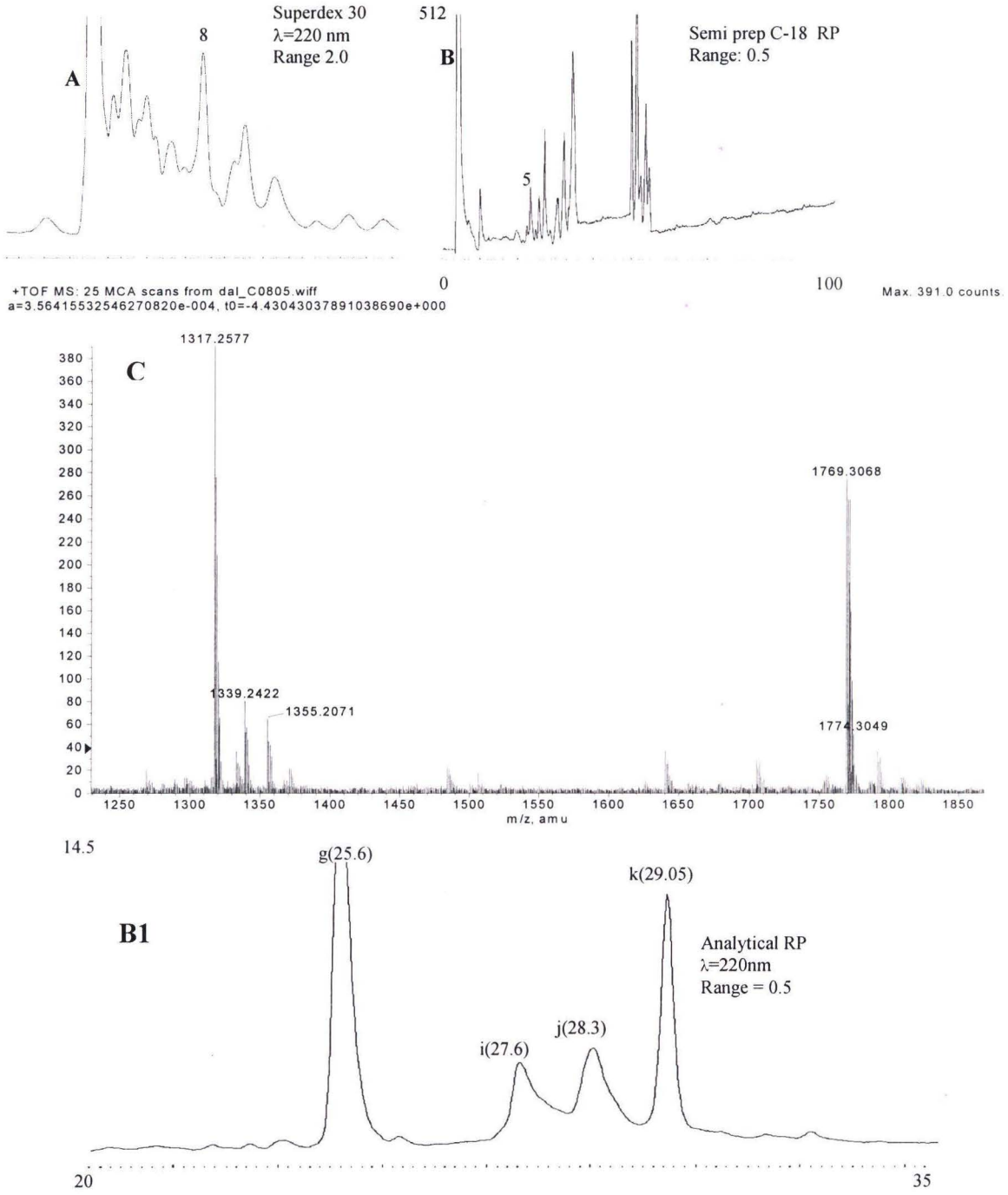

Figure 3.4: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 8. C) MALDI-TOF for fraction dal_C0805. BI) Analytical RP-HPLC for dal_C8005 (λ=220 nm).

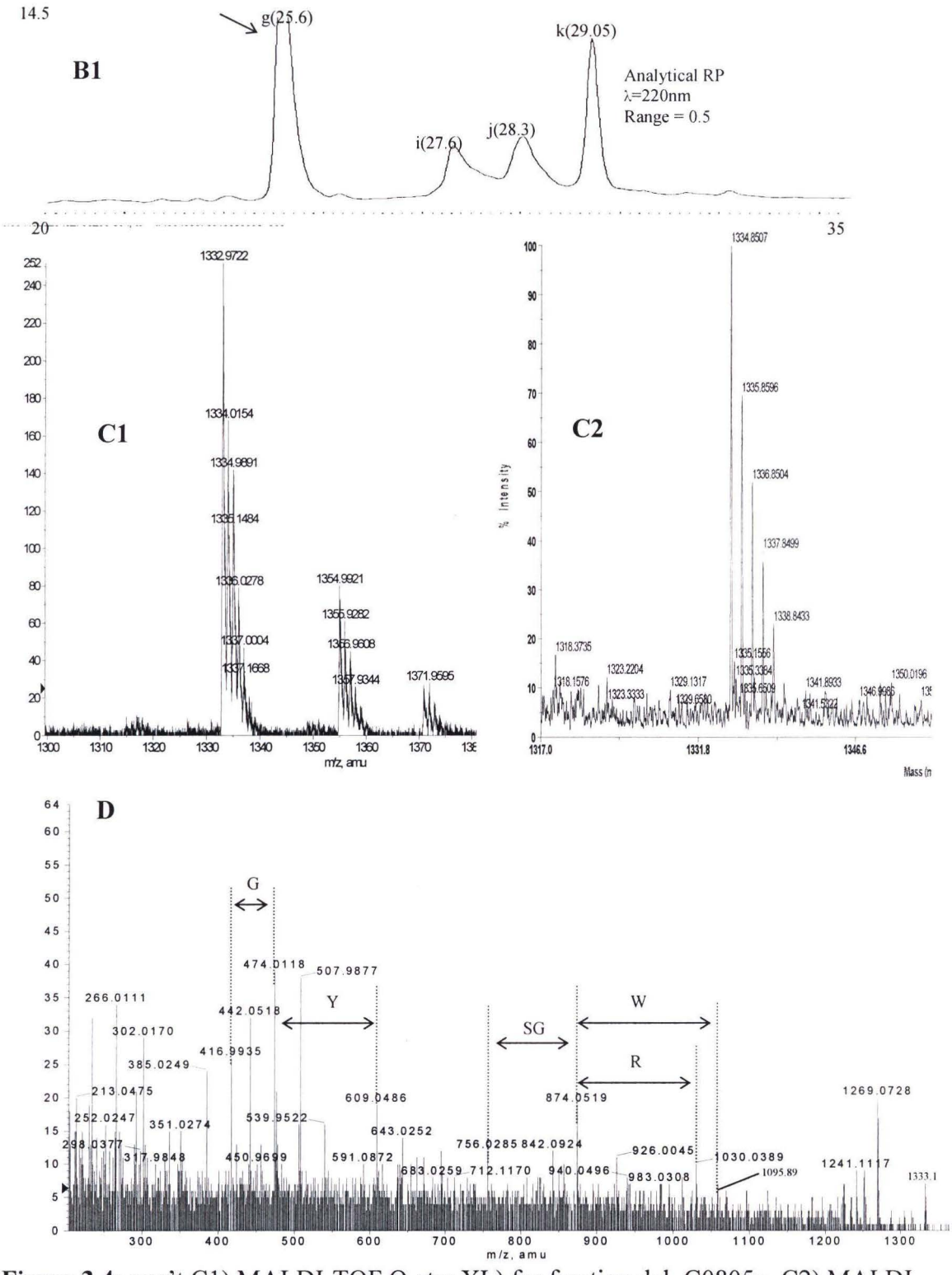

Figure 3.4: con't C1) MALDI-TOF Q-star XL) for fraction dal_C0805g. C2) MALDI-TOF (Voyager) for fraction dal_C0805g. D) Q-TOF MALDI MS/MS

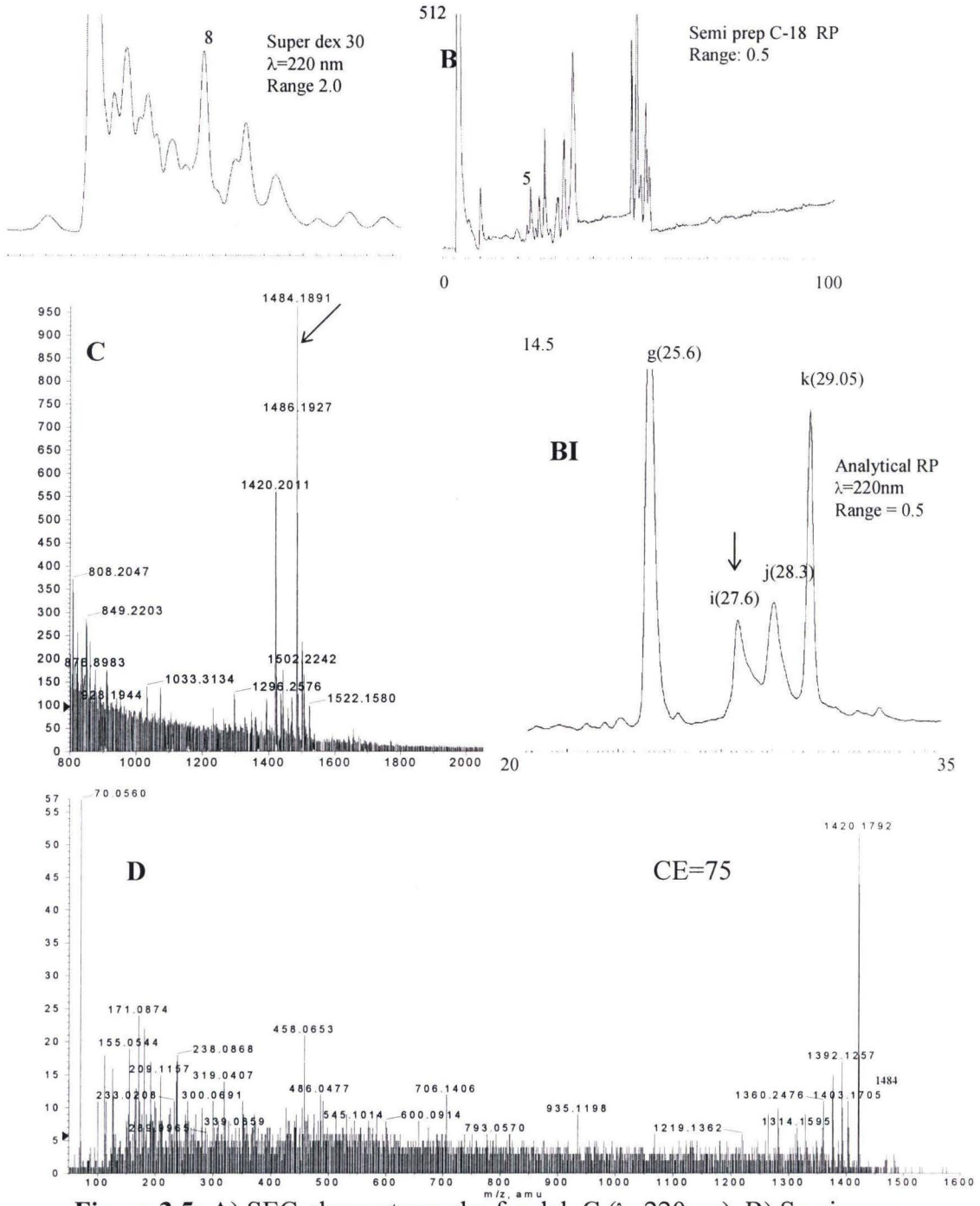

Figure 3.5: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 8. BI) Analytical RP-HPLC for dal_C0805 (λ=220 nm). C) MALDI-TOF for fraction dal_C0805i. D) Q-TOF MALDI MS/MS for dal_C0805i.

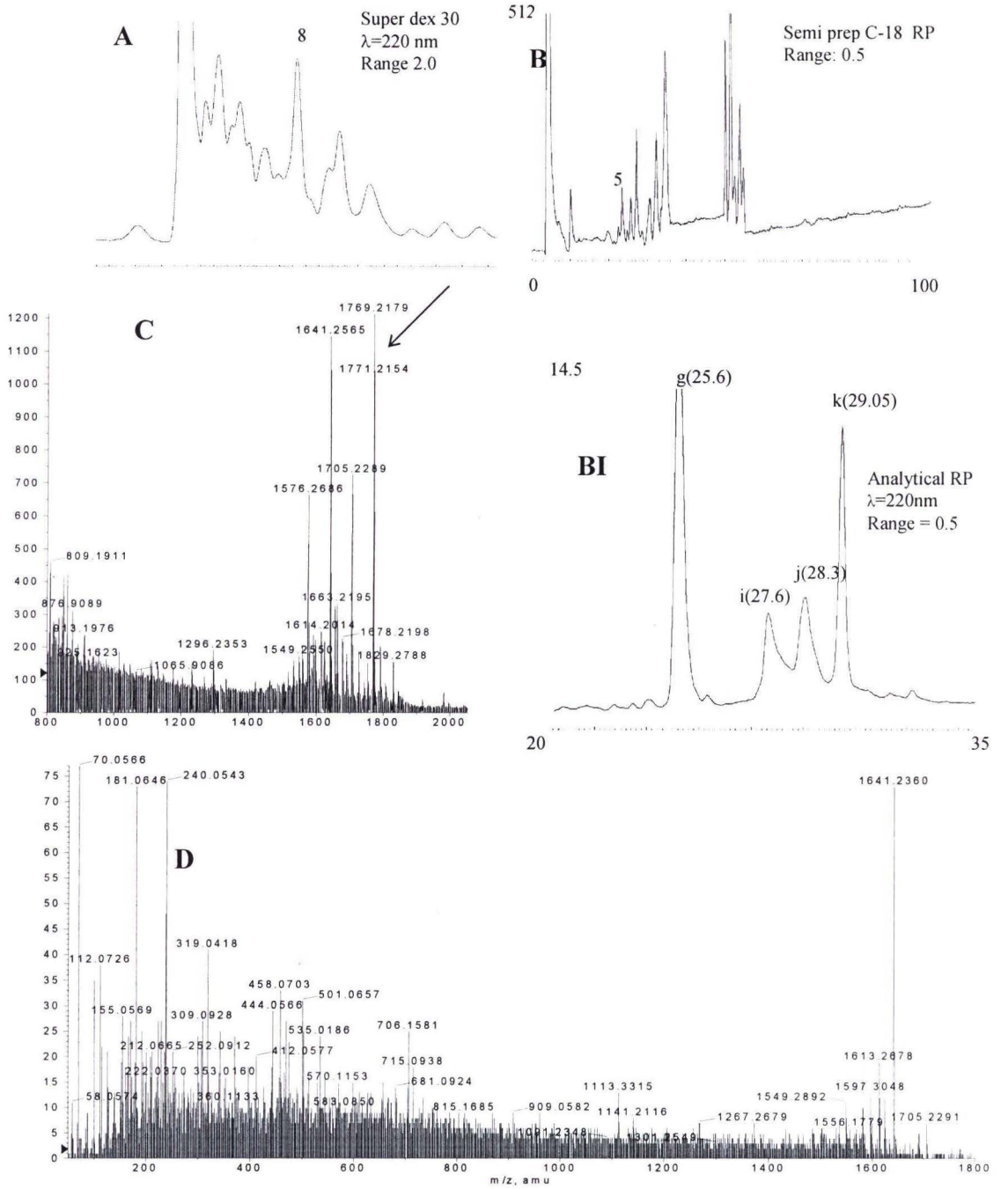

Figure 3.6: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 8. BI) Analytical RP-HPLC for dal_C0805 (λ=220 nm). C) MALDI-TOF for fraction dal_C8005j. D) Q-TOF MALDI MS/MS for dal_C0805j.

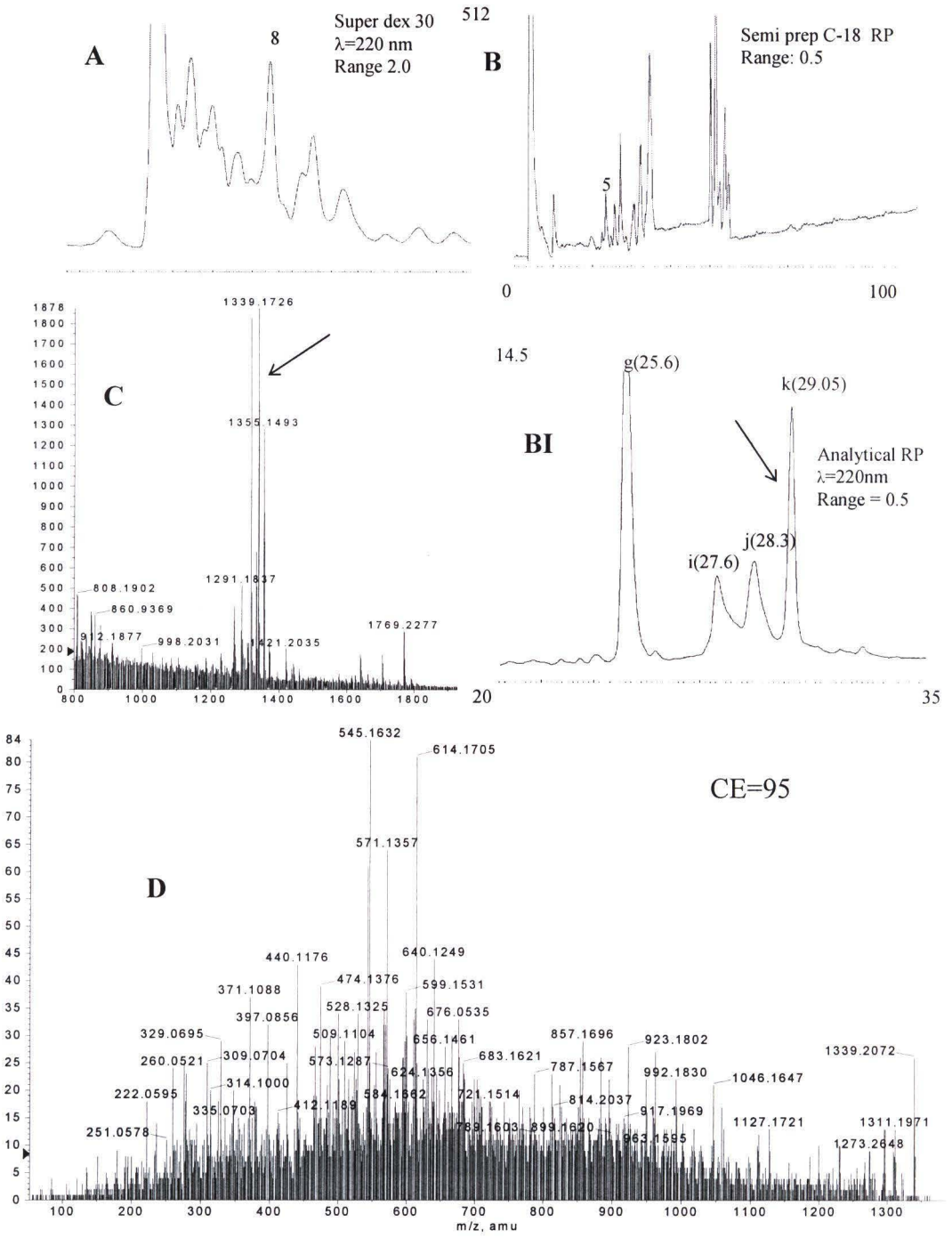

Figure 3.7: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 8. BI) Analytical RP-HPLC for dal_C0805 (λ=220 nm). C) MALDI-TOF for fraction dal_C8005k. D) Q-TOF MALDI MS/MS for dal_C0805k.

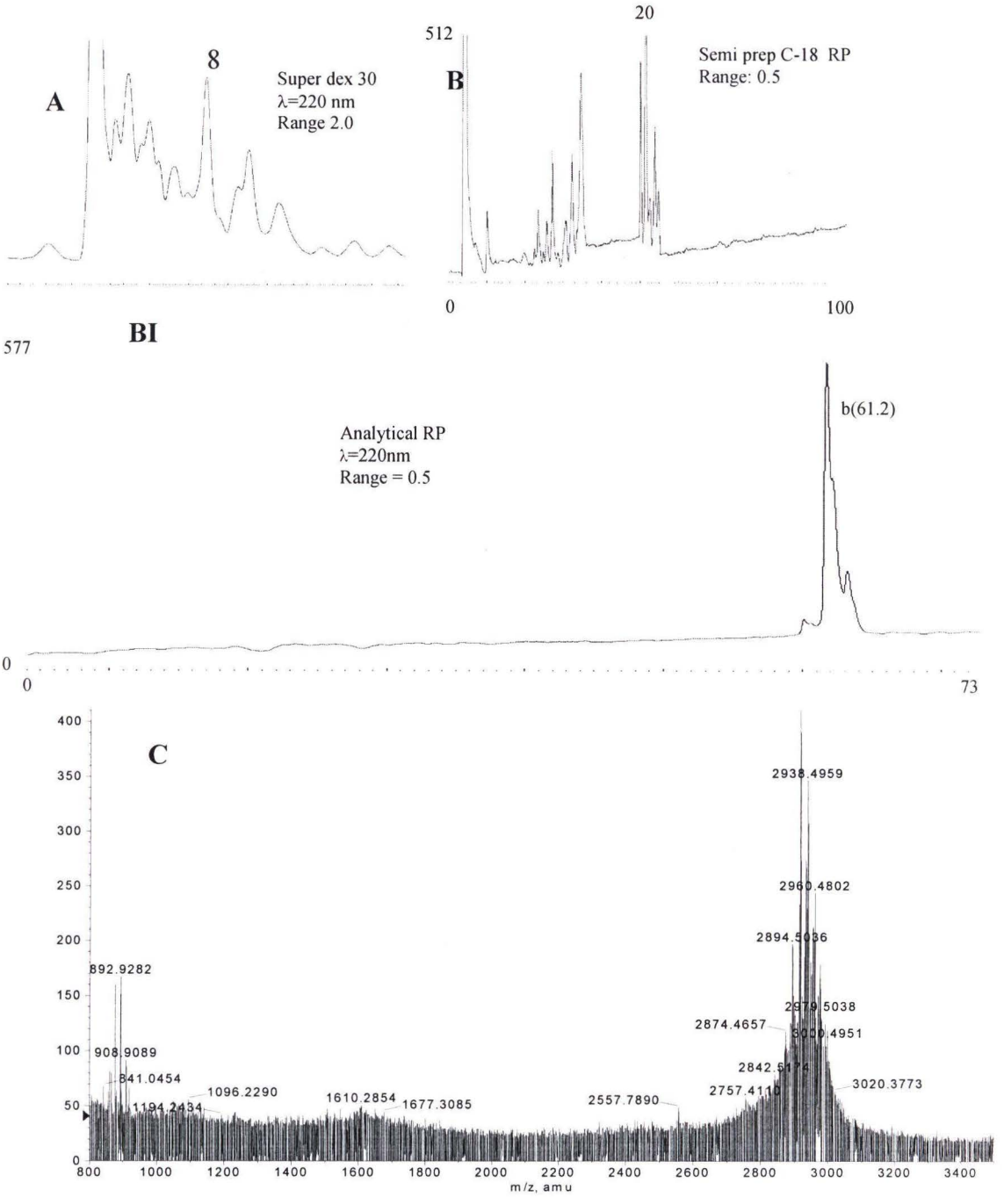

Figure 3.8: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 8. BI) Analytical RP-HPLC for dal_C0820 (λ=220 nm). C) MALDI-TOF for fraction dal_C0820b.

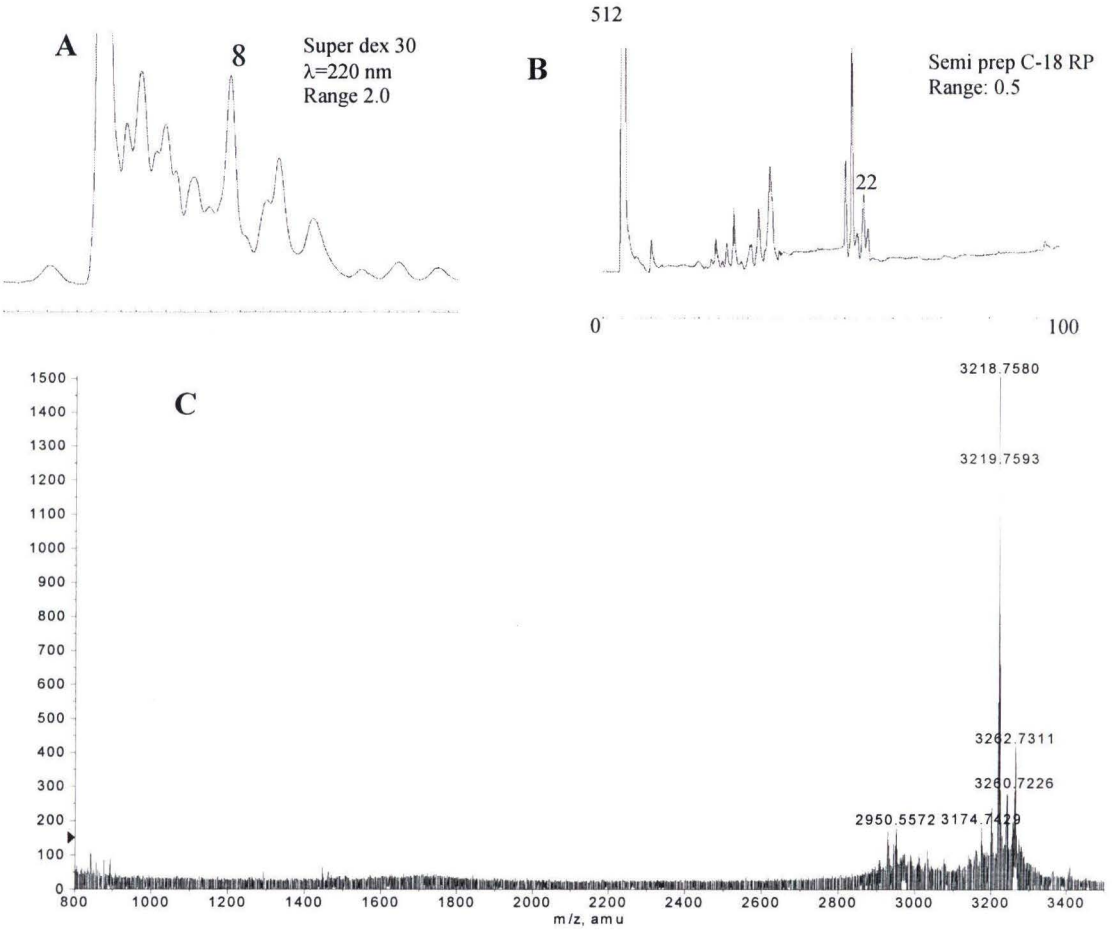

Figure 3.9: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 8. C) MALDI-TOF for fraction dal_C0820.

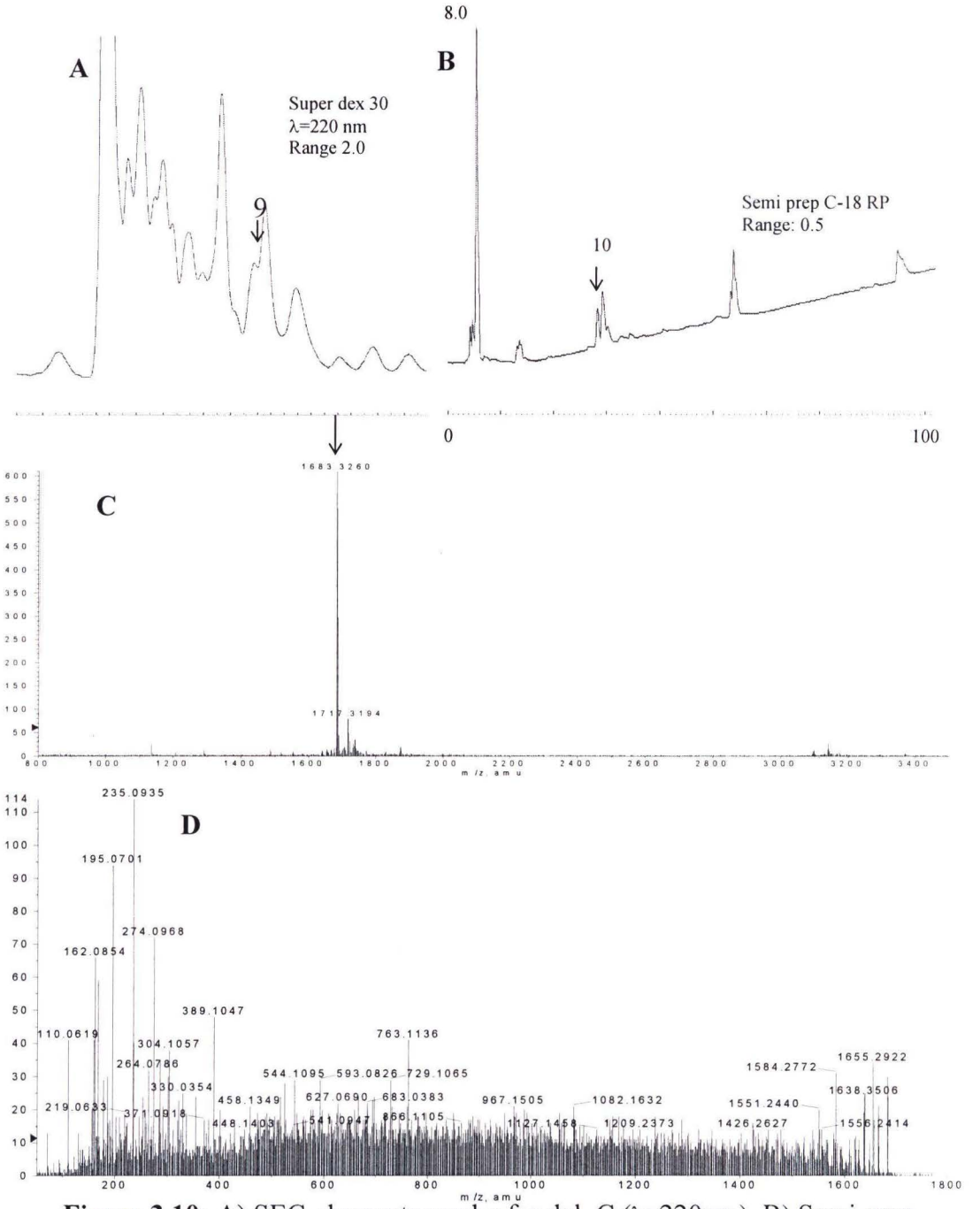

Figure 3.10: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 9. C) MALDI-TOF for fraction dal_C0910. D) Q-TOF MALDI MS/MS for dal_C0910.

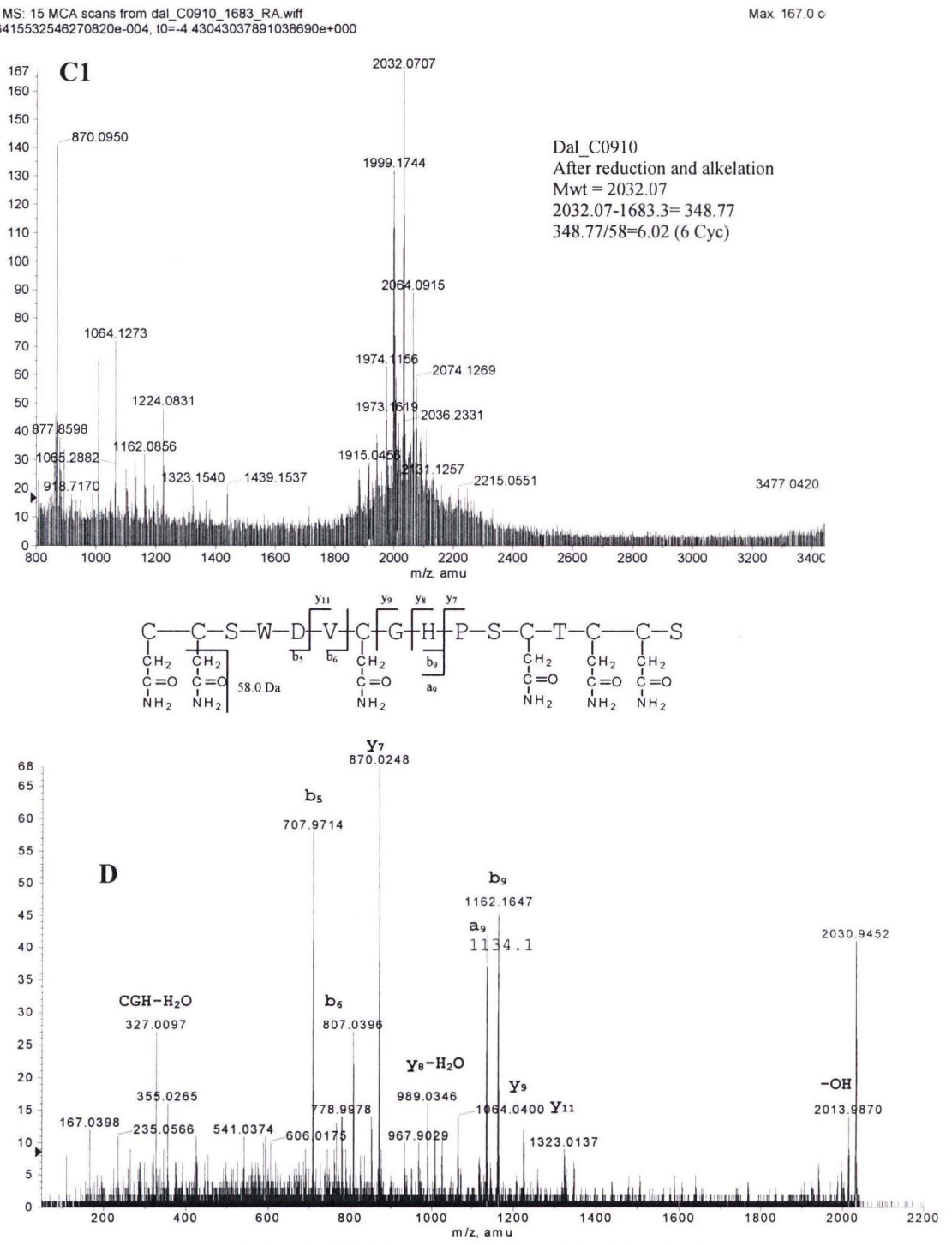

Figure 3.10: con't C) MALDI-TOF for fraction dal_C0910 after reduction and alkylation. D) Q-TOF MALDI MS/MS for dal_C0910 after reduction and alkylation.

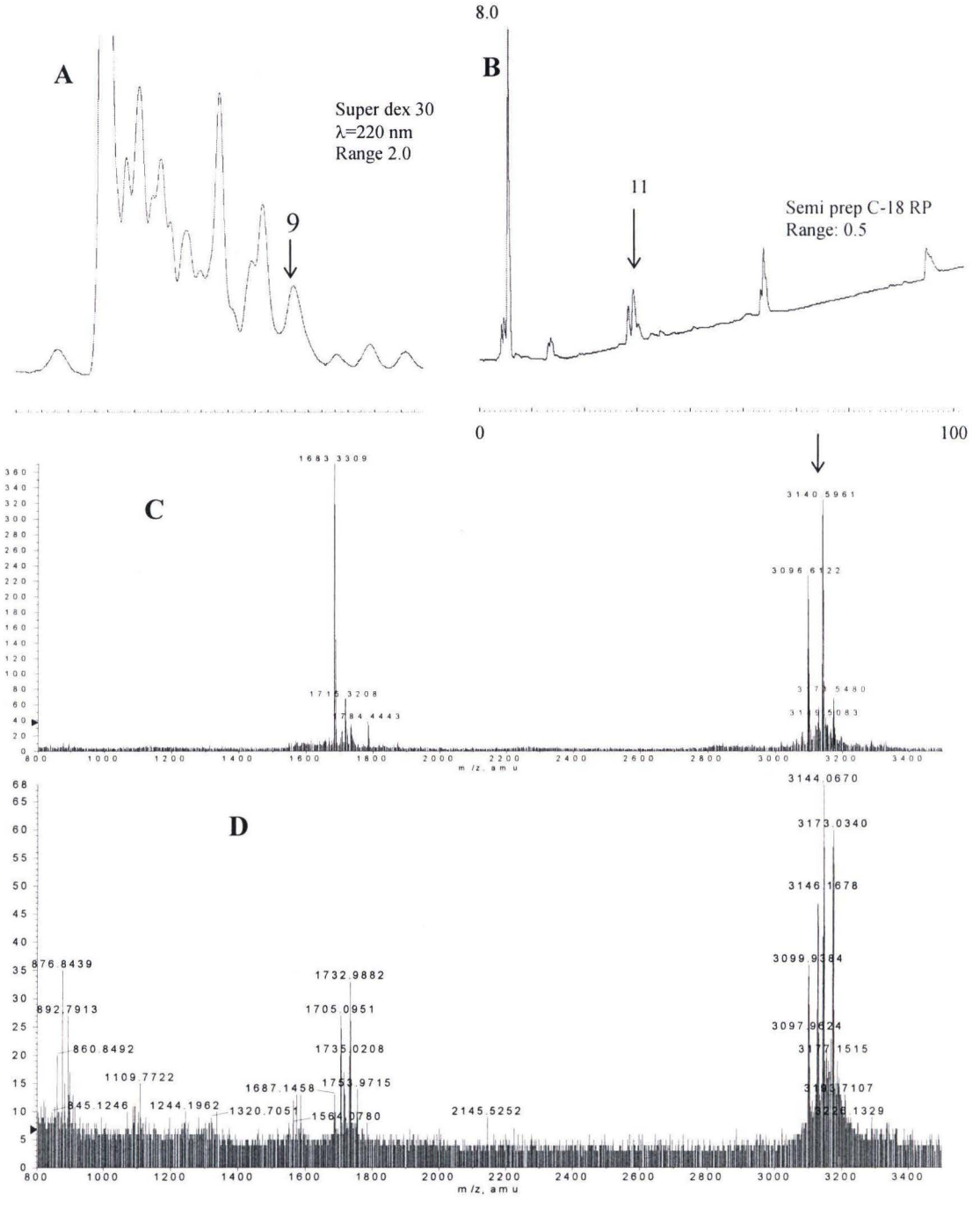

Figure 3.11: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 9. C) MALDI-TOF for fraction dal_C0911. D) Q-TOF MALDI MS/MS for dal_C0911.

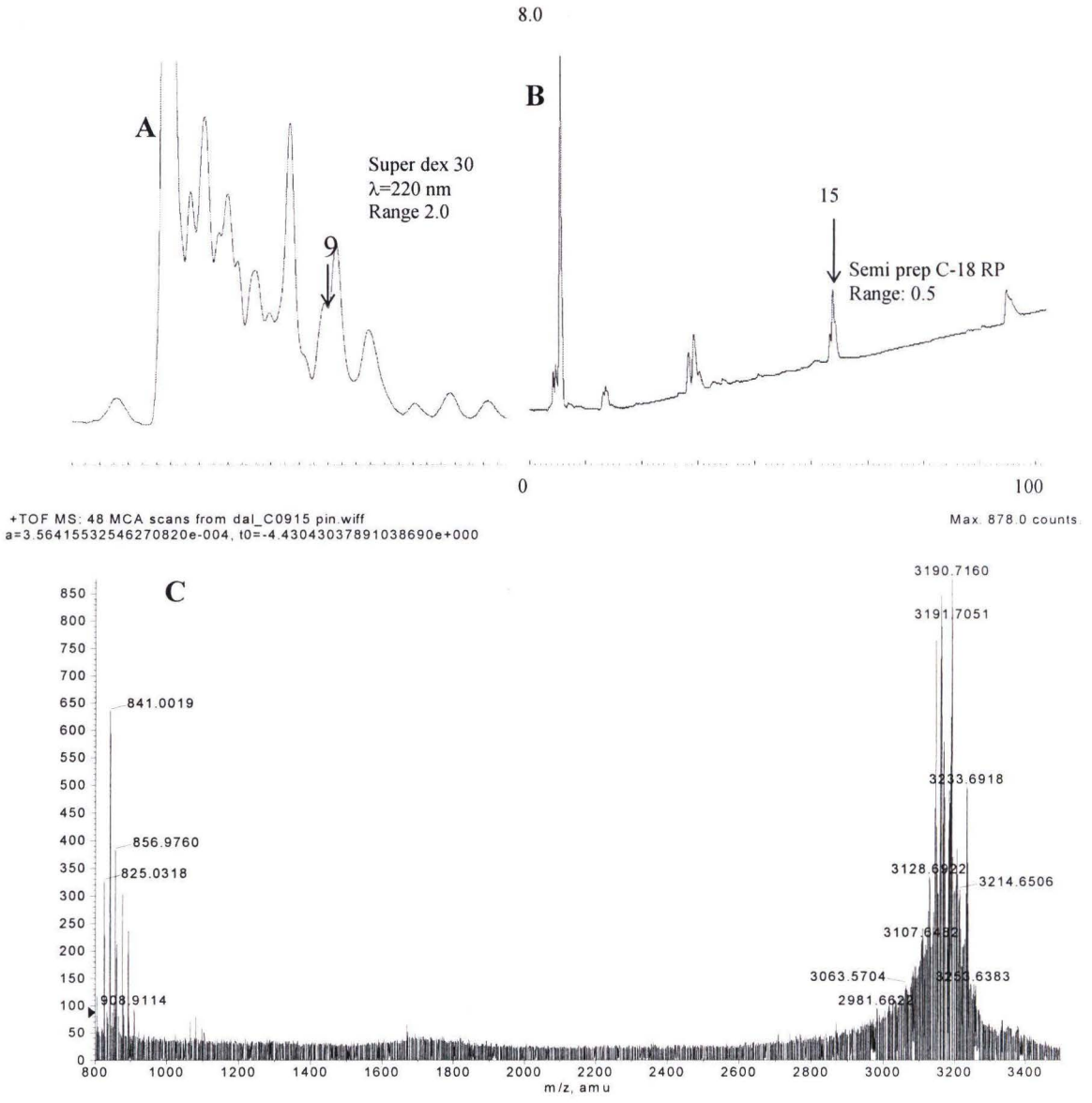

Figure 3.12: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 9. C) MALDI-TOF for fraction dal_C0915.

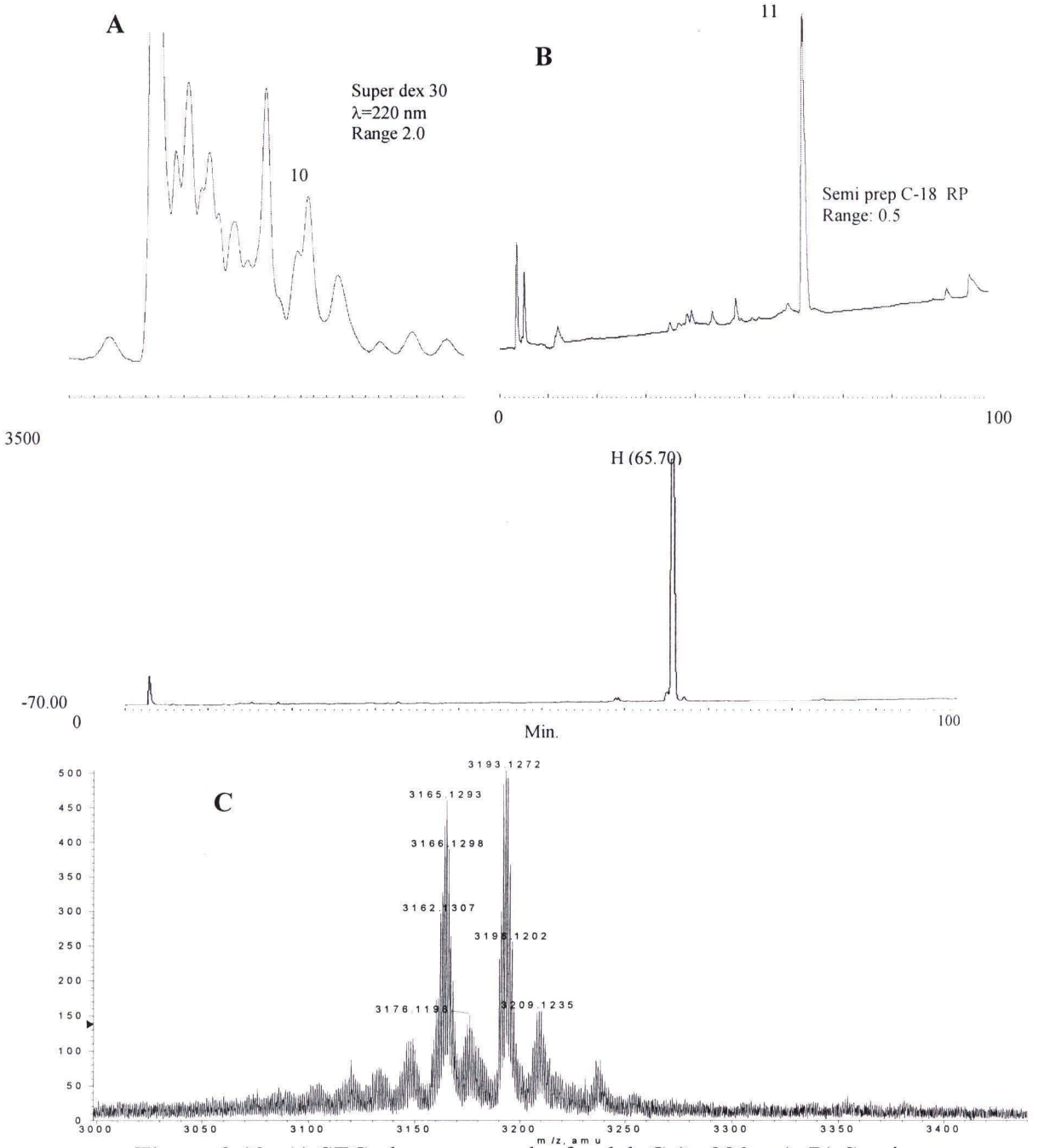

Figure 3.13: A) SEC-chromatography for dal_C (λ=220nm). B) Semi-prep RP-HPLC for fraction 10. BI) Analytical RP-HPLC for dal_C1011 (λ=220 nm). C) MALDI-TOF for fraction dal_C1011h.

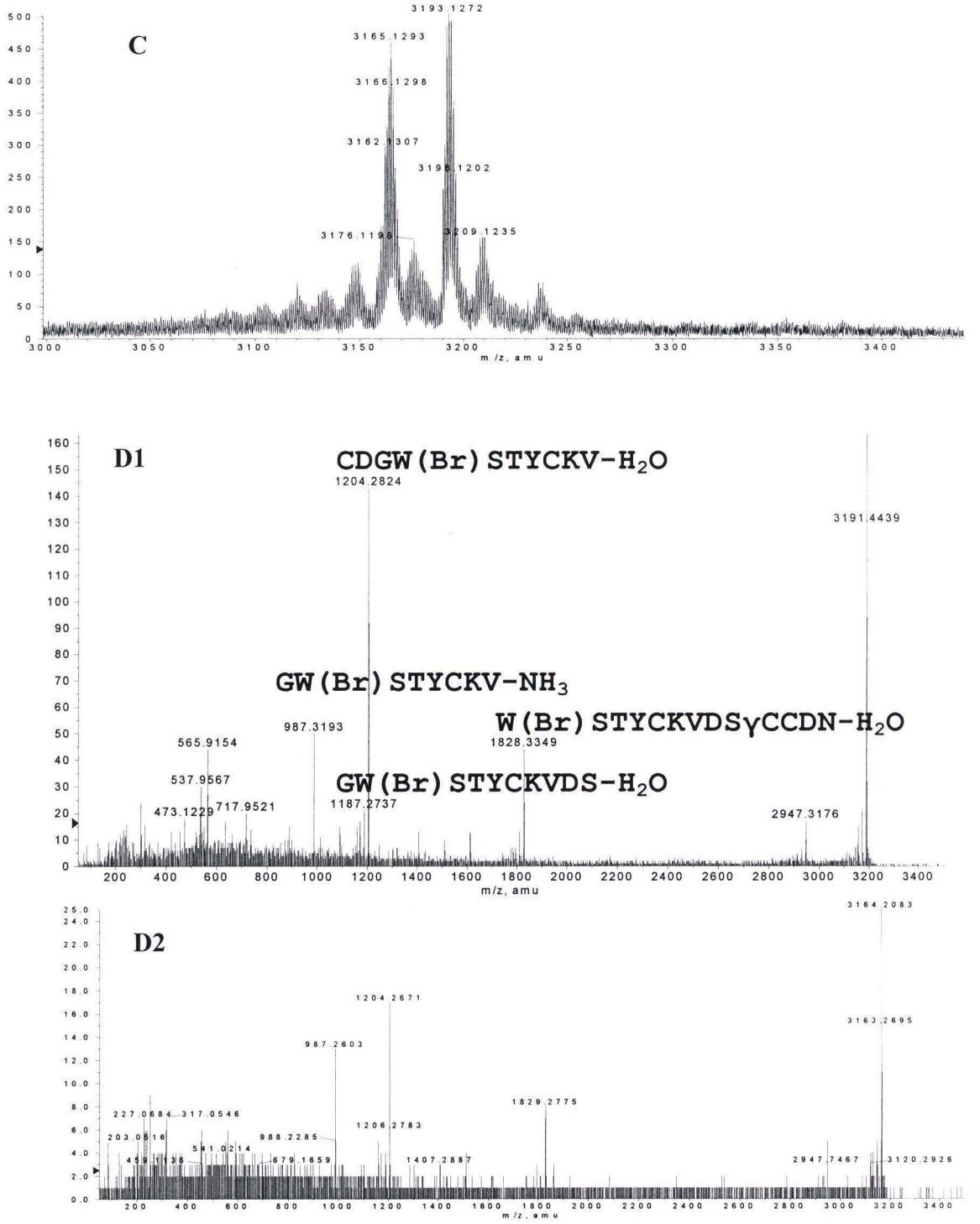

Figure 3.13: con't C) MALDI-TOF for fraction dal_C1011h. D1) Q-TOF MALDI MS/MS for dal_C1011h, molecular weight = 3190.4 Da. D2) Q-TOF MALDI MS/MS for dal_C1011h, molecular weight = 3164.2 Da.

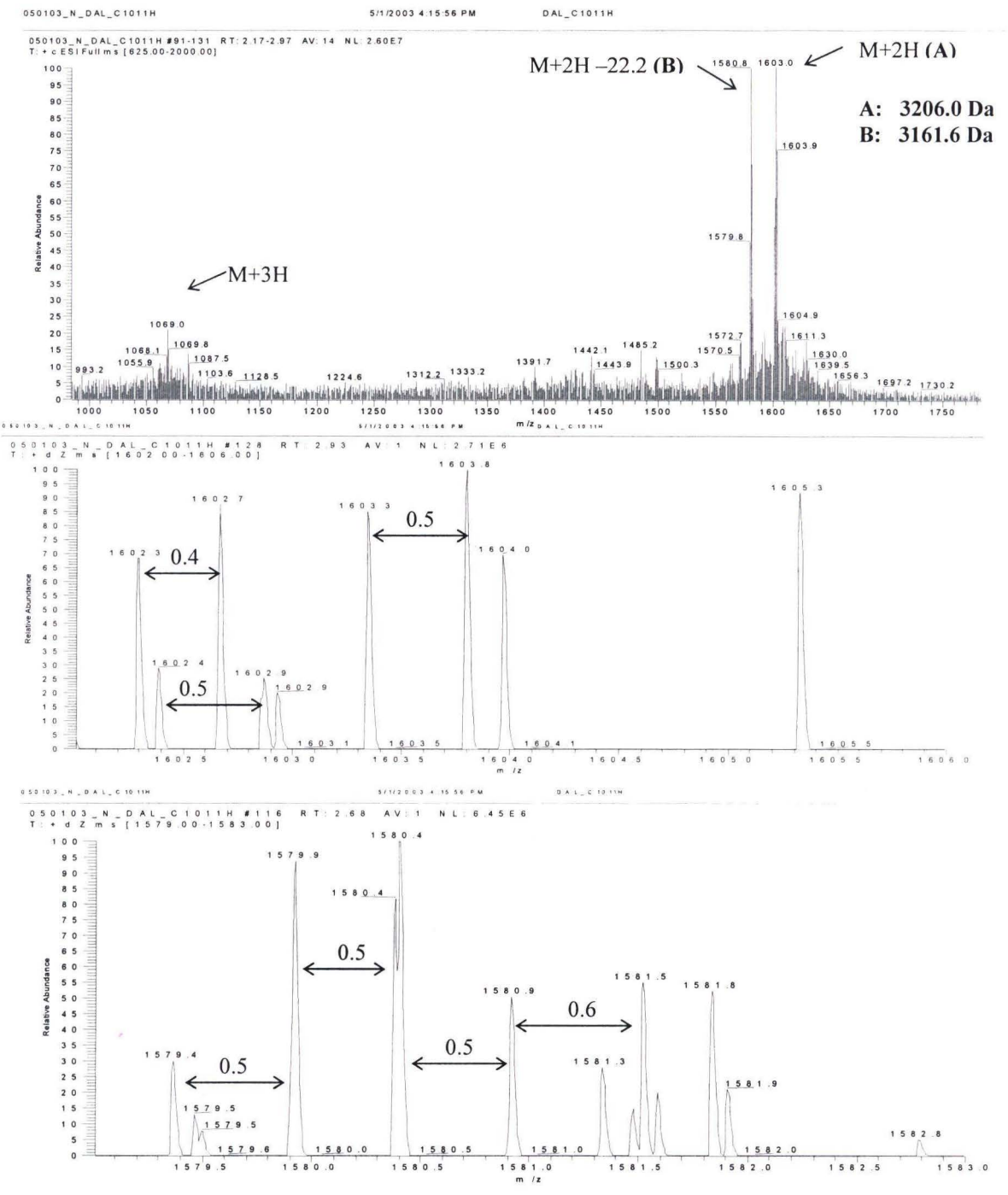

Figure 3.14: ESI-MS spectrum of dal_C1011h, Doubly and triply protonated peptide molecule were observed in the displayed mass range and are marked as [M+2] and [M+3] respectively, where **A** and **B**. Observed average molecular masses (based on the mass of doubly and triply charged ions) for the naturals main component (designated as **A**) is shown in the upper right of the spectrum along with masses

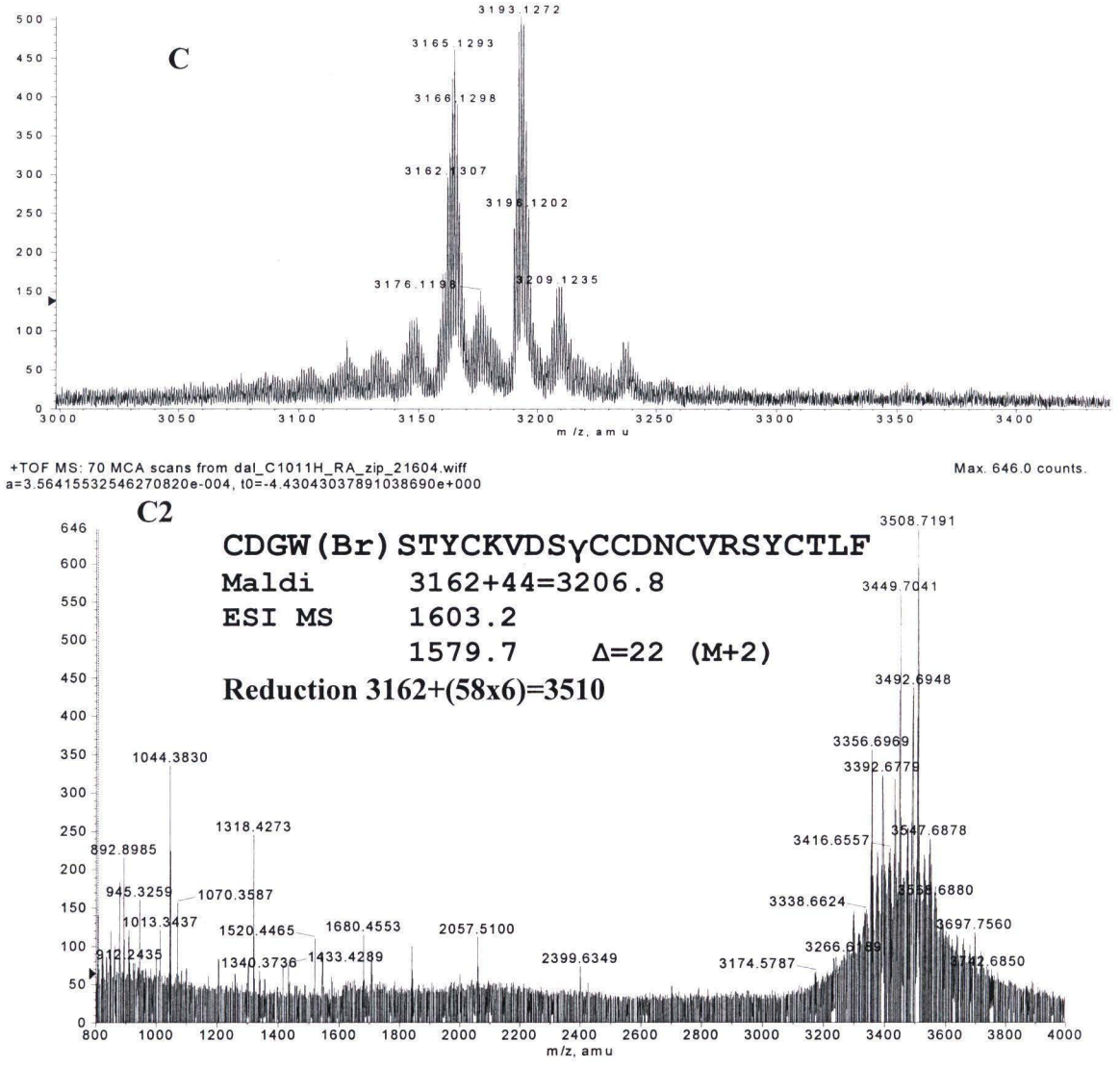

Figure 3.15: C) MALDI-TOF for fraction dal_C1011h. C2) MALDI-TOF for fraction dal_C1011h after reduction and alkylation; the increments of molecular weight show a difference of 348 Da.

Fraction 8, with a retention time of 153.0 min., was separated by size exclusion chromatography using the semi-prep RP-HPLC. A linear gradient was used, as described earlier. A total of 25 fractions were isolated, most of which were screened using MALDI-TOF mass spectrometry. A high variety of molecular weights were found, ranging from 1317 to 3218.7 Da. Analytical RP-HPLC was then applied to many of those fractions. Fraction 0805, with a retention time of 27.0 min., showed a very clear separation with four fractions: g, i, j, and k (Figure 3.4 B1). The molecular weights for fraction g is 1332.98 Da, fraction i is 1420.2 Da, 1486.2 Da (Figure 3.5), fraction j is 1576.3 Da, 1641.26 Da, 1705.2 Da, 1769.18 Da (Figure 3.6) and fraction k is1291.18 Da, 1339.17 Da, 1769 Da (Figure 3.7). Fractions i, j and k show multiple components. The concentration of the single-component fraction g is 60 pmol (Figure 3.16), where the other fractions "i, j, and k" were too low (less than 10 pmol), which make fraction g suitable for more analysis. (Figure 3.4 C1 and C2). Q-TOF MALDI MS/MS data for the 0805g fraction shows many fragments, which leads us to conclude that this fraction is linear, and hence no reduction/alkylation is needed for this fraction. 50 pmol of sample were sequenced by using Procise™ 491A (*Applied Biosystems Foster City, CA*) amino acid sequencer and the result is shown in Table 3.2. Sequence was not completed and ended with at least 3 unknown amino acids in position 2, 12 and 13. The calculated molecular weight of the sequenced peptide is 1033.5 Da. The difference between calculated value and the molecular weight determined by MALDI-TOF is 299.4 Da, also due to high signal to noise ratio (S/N) from data obtained from Q-TOF MALDI MS/MS it was difficult to determine the final sequence. 1D NMR shows a singlet peak at a chemical shifted 7.92 ppm, which is typical of amidated hydrogen. Amidated hydrogens

are present in Asparagine and Glutamine, which they are not present in dal_C0805g. This give strong evidence that dal_C0805g is amidated at the C-terminus.

We suggest that dal_C0805g belongs to the RFamide family because of the presence of glycine, proline, methionine and arginine in positions 4, 5, 7 and 10 which match the same pattern of conorfamide-Sr1 in position number 1, 2, 3, and 10 (Table 3.2). Also the amidated C-terminus of dal_C0805g is one of main characteristics of the conorfamide family.

Conus	Fractions	Sequence
C.dalli	dal-0805g	AXS**GP**R**M**SS----**R**OX-NH_2
C. spurius(48)	Sr1	**GP**-**M**GWVPFY**R**F-NH_2

Table 3.2: *Comparison between dal_C0805g and conorfamide-Sr1*

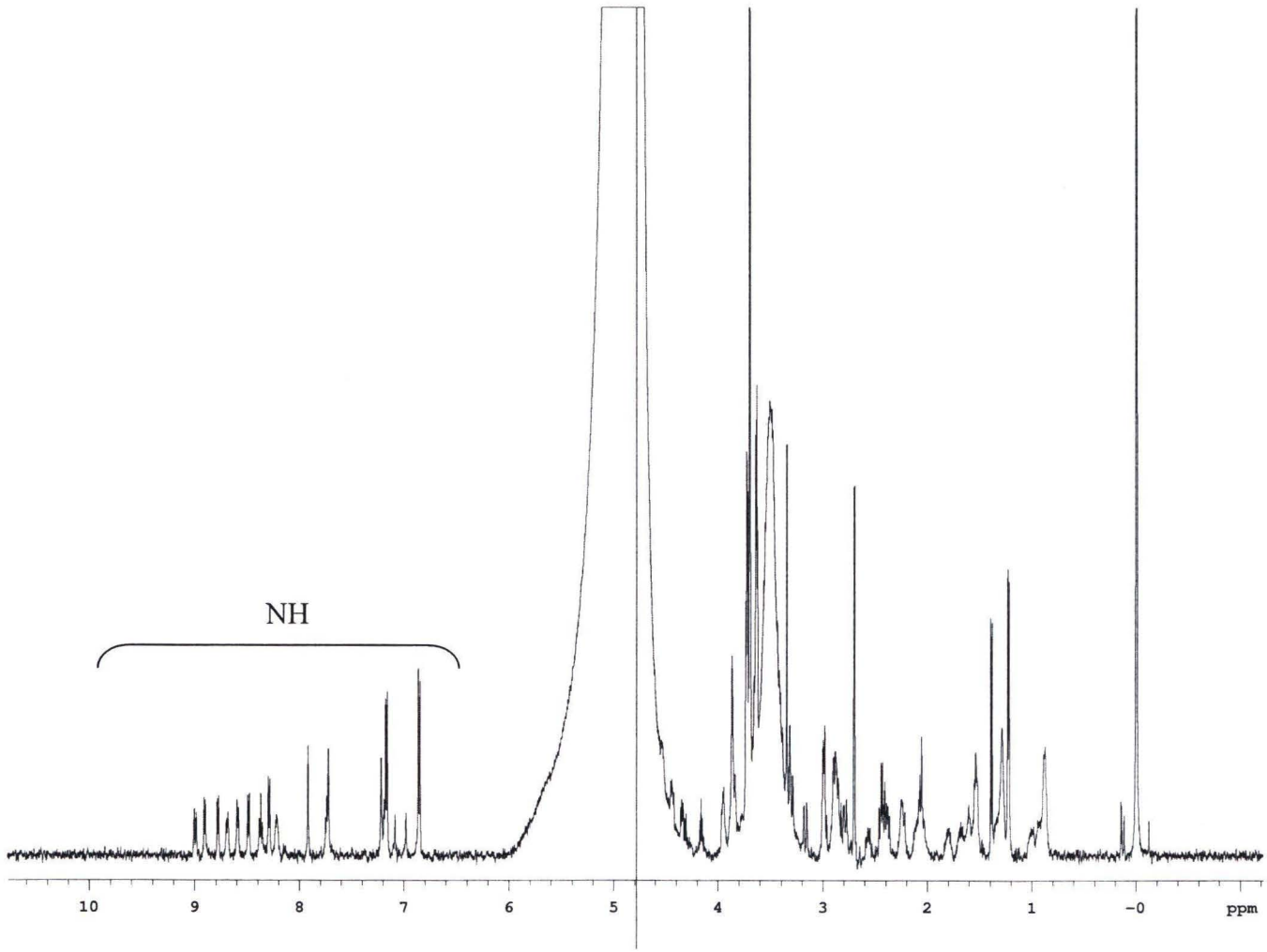

Figure 3.16: 1D NMR of dal_C0805g at pH=3.6 and 25°C.

Size exclusion was used to isolate the second fraction from fraction 9 (retention time = 177.05 min). Further separation was applied to fraction 9 using the semi-prep RP-HPLC and a total of 6 fractions were collected by this method. Three fractions (numbers 10, 11, and 15) showed significant amounts. After scanning those fractions by MALDI-TOF (Figure 3.10 and 3.12), only fraction 10 showed high purity of one component with a molecular weight of 1683.32 Da (Figure 3.10 C). Q-TOF MALDI MS/MS shows a small number of fragments (Figure 3.10 D), which gives strong indication regarding the availability of cysteine frame networks. Reduction and alkylation was performed for small portions of fraction dal_C0910. The molecular weight of the alkylated sample was 2032.07 Da with a molecular weight increment of 348.77 Da from the original molecular weight of the sample. This molecular weight increment of 348.77 Da represents 6 groups of iodoacetamide, the molecular weight of the iodoacetamide group being 58 Da (Figure 3.10 C1), which gives strong indication of the presence of 6-cysteine residues. Q-TOF MALDI MS/MS data of the reduced/alkylated peptide shows many fragments, which gives strong indication of breaking cysteine frame network systems. The concentration of dal_C0910 was 30 pmol (Figure 3.18). The sequence was determined by using Procise^{TM} 491A (*Applied Biosystems Foster City, CA*) amino acid sequencer. The amount of sample used was 25 pmol. Results are shown in Table 3.3. The molecular weight of the sequenced peptide is 1684.52 Da; the difference between the sequenced peptide and the molecular weight determined by MALDI-TOF is 1.2 Da. On the other hand, the difference between the theoretical molecular weight of the reduced and alkylated peptide calculated from the Edman degradation sequencer and the actual molecular weight is 0.58 Da. The following results were obtained by analyzing Q-TOF

MALDI MS/MS data (Figure 3.10 D) of the reduced and alkylated peptide: b_2 = 320.00 Da, b_5 = 708 .00 Da, b_6 = 807.15 Da, y_7 = 870.10 Da, y_9 = 1065.04 Da, a_9 = 1134.09 Da, b_9 = 1162.10 Da, y_{10} = 1225.13 Da, y_{11} = 1324.15 Da, and b_{11} = 1246.00 Da. The previous fraction gives another confirmation of dal_C0910's sequence.

The isolated conopeptide, dal_C0910 has the characteristic six cysteines arrangement (-CC-C-C-CC) of the M-superfamily. As mentioned earlier the M-superfamily is comprised of four families of conotoxins, the μ-conotoxins and ψ-conotoxins, κM-Conotoxins and Mini-M-Conotoxins. The cysteine frame network of dal_C0910 (CC---C---CXCC--) belongs to the Mini-M-Conotoxins with 16 residue and mass of 1683.32 Da. The three other conopeptides that have been identified as Mini-M conopeptide so far are from *Conus nux and Conus marmoreus*. Dal_C0910 is the first peptide from *Conus dalli* to be isolated, characterized and sequenced. By reviewing the sequence of dal_C0910 with peptides from the Swiss-port data bank using blast as search engine, it has been found that there is a high similarity between the isolated peptide of dal_C0910 and scaffold III/IV (Table 3.3), which is a precursor isolated from *Conus textile* (43).

Conus	Fraction.	Sequence	Target
C.dalli	dal_C0910	**CC**SWDV**C**GHPS**C**T-**CC**S	Unknown
C.textile	scaffold III/IV	--RREIILHALGTR**CC**SWDV**C**DHPS**C**T-**CC**G	Unknown
C.nux	Nux3a	R**CC**OOQR**C**STH-**C**RS**CC**G	Unknown
C.nux	Nux3b	R**CC**ORRR**C**STH-**C**RS**CC**Q	Unknown
C.marmoreus(48)	mr3a	G**CC**GSFA**C**RFG-**C**VO**CC**V	Unknown

Table 3.3: Comparison between dal_C0910, scaffold III/IV and other conopeptides belong to mini-M conotoxin

The third fraction was isolated by size exclusion from fraction 10, which had a retention time of 185.4 min. (Figure 3.13 A). Further separation was applied to fraction 10 using semi-prep RP-HPLC. A total of 12 fractions were collected from the semi-prep RP-HPLC (Figure 3.13 B), with only one fraction (fraction 11) showing a significant amount. Further purification was applied to dal_C1011 using analytical RP-HPLC; only one fraction showed a considerable amount, which is fraction "h", having retention time of 65.7 min. The averages molecular masses of dal_C1011h by MALDI-TOF, was found to be 3207.3 Da. In addition the MALDI-TOF spectrum revealed the presence of two other significant compounds whose molecular weights are 17 and 44 Da lower than the main component (Figure 3.13 C), which gives strong evidence that there is an availability of hydroxyl and carboxyl groups. The MALDI-TOF spectrum was supported by additional measurements using ESI-MS. In the spectrum of dal_C1011h showing doubly and triply protonated peptide molecule were observed and marked as [M+2H] and [M+3H] (Figure 3.14). The native molecular masses for fraction A and B are 3206.0 Da and 3161.8 Da, respectively (which gives a difference of 44-unit by mass per Gla residue between fraction A and B). Also, Q-TOF MALDI MS/MS was applied for the three previous fractions. The last two fractions show weak fragments with very low ion numbers (Figure 3.13 D1, D2). By comparing Figures 3.13 D1 and 3.13 D2, a high similarity can be found between them, indicating that those two fragments belong to the same source. Q-TOF MALDI MS/MS showed a small number of fragments (Figure 3.13 D1, D2), which gives strong indication about the availability of cysteine frame networks.

Reduction/alkylation was done for small portions of fraction dal_C1011h. The molecular weight of alkylated sample was 3508.72 Da with a molecular weight difference of 344.44 Da from the original molecular weight of dal_C1011h minus the carboxyl group (MW = 3164.34 Da). The molecular weight difference of 348.77 Da represents 6 groups of iodoacetamide (Figure 3.15 C2), which gives strong evidence of the presence of 6-cystine residues. Q-TOF MALDI MS/MS of the reduced/alkylated peptide didn't show any fragments due to the presence of the carboxyl group, which absorbed most of the collision energy

The concentration of the dal_C1011h fraction was determined to be 30 nmol (Figure 3.19). This concentration was sufficient to allow more 2D NMR experiments to be done. TOCSY was applied to dal_C1011h, which gave the opportunity to determine the amino acid composition for the dal_C1011h fraction (Table 3.5). dal_C1011h was sequenced by using Procise^{TM} 491A (*Applied Biosystems Foster City, CA*) amino acid sequencer, the results showed unique peaks in positions 1, 8, 14, 15, 19, and 24 after Glutamic acid and before Histidine which represented alkylated cysteines (Figure 3.17). Residue no. 4 shows a very hydrophobic peak at a late retention time (17.7 min.), which does not match the retention time of any unmodified amino acids. It has been published that bromotryptophan shows hydrophobic behavior in the Edman degradation sequencer (Figure 3.17) (45). TOCSY data also shows a strong correlation at 10.39 ppm and 7.31 ppm, representing a correlation between aromatic C-H and aromatic N-H, which is one of the unique characteristics of tryptophan (Figure 3.20b and Table 3.5). Also at chemical shift 8.35 ppm the following correlations had been observed: δCH_3-αH 4.24 ppm, δ CH_3-βH 1.88 ppm, δ CH_3-γH 0.96 ppm, and δ CH_3-δ $CH_3$0.83 ppm. This unique

correlation represents Leucine (Figure 3.20a, and Table 3.5), which is one of the last two residues.

The molecular weight of dal_C1011h after determining 8 unknown residues out of 9 is 3056.5 Da with a difference of 152.5 Da from the original molecular weight. By reviewing the sequence of dal_C1011h with Swiss-port data bank using blast as search engine it has been found that there is high similarity between dal_C1011h and TxVIIA (43) (Table 3.4) with differences in the following amino acids positions: 2, 4, and 9, which are glycine instead of aspartic acid, tyrosine instead of bromotryptophan and γ-carboxyglutamic acid instead of lysine, respectively, from TXVIIA and dal_C1011h. By substituting the last unknown residue in dal_C1011h with phenylalanine, the molecular weight will be 3205.99 Da, matching the molecular weight of dal_C1011h. The final sequence of dal_C1011h is CDGWSTYCKVDSγCCSDNCVRSYCTLF .

Conus	Fraction	Sequence	Target
C.dalli	dal_C1011h	**C**DGWSTY**C**KVDSγ--**CC**SDN**C**VRSY--**C**TLF	Unknown
C.textile	TxVII(31)	**C**KQADEP**C**DVFSLD-**CC**TGI**C**LGV---**C**MW	Ca^{+2} Channel
C.textile	TXVIIA(44)	**C**GGYSTY**C**γVDSγ--**CC**SDN**C**VRSY--**C**TLF	Unknown
C.pennaceus	PnVIIA(15)	D**C**TSWFGR**C**TVNSγ--**CC**SNS**C**DQTY--**C**γLYAFOS	Unknown
C.delessertii	Da7a(52)	A**C**KOKNNL**C**AITγMAγ**CC**SGF**C**LIYR--**C**S	Unknown
C. geographus	GVIC(3)	**C**KSOGSS**C**SOTSYN-**CC**-RS**C**NHWTKR**C**	Ca^{+2} Channel
C. tulipa	TVIA(10)	**C**LSOGSS**C**SOTSYN-**CC**-RS**C**NOYSRK**C**Y	Ca^{+2} Channel
C. catus	CVID(48)	**C**KSKGAK**C**SKLMYD-**CC**SGS**C**SGTVGR**C***	Ca^{+2} Channel
C. consors	CnVIIA(49)	**C**KGKGAO**C**TRLMYD-**CC**HGS**C**SSSKGR**C***	Ca^{+2} Channel
C. magus	MVIIC(50)	**C**KGKGAP**C**RKTMYD-**CC**SGS**C**GRR-GK**C***	Ca^{+2} Channel
C. striatus	SVIB(51)	**C**KLKGQS**C**RKTSYD-**CC**SGS**C**GRS-GK**C***	Ca^{+2} Channel
C. catus	CVIC(48)	**C**KGKGQS**C**SKLMYD-**CC**TGS**C**SRR-GR**C***	Ca^{+2} Channel
C. magus	MVIIC(50)	**C**KGKGAP**C**RKTMYD-**CC**SGS**C**GRR-GK**C***	Ca^{+2} Channel
C. catus	CVIC(48)	**C**KGKGQS**C**SKLMYD-**CC**TGS**C**SRR-GR**C***	Ca^{+2} Channel
C. striatus	SVIB(51)	**C**KLKGQS**C**RKTSYD-**CC**SGS**C**GRS-GK**C***	Ca^{+2} Channel
C. striatus	SVIA(51)	**C**RSSGSO**C**GVTSI--**CC**GR-**C**YR--GK**C**T*	Ca^{+2} Channel

Table 3.4: Comparison between dal_C1011h, TXVIIA and other conopeptides belong to ω- conotoxin.

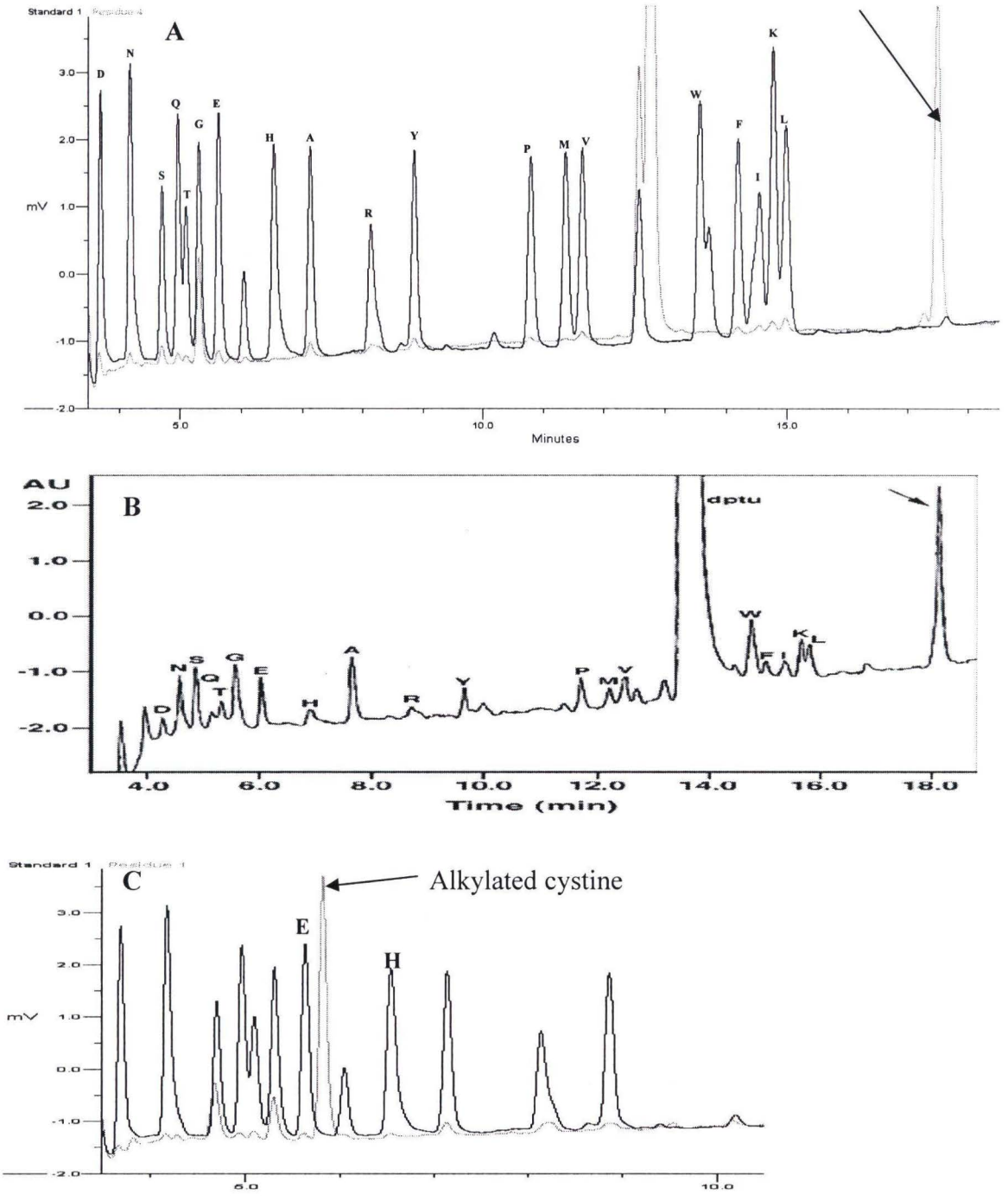

Figure 3.17: A) Residue number 4 of dal_C1011h determined by Edman degradation method. B) Published result of Bromo-tryptophane residue from Edman degradation method (45). C) A distinct peak of alkylated cystine residue appear between glutamic acid and histidine residue.

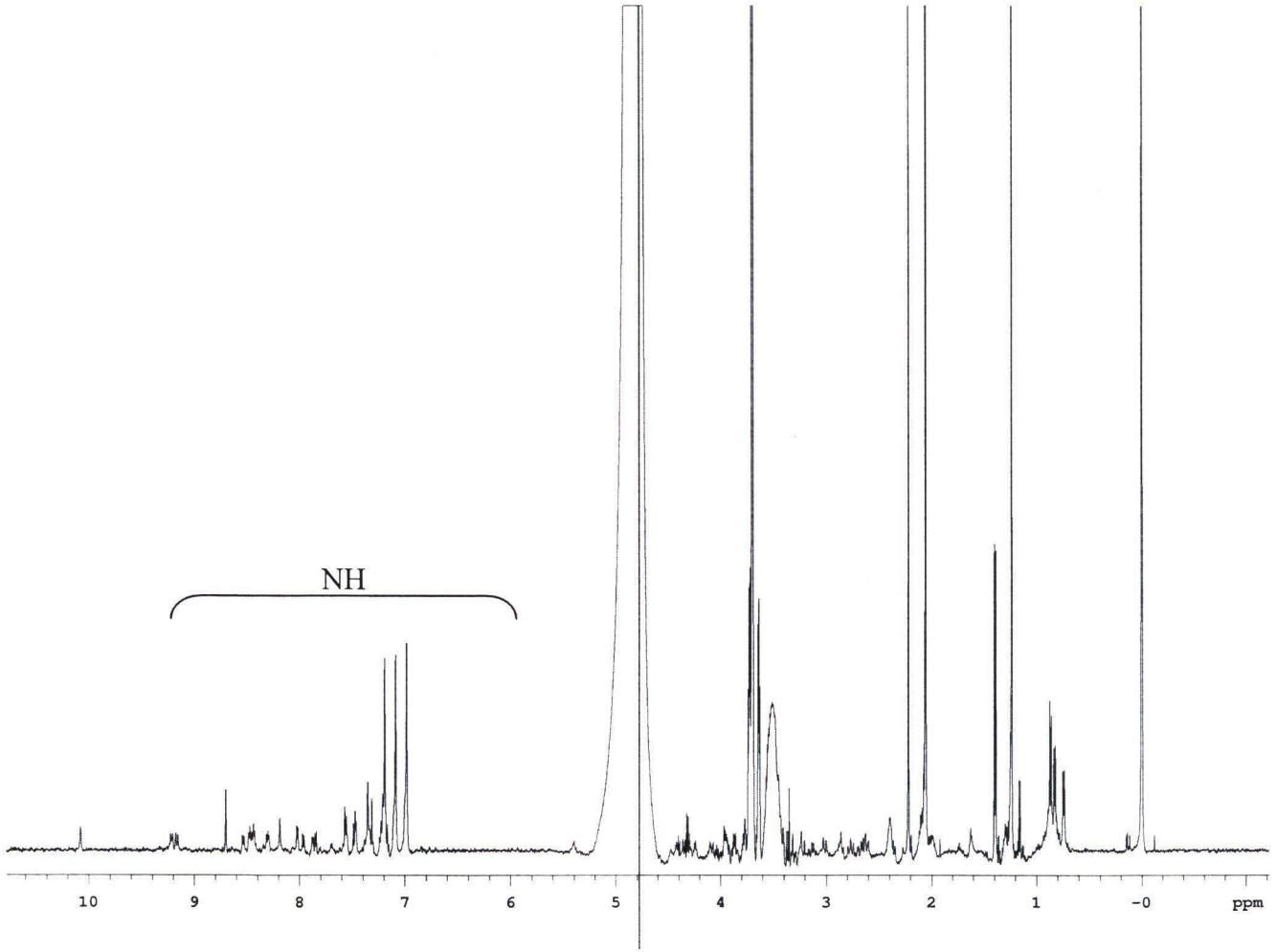

Figure 3.18 1D NMR of dal_C0910 at pH=3.6 and 25°C

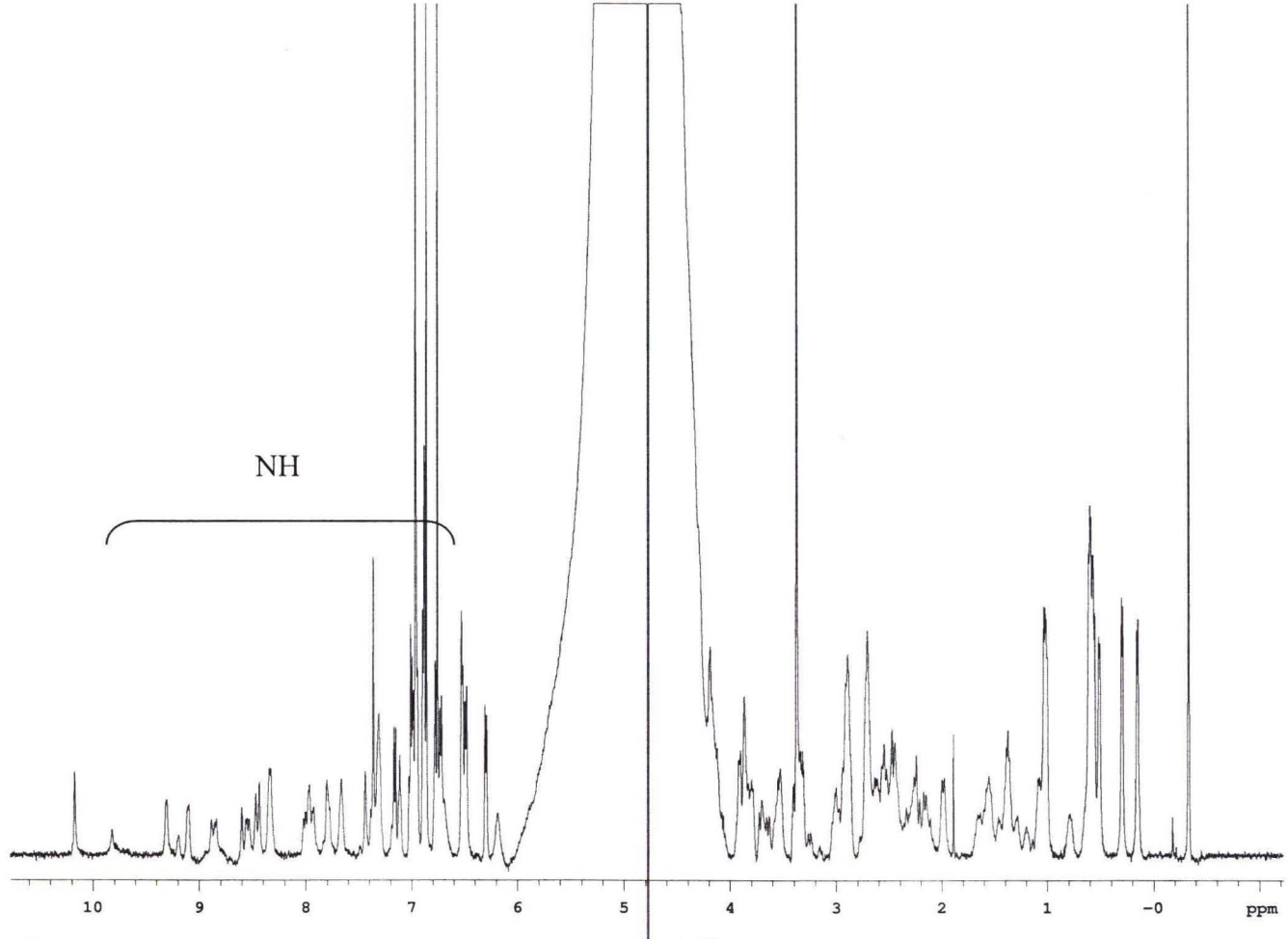

Figure 3.19: 1D NMR of dal_C1011h at pH=3.6 and 0°C.

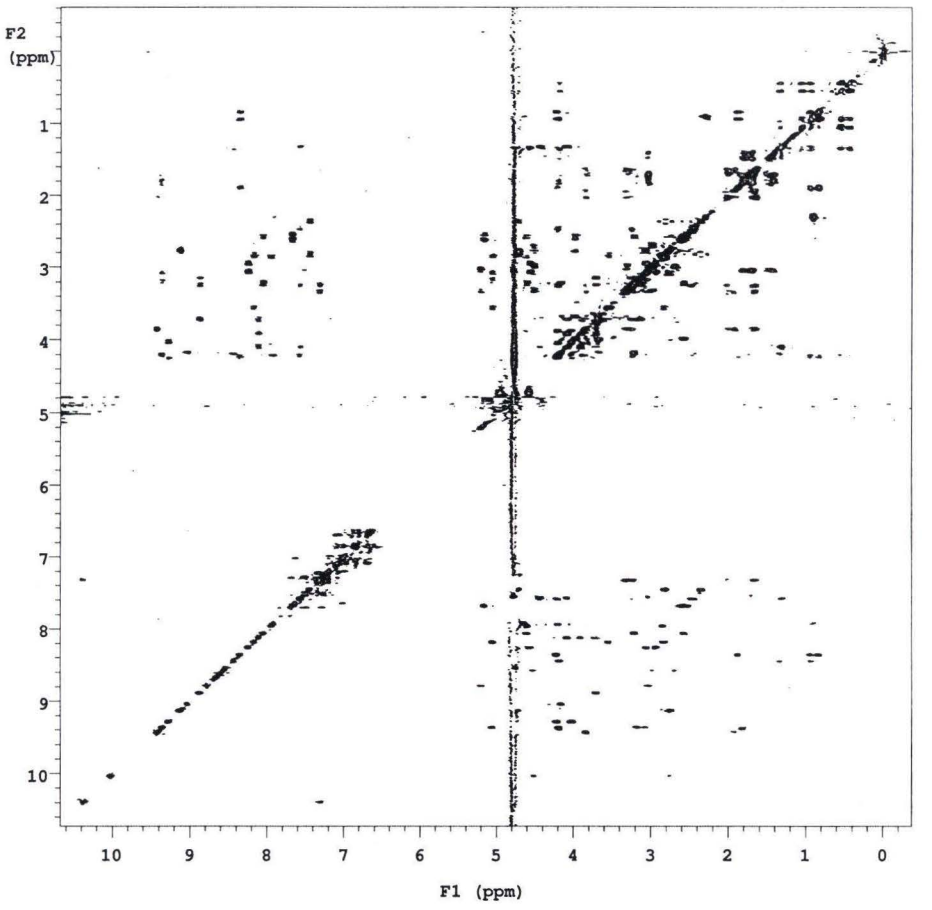

Figure 3.20: TOCSY of dal_C1011h at pH=3.6 and 25°C.

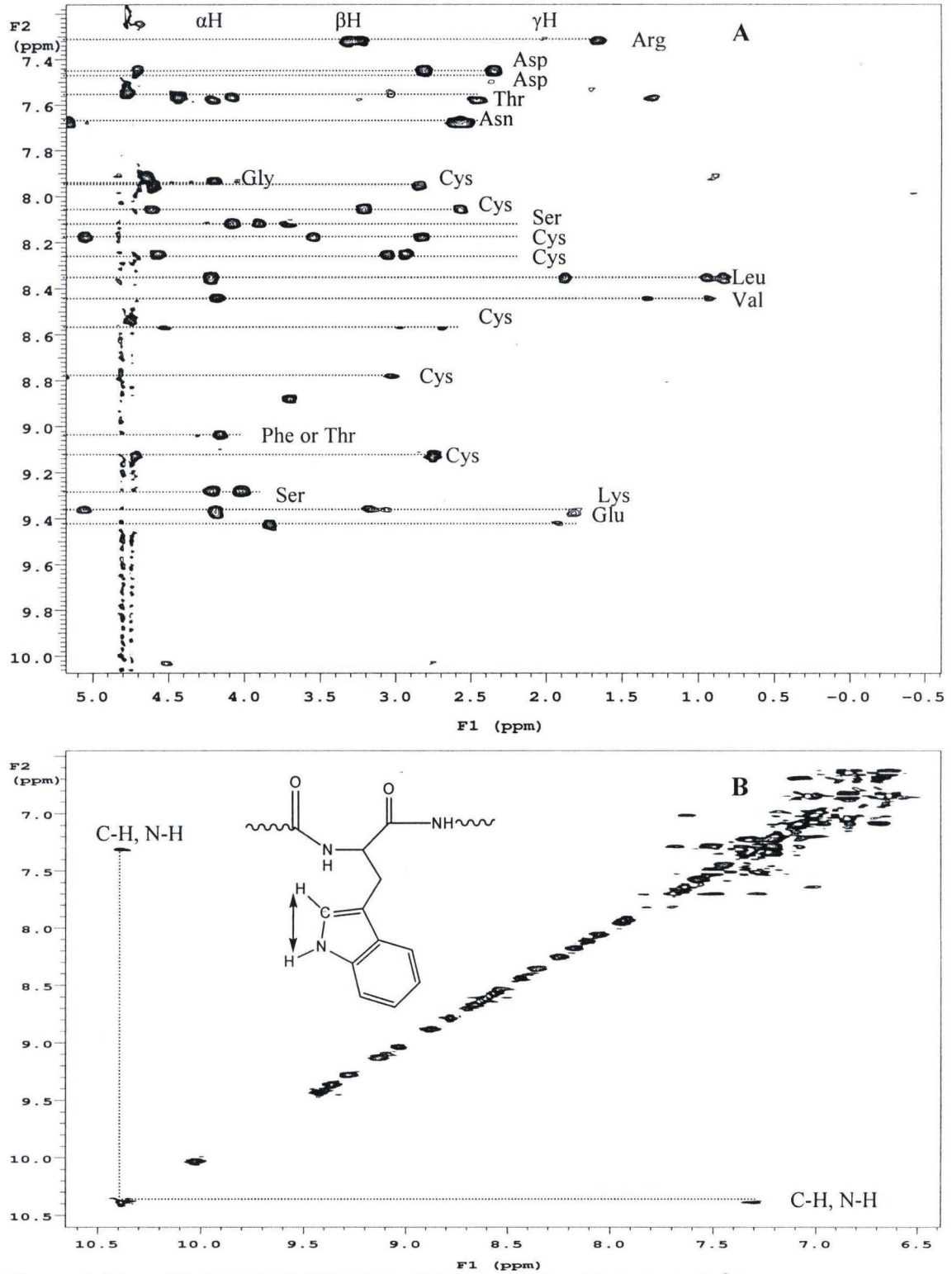

Figure 3.21: a, b) 2D NMR TOCSY of dal_C1011h, pH=3.6, T=25°C.

#	Amino acids		NH	α-H	β-H		γ-H		Other
1	Arg	R	7.31	3.31	3.23		1.66		
2	Asp	D	7.43	4.65	2.81	2.34			
3	Asp	D	7.45	4.76	2.81	2.34			
4	Thr	T	7.58	4.44	4.22		1.23		
5	Asp	D	7.59	4.09	2.46				
6	Asn	N	7.65	5.17	2.57				
7	Gly	G	7.93	4.23					
8	Cys	C	8.07	4.61	3.22	2.57			
10	Ser	S	8.12	4.08	3.89	3.71			
11	Cys	C	7.94	4.61	2.84				
13	Cys	C	8.17	5.06	3.55	2.82			
14	Cys	C	8.25	4.56	3.08	2.94			
15	Leu	L	8.35	4.24	1.88		0.96	0.83	
16	Val	V	8.44	4.17	1.33		1.03	0.93	0.55
17	Cys	C	8.57	4.53	2.97	2.69			
18	Cys	C	8.78	5.2		3.01			
19	Ser	S	8.88	3.7	3.30'	3.15'			
20	Phe or Try		9.03	4.39	4.15				
21	Cys	C	9.13	4.72	2.75				
22	Ser	S	9.29	4.24	4.03				
23	Lys	K	9.38	4.18	1.84'	1.82'	1.77'		
24	Ser or Cys		9.36	3.21	3.17'	3.08'			
25	Gla	γ	9.41	3.84	1.94				
26	Trp	W	Between 10.39 And 7.31 there is strong correlation which represent N(1) & H (10.22) and H aromatic (7.24)						
27	Also there is strong correlation in the following points 7.65& 7.28						Represent aromatic region which give strong indication of Tyr's		
28	Also there is strong correlation in the following points 7.08& 6.70								
29	Also there is strong correlation in the following points 6.84& 6.65								

Table 3.5: Tentative 2D NMR Assignments of dal_C1011h

4. Conclusion

This work describes for the first time the isolation and characterization of the components of the venom of *Conus dalli,* the only of the Panamic cone snail species. The venom of *Conus dalli* is quite complex. From the initial method of separation, using Semi-preparative RP-HPLC, a total of 68 fractions were collected (dal_A), each having multiple components of different molecular weights.When Size Exclusion Chromatography (second method of separation) was applied, 14 fractions were collected for each run (dal_C). The Semi-preparative RP-HPLC separations of each Size Exclusion Chromatography fraction had fewer components than the initial dal_A chromatogram. After applying both chromatographic techniques, each final fraction required fewer purification steps, which is important when working with limited amounts of sample. The selected fractions for sequencing, dal_0805g, dal_C0910, and dal_C1011h were further purified using analytical RP-HPLC.

The sequence of dal_C0805g is not completed with three amino acids missing; dal_C0805g reported in this thesis is one of three sequences ever isolated from the cone snail *Conus dalli.* Due to the absence of a cystine residue, dal_C0805g is considered a member of the one of the linear peptide families, most closely the Conorfamide superfamily.

Conorfamide superfamily is biologically active and from *Conus* venom. It is surprisingly potent in the mammalian central nervous system (CNS). The RFamide peptide family has host of diverse function in the inveretbrate nervous systems. Also it has been reported that RFamide peptide has effect on H^+ -gated EnaC channel (48).

The second isolated peptide is dal_C0910. The sequence of dal_C0910 is **CC**SWDV**C**GHPS**CTCC**S dal_C0910 is a novel member of the M-superfamily of Mini-M conotoxins. A high similarity was observed between dal_C0910 and a precursor isolated from *Conus textile* (scaffold III/IV, (34)). M-superfamily conopeptides, the μ-conotoxins targeted and block voltage-gated Na^+ channels, and ψ- conotoxins are noncompetitive antagonists of the nAChR. The Mini-M conotoxins enticed different behavior in rates than did the μ-conotoxin. The exact target of the Mini-M conotoxins is currently unknown.

The third isolated peptide is dal_C1011h. The sequence of dal_C1011h is **C**DGW(Br)Y**C**KVDSγ**CC**SDN**C**VRSY**C**TLF, dal_C1011h is a novel member of O-superfamily of conotoxins, a high similarity was observed between dal_C1011h and conopeptide (TXVIIA) isolated from *Conus textile* (44).

TXVIIA did not show any biological activity. However, γGlu residues can form a secondary stabilizing Ca^{+2} chelat ion under physiological conditions (44,46).

By comparing the three fractions isolated from *Conus dalli* (dal_C1011h, dal_0910, and dal_C0805g) with direct injection of crude venom using RF-HPLC a high similarity have been found between dal_C0805g and fraction 5, also between dal_0910 and fraction 13 and finally between dal_C1011h and fraction 39. As shown in table 3.6 it is it is difficult to determine the biological activity of each of three fractions by using

fluorescence bioassays method due to the presents of more than one compound in each fraction. Also fluorescence bioassays method gives only hint about bioactivity of the component with law accuracy of targeting specific ion channel.

Size Exclusion chromatography method			Direct injection method using RF-HPLC					
Fraction	Retention time(min)	Mwt(Da)	Peak No	Retention time (min)	Mwt (Da)	Na^+	K^+	Ca-Fluo3
dal_C0805g	25.60	1332.97	5	28.68	1319.2 1343.9	NA	NA	NA
dal_C0910	34.7	168.3.0	13	36.05	1213.6 1235.0 1527.2 1704.1 1811.1 2905.7	6.3	1.6	2.1
dal_C1011h	65.7	3165.2	39	63.1	3182.2 6517.7 9738.7 12970.2	3.2	3.0	3.2
			40	64.71	2926.4	4.8	-4.2	3.6

Table 3.6: Comparison between Size Exclusion chromatography method and Direct injection method using RF-HPLC

Due to the similarity of cyc arrangement in 4 conotoxins isolated from *C.textile, C.pennaceus, C.delessertii* and *C.dalli*. However dal_C1011h contain a motif (-γCCS-) (Table3.7) that has previously only found in both molluscivorois cone snail: TXVIIA(44) from *C.textile,* γ-PnVIIA(15) from *C.pennaceus* and vermivorous cone snail: de7a (52) from *C.delessertii.* The first two toxins (TXVIIA and γ-PnVIIA) cause depolarization and increase firing of action potentials in molluscan neural systems, and toxin γ-PnVIIA has been shown act as an agonist of neuronal pacemaker cation currents (15). The similarities

to toxin TXVIIA and γ-PnVIIA suggests that dal_C1011h peptide might also affect voltage gated nonspecific cation pacemaker.

Conus	*Fraction*	*Sequence*
C.dalli	dal_C1011h	CDGWSTYCKVD--SγCCSDNCVRSY-CTLF * * * **** * * * *
C.textile	TXVIIA (44)	CGGYSTYCγVD--SγCCSDNCVRSY-CTLF * * * **** * * * *
C.pennaceus	γ-PnVIIA(15)	DCTSWFGRCTVN--SγCCSNSCDQTY-CγLYAFOS * * * **** * * * *
C.delessertii	de7a (52)	ACKOKNNLCAITγMAγCCSGFCLI-YRCS * * **** * * *

Table 3.7: Comparison between Dal_C1011h fraction and TXVIIA, γ-PnVIIA, and da7a

Future work could include the synthesis and characterization of dal_C0805g, dal_C0910, and dal_C1011h to determine the precise bridging pattern of the disulfide bonds and electrophysiological studies to determine the specific type of ion channels that they target.

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