DBT PDF Research Progress Report for the Period JAN 2011 to DEC 2011

Title

ISOLATION, PURIFICATION AND CHARACTERIZATION OF POTENTIAL BIOACTIVE PEPTIDES FROM STIMULATED FROG SKIN SECRETIONS

by

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ISOLATION, PURIFICATION AND CHARACTERIZATION OF POTENTIAL BIOACTIVE PEPTDES FROM STIMULATED FROG SKIN SECRETIONS

Amphibians secrete a complex chemical cocktail from highly specialized skin structures, as a result of contraction of mycocytes surrounding the glands namely the venom or granular glands as a defense mechanism from predators (Simmaco *et al.*, 1998). These secretions contain biologically active components including alkaloids, biogenic amines, peptides and proteins (Lazarus & Atilla, 1993). Most of these peptides share common properties, such as an overall cationic character and the tendency to adopt a helical conformation often resulting in amphipathic behaviour. These properties are believed to play important roles in the interaction of the peptides with the membrane of target cells and in the mechanism that eventually causes cell lysis (Hancock *et al.*, 1995, Hancock & Lehrer, 1998). Despite efforts directed towards the structural and functional analysis of frog skin peptides for several decades, the vast majority of species remain unknown. In our laboratory screening of bioactive peptides, from the skin secretions of some species of frogs and toads, collected from Orissa, Dehradun and Andaman is in progress.

Materials and Methods

Frog species selected for the study

Uperodon globulosus (collected from North Orissa University, Orissa)

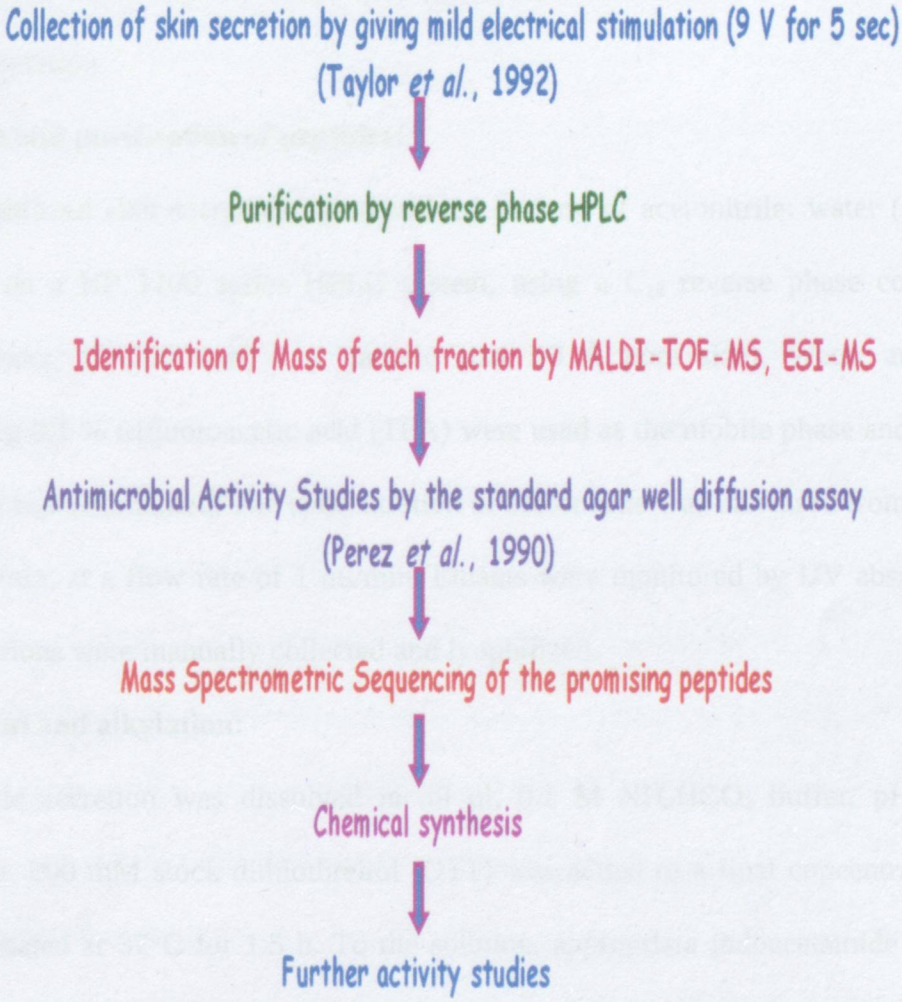
U. systoma ””

Kaloula taprobanica ””

Duttaphrynus himalayanus (collected from Wild life Institute of India, Dehradun)

D. melanostictus (collected from Andaman Islands)

Outline of the work/Methodology



Collection of frog skin secretions:

The skin secretions of *Uperodon globulosus, U. systoma, Kaloula taprobanica, Duttaphrynus himalayanus and D. melanostictus* were collected by giving mild electrical stimulation of 9 V for 5 seconds, a non-invasive technique which is not causing much discomfort to the animal and most importantly without sacrificing the animal, following Taylor *et al.*, (1992). The identification was confirmed by Prof. Sushil Dutta, Head of the Department of Zoology, North Orissa University. The frogs were released into their natural habitat after the collection of skin secretion.

Isolation and purification of peptides:

The lyophilized skin secretion was dissolved in 1 ml of acetonitrile: water (50 % v/v) and purified on a HP 1100 series HPLC system, using a C_{18} reverse phase column (Jupiter, Phenomenex, 10×250 mm, 4 µ particle size, 90 Å pore size). Water and acetonitrile containing 0.1 % trifluoroacetic acid (TFA) were used as the mobile phase and a flow rate of 1 ml/min was maintained. The concentration of acetonitrile was increased from 10 % to 95 % over 55 min, at a flow rate of 1 ml/min. Eluants were monitored by UV absorbance at 226 nm. Fractions were manually collected and lyophilized.

Reduction and alkylation:

The crude secretion was dissolved in 30 µl, 0.1 M NH_4HCO_3 buffer, pH 8.0. For the reduction, 200 mM stock dithiothreitol (DTT) was added to a final concentration of 8 mM and incubated at 37°C for 1.5 h. To the solution, appropriate iodoacetamide stock solution was added to get a final concentration of 40 mM and the mixture was incubated at room temperature (28±2°C) in the dark, for 45 min (Sudarslal *et al.*, 2003). The reaction mixture was analyzed by MALDI-TOF-MS.

Esterification: Methanolic HCl was prepared by dropwise addition of 20 µl acetyl chloride to 100 µl of ice cold, anhydrous methanol. Four µl of peptide was added to 16 µl of

methanolic HCl. The esterification was allowed to proceed at 25°C. The reaction mixture was analyzed by MALDI-MS.

Acetylation: To 5 µl of peptide, 2 µl of 0.5 M NH_4HCO_3 pH 8.0, and 2 µl acetic anhydride were added and the volume was made up to 20 µl with distilled water. After incubating the reaction mixture for 1 h at 25°C, the product was analyzed in MALDI-MS.

Mass spectrometry

MALDI-TOF-MS analyses were performed using an Ultra flex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron (positive ion) mode equipped with a nitrogen laser of wavelength 337 nm. The samples were prepared by mixing an equal amount of peptide (0.5 µl) with a matrix solution (α-cyano-4-hydroxy cinnamic acid) in 1:1 (v/v) acetonitrile-water mixture. MS/MS Spectra were acquired and fragments were generated in post-source decay mode (PSD). Mass spectra were analyzed using Flex-Analysis software (Bruker Daltonics).

Electrospray ionization (ESI) mass spectra were recorded using a Hewlett Packard single quadrupole mass spectrometer (HP 1100 MSD series). The samples were infused into the mass spectrometer through a reverse phase C_{18} column (Zorbax, 4.6×150 mm) with solvent A (Water with 0.1 % TFA) and solvent B (acetonitrile with 0.1% TFA) at a flow rate of 0.25 ml/min. The data were acquired over the range *m/z* 50–3000 in positive ion mode and were analyzed using HPLC/MSD Chemstation software.

Antibacterial Assay:

The test organisms included the Gram-positive bacteria *Bacillus cereus* (NCIM 2217), *Staphylococcus aureus* (NCIM 2079) and Gram-negative bacteria *Klebsiella pneumoniae* (NCIM 5082), *Escherichia coli* (NCIM 2574), *Salmonella typhimurium* (NCIM 2501). All the bacterial strains were obtained from National Chemical Laboratory (NCL), Pune, India. The bacteria were grown in the nutrient broth at 37°C and maintained on nutrient agar slants

at 4°C. One mg of crude lyophilized skin secretion and 1 mg of HPLC fraction was resuspended in 50 µl of sterile deionized water. The aqueous extract was screened for potential antibacterial activity by the standard agar well diffusion assay (Perez *et al*., 1990).

For all the bacterial strains, overnight cultures grown in nutrient broth and 200 µl of bacterial cultures with OD 1.000 was spread plated onto Nutrient agar media. After that wells were made on the agar media using a sterile well puncher. Sterile deionized water was taken as negative control and Ampicillin with concentration of 1 mg/ml was used as positive control. The plates were allowed to dry for 3–5 minutes after which 10 µl of the test samples and the controls were dispensed into each well. Following an incubation period of 24 h at 37°C, antibacterial activity was evaluated by quantifying zones of inhibition of bacterial growth. The experiment was done on triplicate.

Results

Uperodon globulosus

Figure 1 is showing *U. globulosus* before and after giving the electrical stimulation. HPLC profile of its skin secretion is given in Figure 2. Nineteen fractions were collected and all these were further concentrated by lyophilization and afterwards subjected to MALDI MS. The fractions which gave masses were then subjected to ESI MS/MS and tried to sequence the peptides. Fraction 3 contained peptides 1054.4 m/z, 1161.5 m/z, 1601.5 m/z and 1659.5 m/z. Peptides with masses 1226.7, 1297.9 were present in fraction 12; 1142.7, 1226.8, 1297.8 in fraction 13; 1142.8 m/z, 1155.8 m/z, 1213.8 m/z, 1226.8 m/z, 1240.9, 1297.9 m/z in fraction 14; 1254.9 m/z and 1325.9 in fraction 16; 1142.9 and 1315 m/z in fraction 17. The peptides 1298 m/z, 1227 m/z, 1142.8 m/z and 1161.5 m/z were the peptides which fragmented well after ESI MS/MS (Figures 4, 7, 9 and 13). The MALDI MS of these peptides are shown in Figures 3, 8 and 12 respectively.

In order to determine the number of Cysteine (Cys) residues in the observed peptides, the crude secretion was subjected to global reduction and alkylation with DTT and iodoacetamide. Carboxamidomethylation leads to an additional mass of 58 Da for each Cys residue in general. The MALDI-TOF-MS showed no increment in mass in any of the peptides that indicated the absence of disulphide bonded peptides. This confirmed the linear nature of both these peptides.

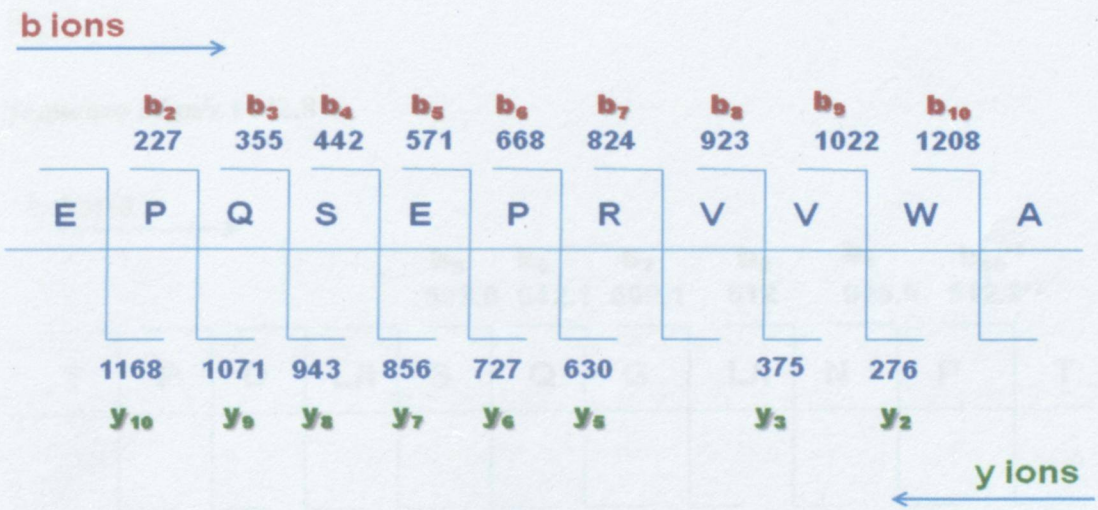
Global esterification and acetylation helps in determining the number of free carboxylic and amino groups present in the peptides. The increment of number of times of 14 Da after esterification to the peptide corresponds to the number of free carboxylic groups present.

Three times addition of 14 Da to peptides of m/z 1298 and 1227 (Figures 5, 5.1, and 5.2), suggests the presence of 3 carboxylic groups in the sequence. The esterified peaks were

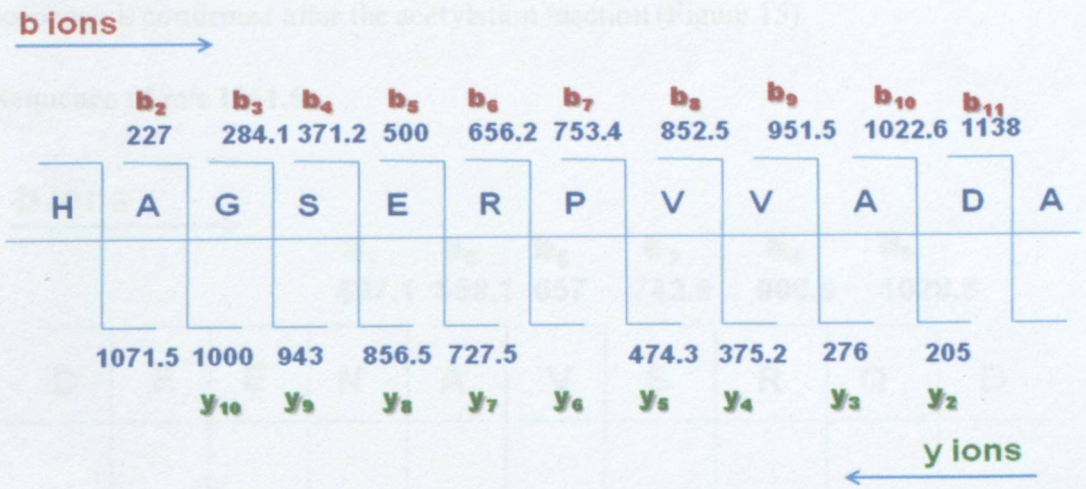
fragmented in MALDI MS and confirmed one of these is because of the free carboxy terminus, while the other two are because of the presence of two acidic amino acids in the sequence; two glutamic acid residues in m/z 1298 and one glutamic acid and one aspartic acid in m/z 1227.

In an acetylation reaction, the $-COCH_3$ group gets attached to the free $-NH_2$ groups present on the peptide, replacing one amino hydrogen. Thus increment of multiples of 42 Da represents the number of free amino groups present in the peptides. There is an increase of 42 Da to both the peptides, m/z 1298 and 1227 (Figures 6, 6.1 and 6.2), suggesting presence of one free amino group on both peptides. The acetylated peaks then fragmented in the MALDI and confirmed this is due to free amino terminus in the sequence.

Sequence of m/z 1298

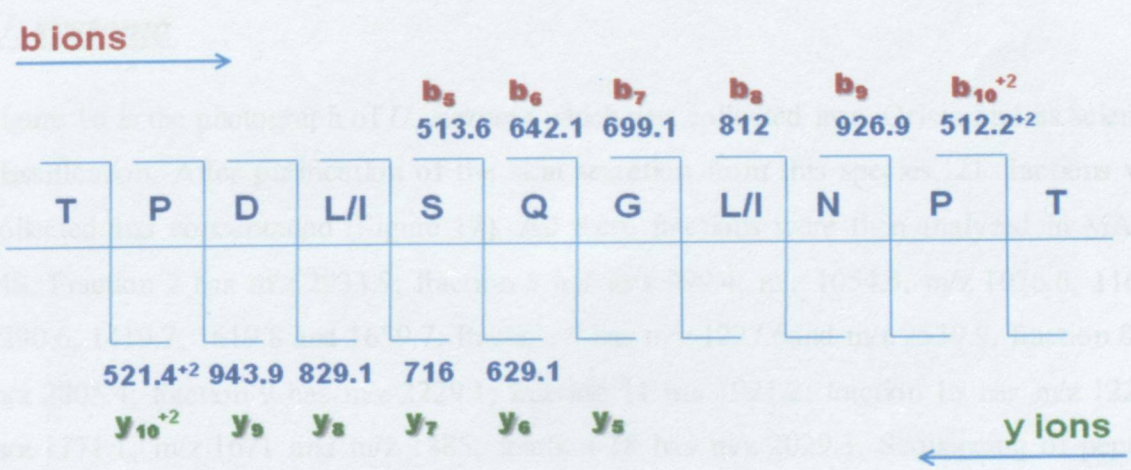


Sequence of m/z 1227



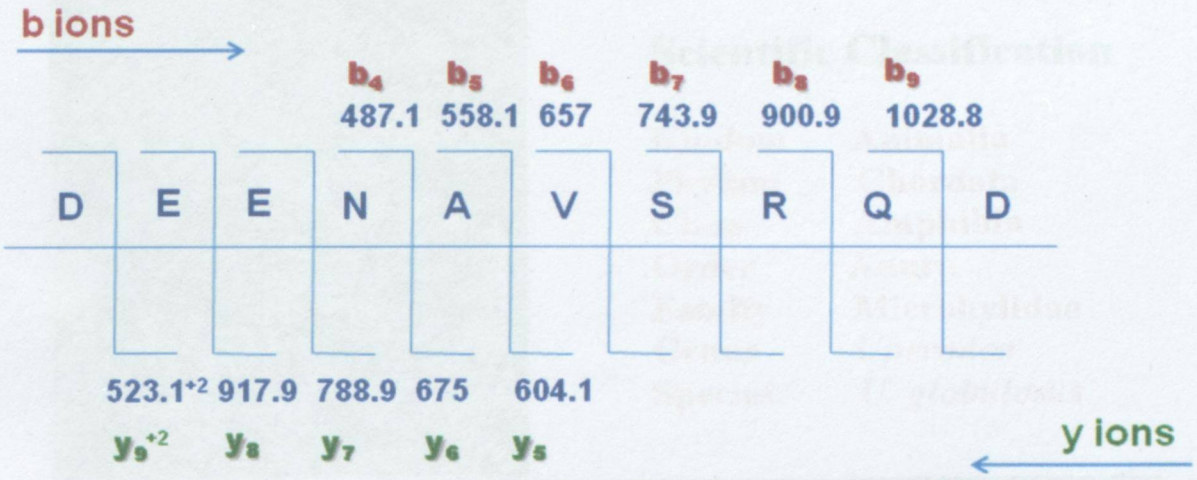
Presence of one free carboxy terminus and one aspartic acid is confirmed in the sequence of m/z 1142.8 after the esterification reaction (Figure 10 and 10.1). Addition of one 42 Da after acetylation is confirmed due to the presence of free amino terminus in the sequence (Figure 11).

Sequence of m/z 1142.8



Esterification of m/z 1161.5 resulted in the addition of five 14 Da additions and that is confirmed because of two aspartic acid residues, two glutamic acid residues and free carboxy terminus in the sequence (Figure 14). Confirmation of the free amino terminus in the sequence is confirmed after the acetylation reaction (Figure 15).

Sequence of m/z 1161.6



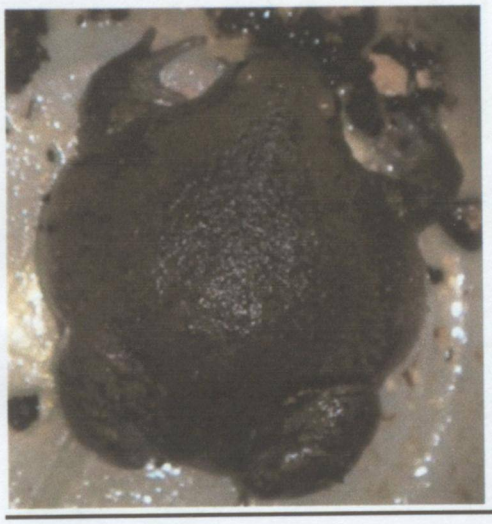
Sequencing of 1325 m/z, 1054 m/z, 1314 m/z, 1659 m/z, 1161 m/z is in progress.

U. systoma

Figure 16 is the photograph of *U. systoma* which was collected from Orissa and its scientific classification. After purification of the skin secretion from this species, 21 fractions were collected and concentrated (Figure 17). All these fractions were then analyzed in MALDI MS. Fraction 2 has m/z 2933.9; fraction 5 has m/z 999.4, m/z 1054.5, m/z 1076.6, 1161.6, 1290.6, 1419.7, 1619.8 and 1659.7; fraction 6 has m/z 1227.6and m/z 2539.9, fraction 8 has m/z 2805.4; fraction 9 has m/z 2229.1; fraction 11 has 1921.2; fraction 15 has m/z 1226.9, m/z 1771.1, m/z 1671 and m/z 1885; fraction 18 has m/z 2029.3. Sequencing of peptides from this species is in progress.

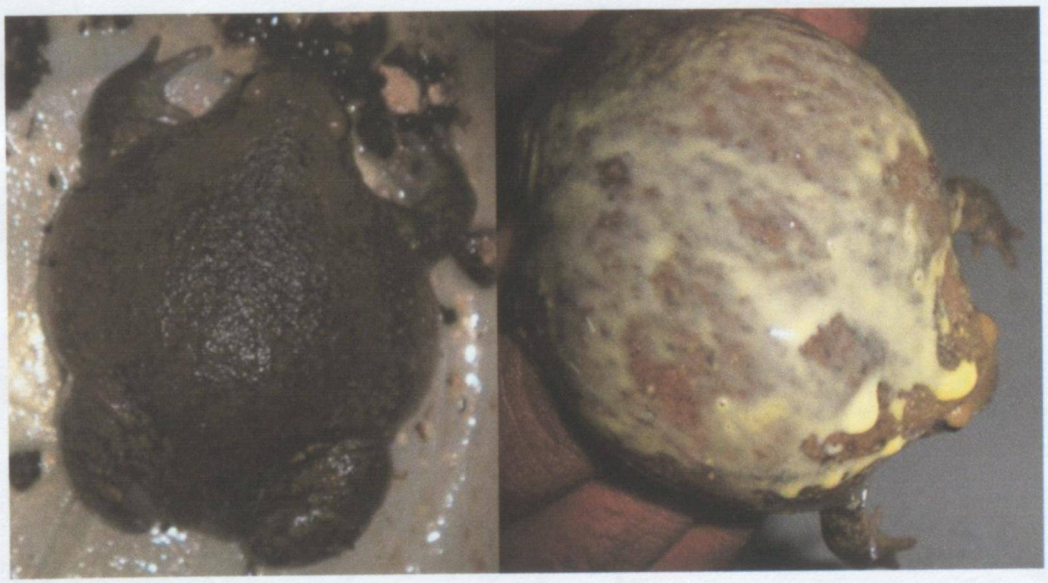
Results

Uperodon globulosus (Gunther, 1864)
(Indian Balloon frog)



Scientific Classification

Kindom	**Animalia**
Phylum	**Chordata**
Class	**Amphibia**
Order	**Anura**
Family	**Microhylidae**
Genus	*Uperodon*
Species	*U. globulosus*



Before Stimulation **After Stimulation**

Figure 1: ***Uperodon globulosus***

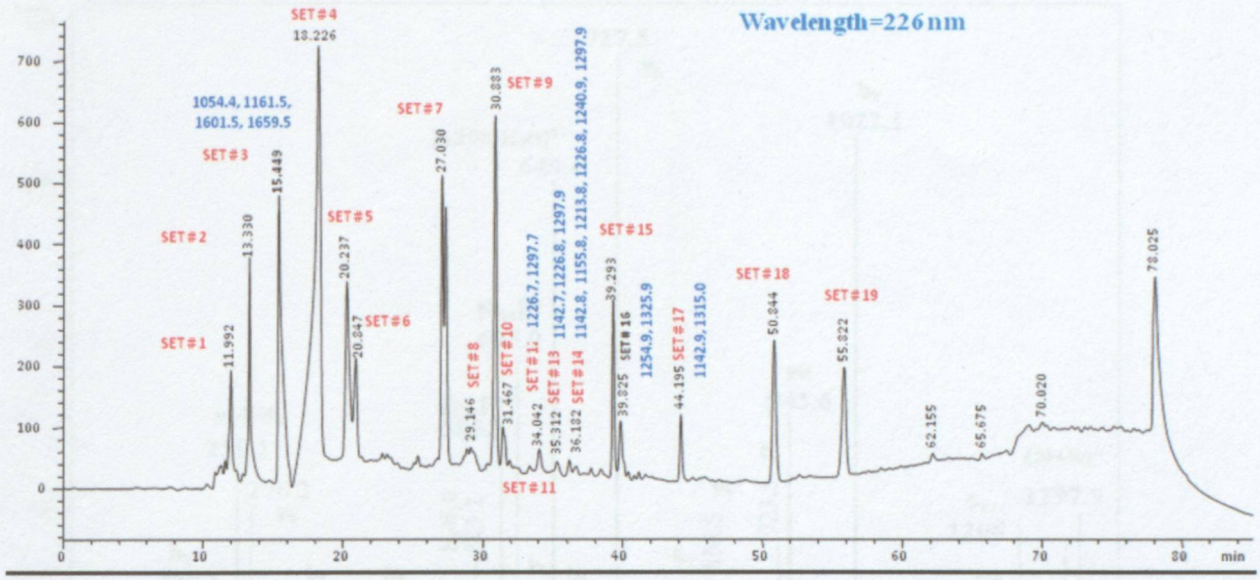


Figure 2: Reverse Phase HPLC Profile of *Uperodon globulosus* skin secretion

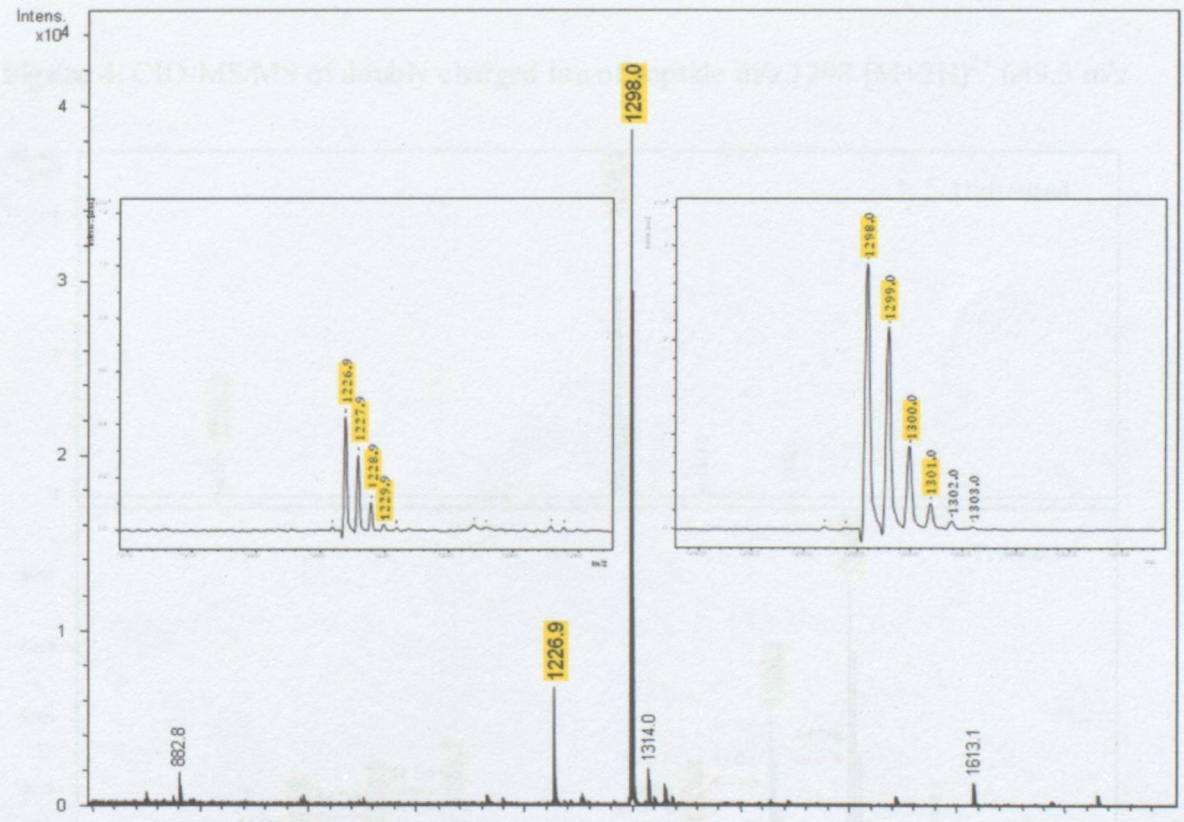


Figure 3: MALDI MS of HPLC fraction 12

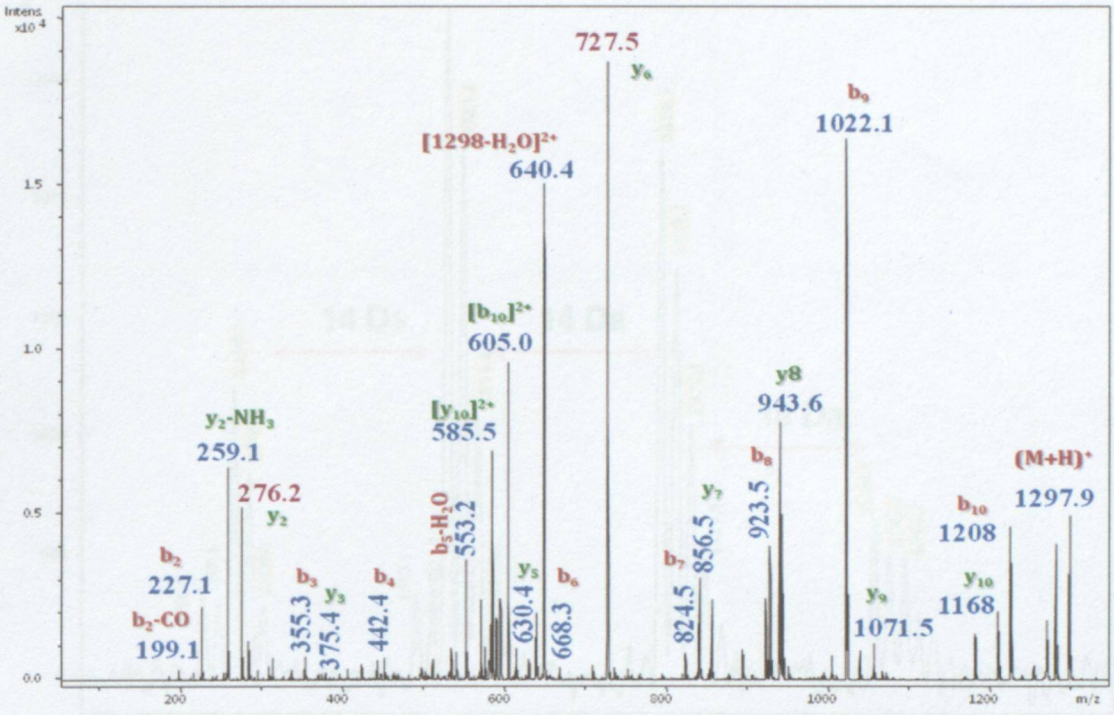


Figure 4. CID-MS/MS of doubly charged ion of peptide m/z 1298 $[M+2H]^{2+}$ 649.5 m/z

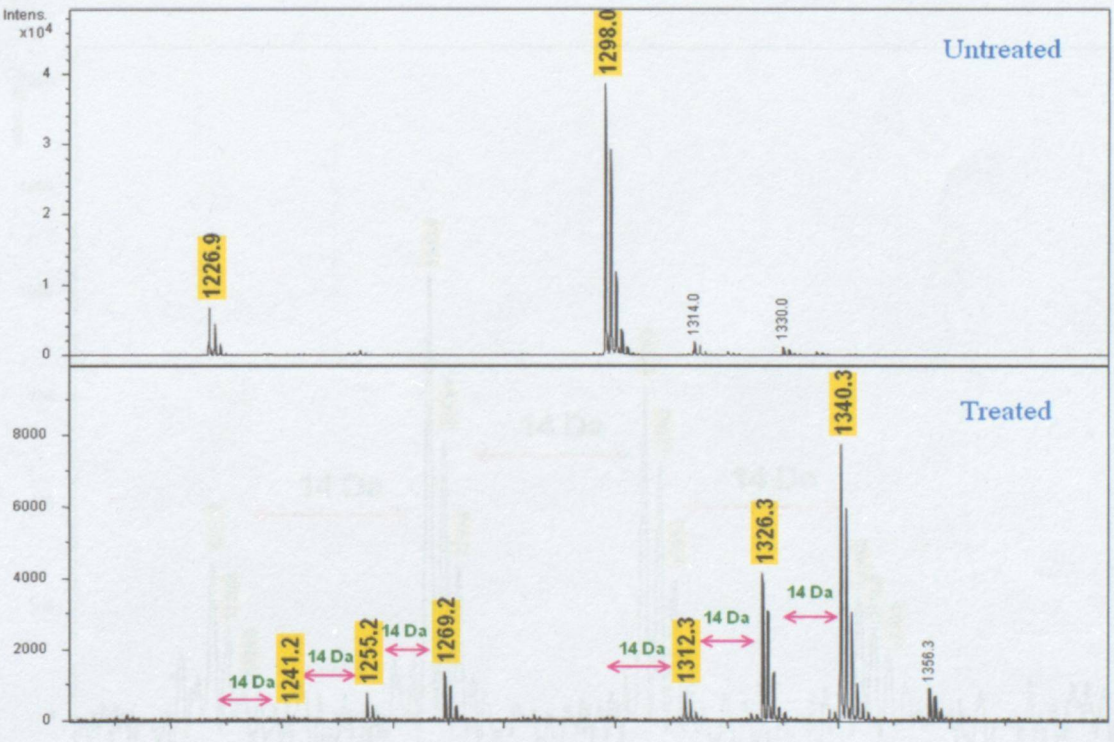


Figure 5: Esterification of HPLC fraction 12

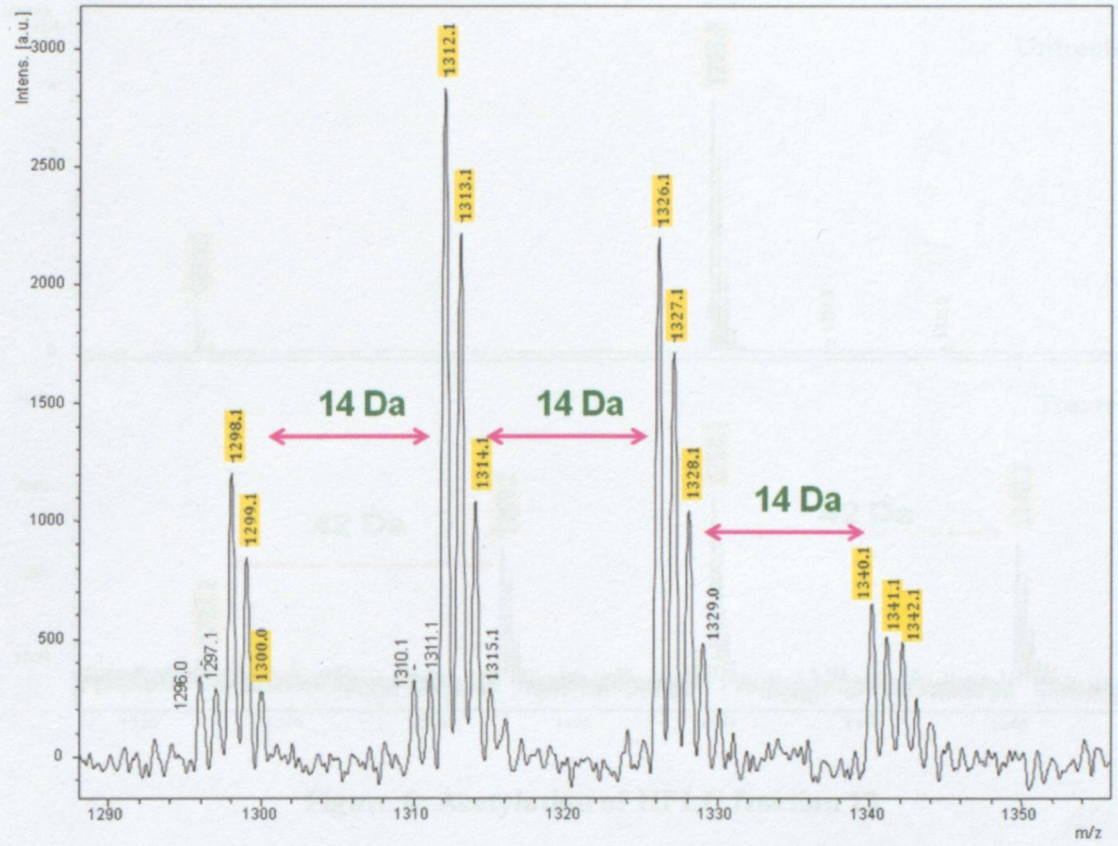


Figure 5.1: Expanded spectrum showing the esterification of 1298 m/z

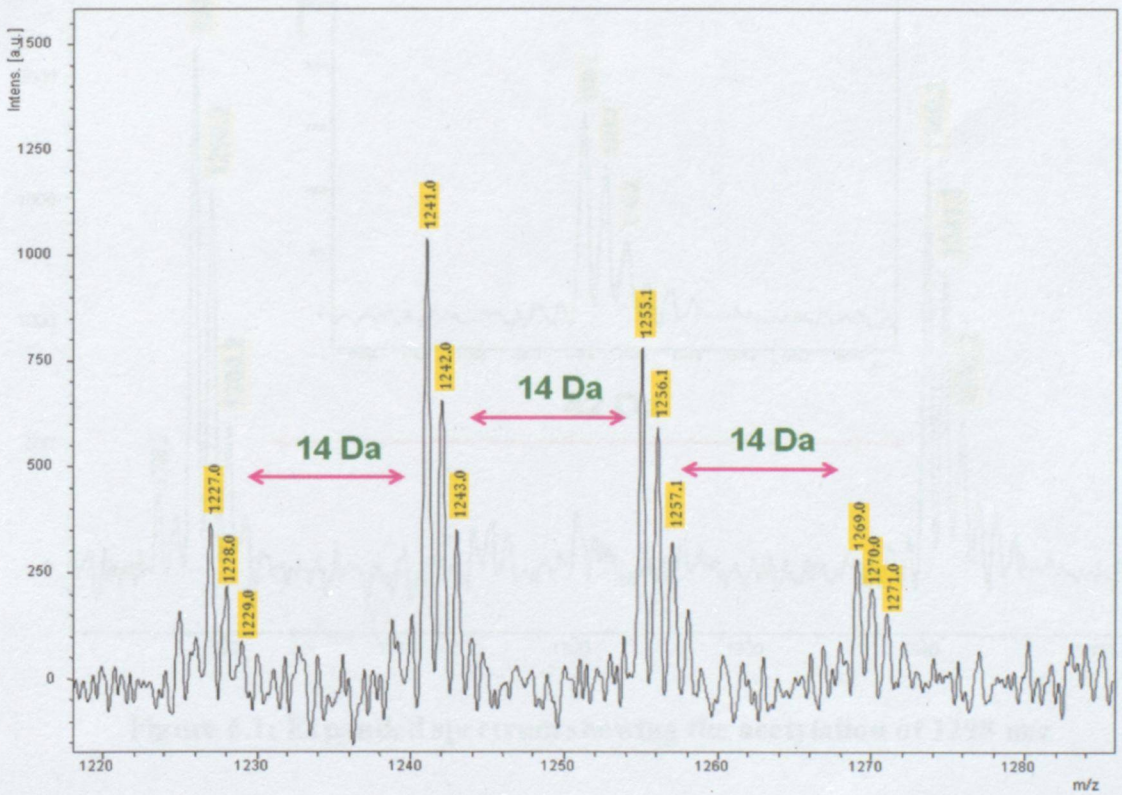


Figure 5.2: Expanded spectrum showing the esterification of 1227 m/z

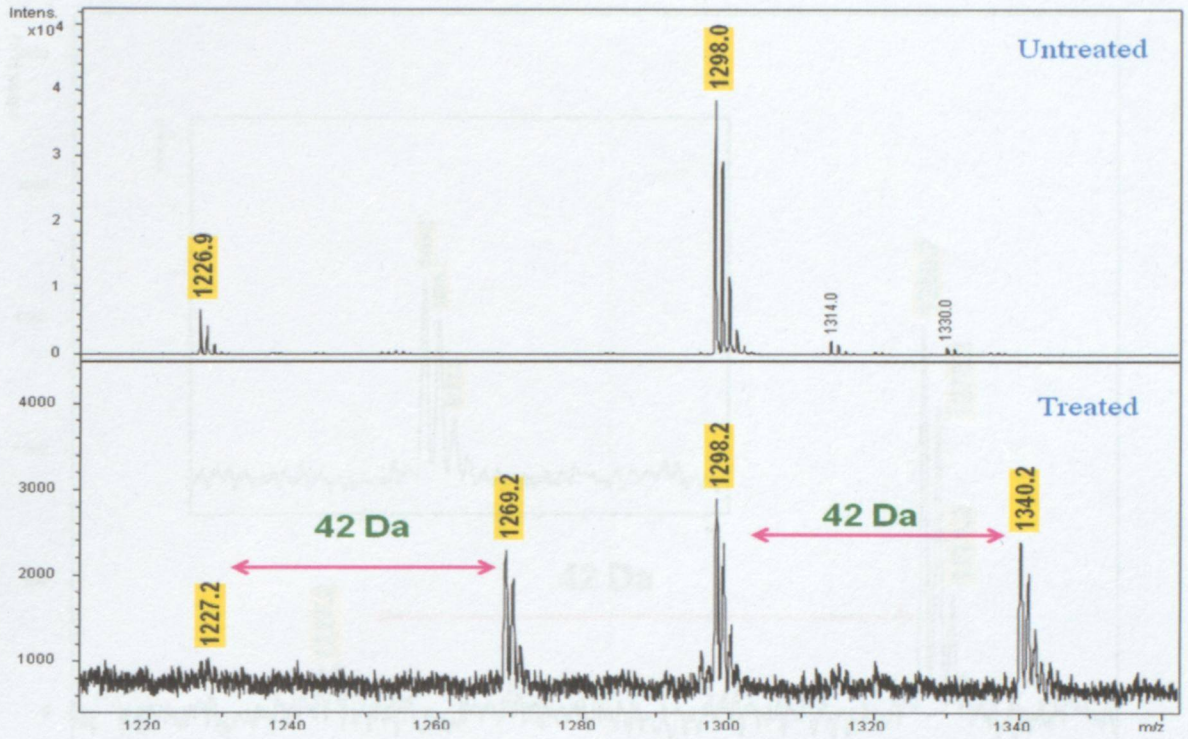


Figure 6: Acetylation of HPLC fraction 12

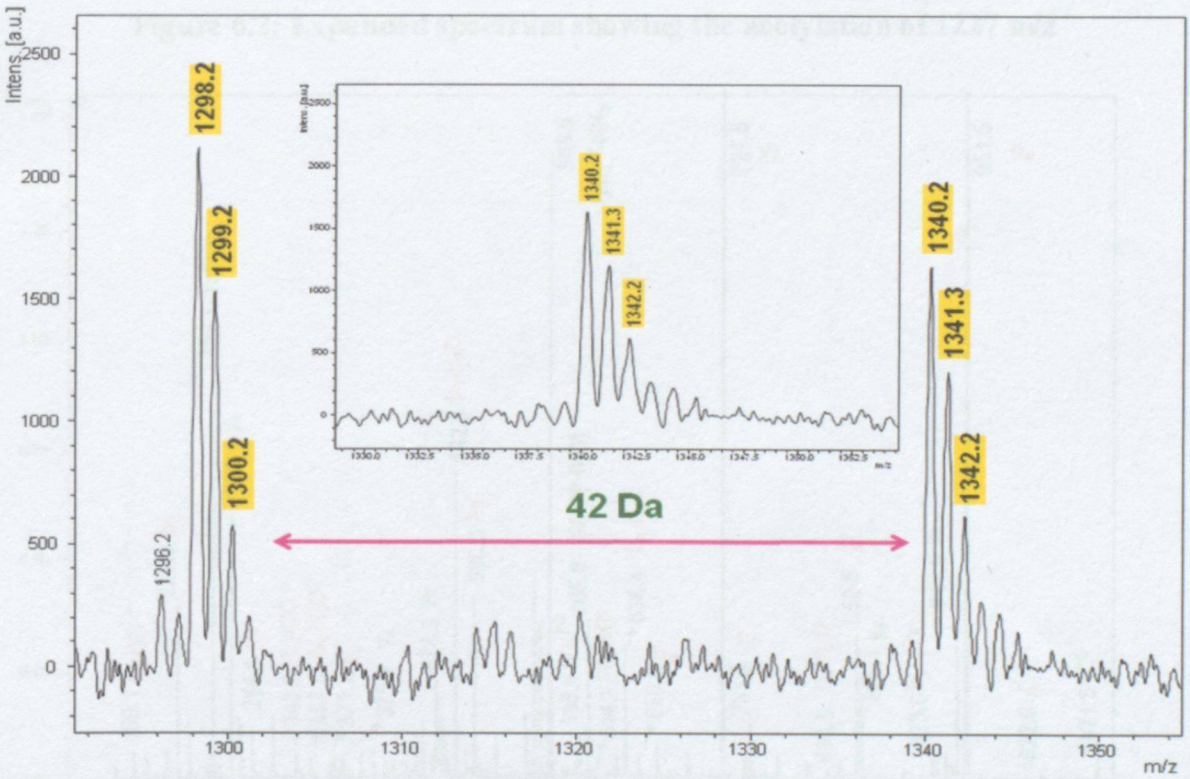


Figure 6.1: Expanded spectrum showing the acetylation of 1298 m/z

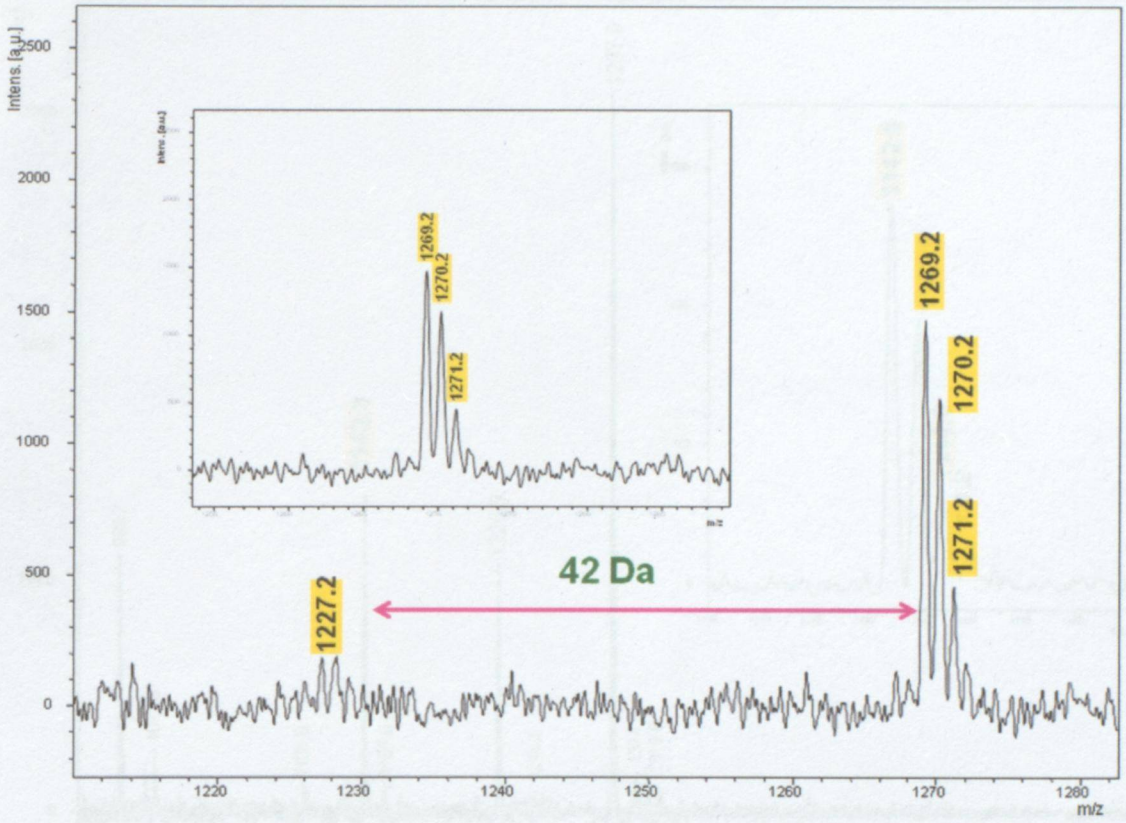


Figure 6.2: Expanded spectrum showing the acetylation of 1227 m/z

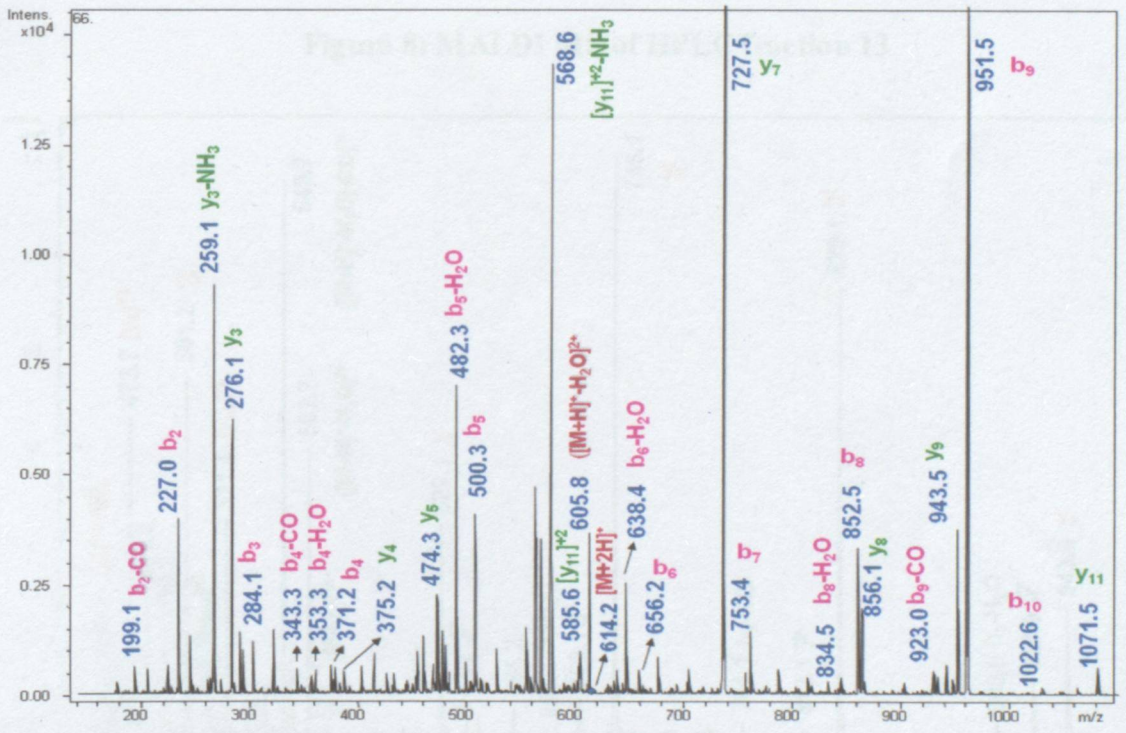


Figure 7. CID-MS/MS of doubly charged ion of peptide m/z 1227 $[M+2H]^{2+}$ 614.7 m/z

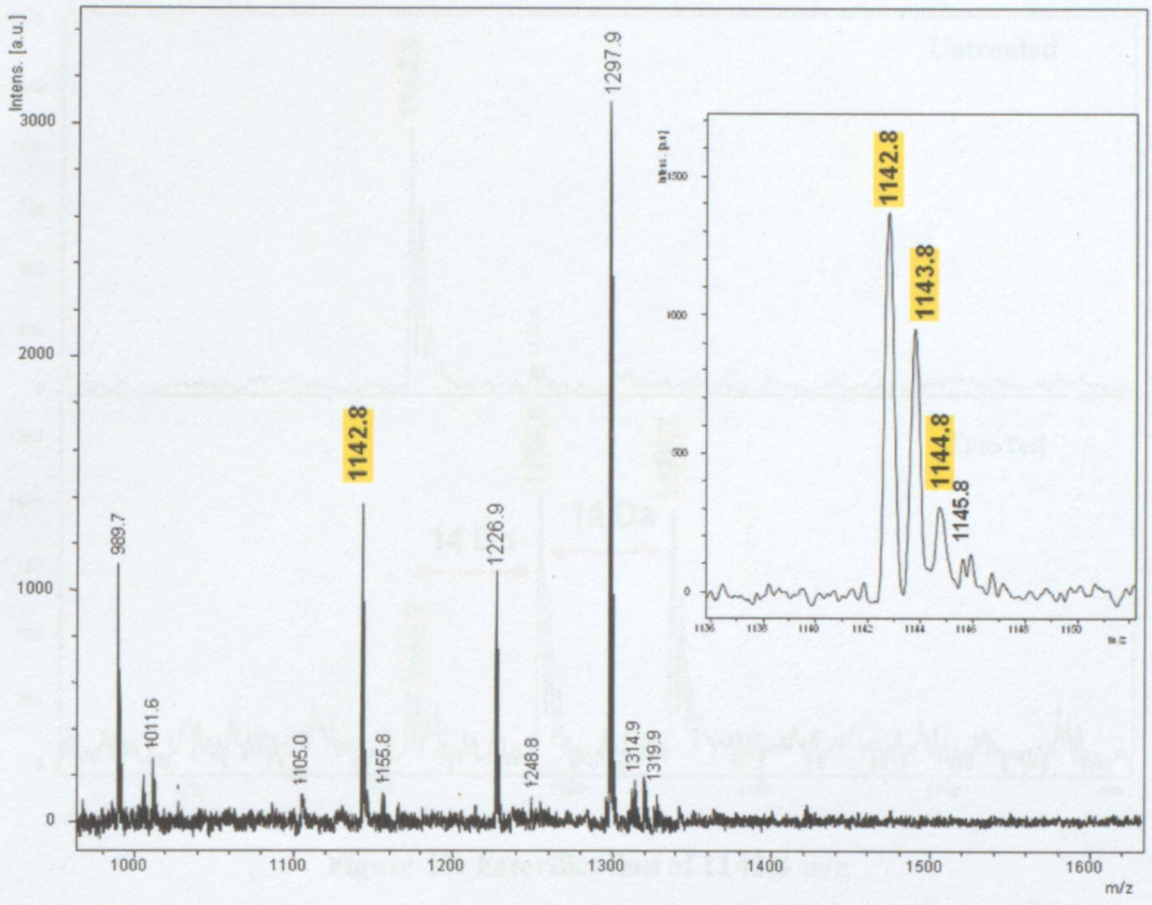


Figure 8: MALDI MS of HPLC fraction 13

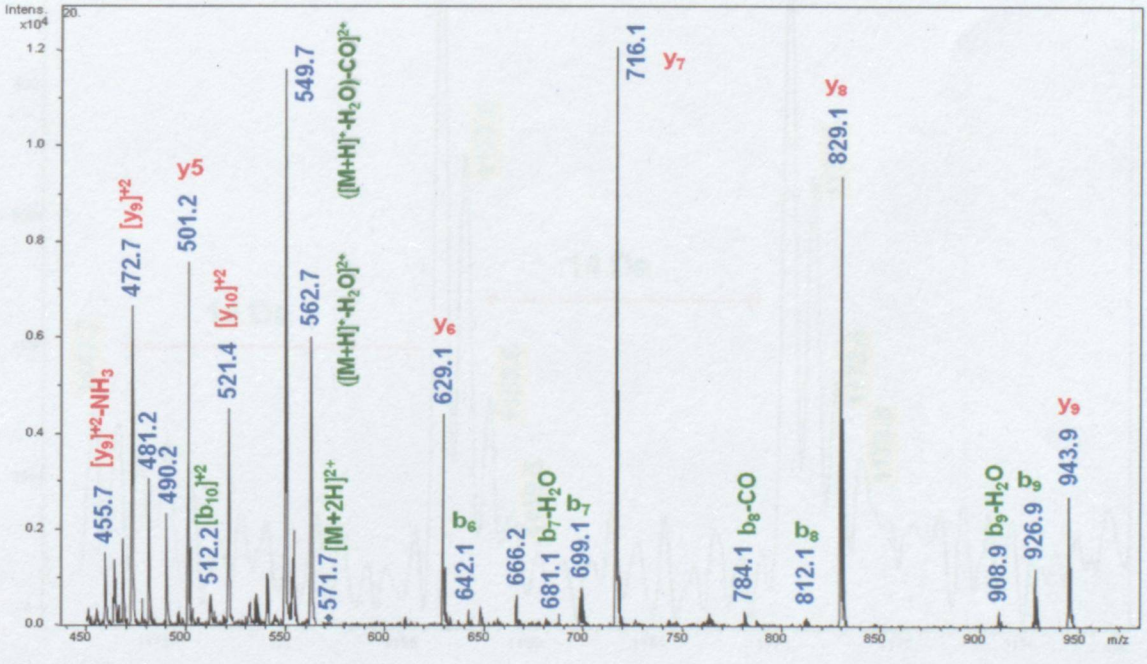


Figure 9: CID-MS/MS of doubly charged ion of peptide m/z 1142 $[M+2H]^{2+}$ 571.7 m/z

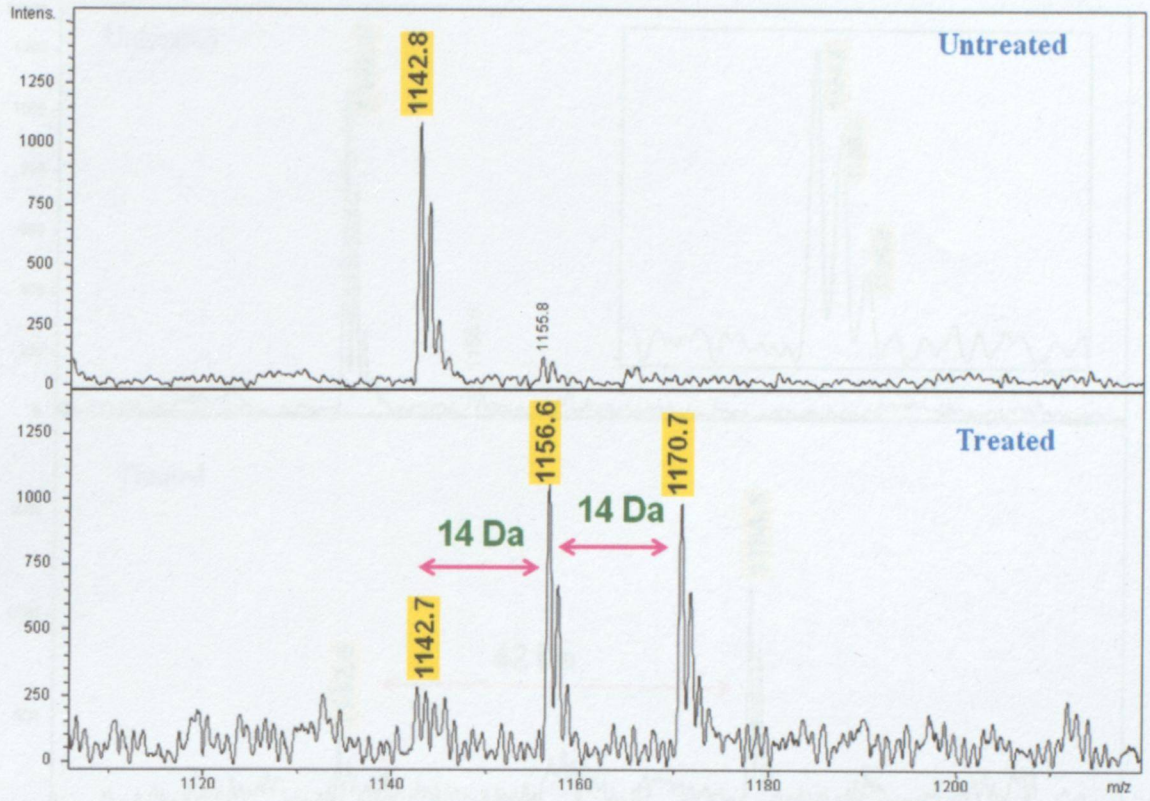


Figure 10: Esterification of 1142.8 m/z

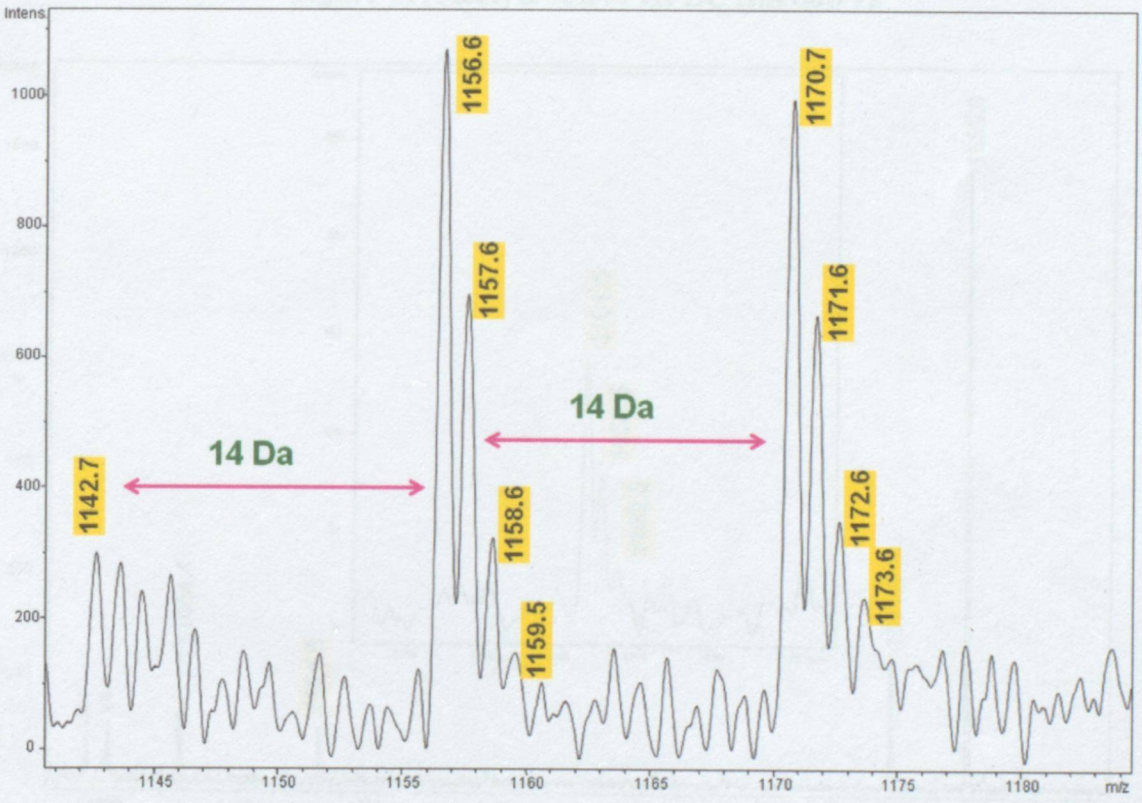


Figure 10.1: Expanded spectrum showing the esterification of 1142.8 m/z

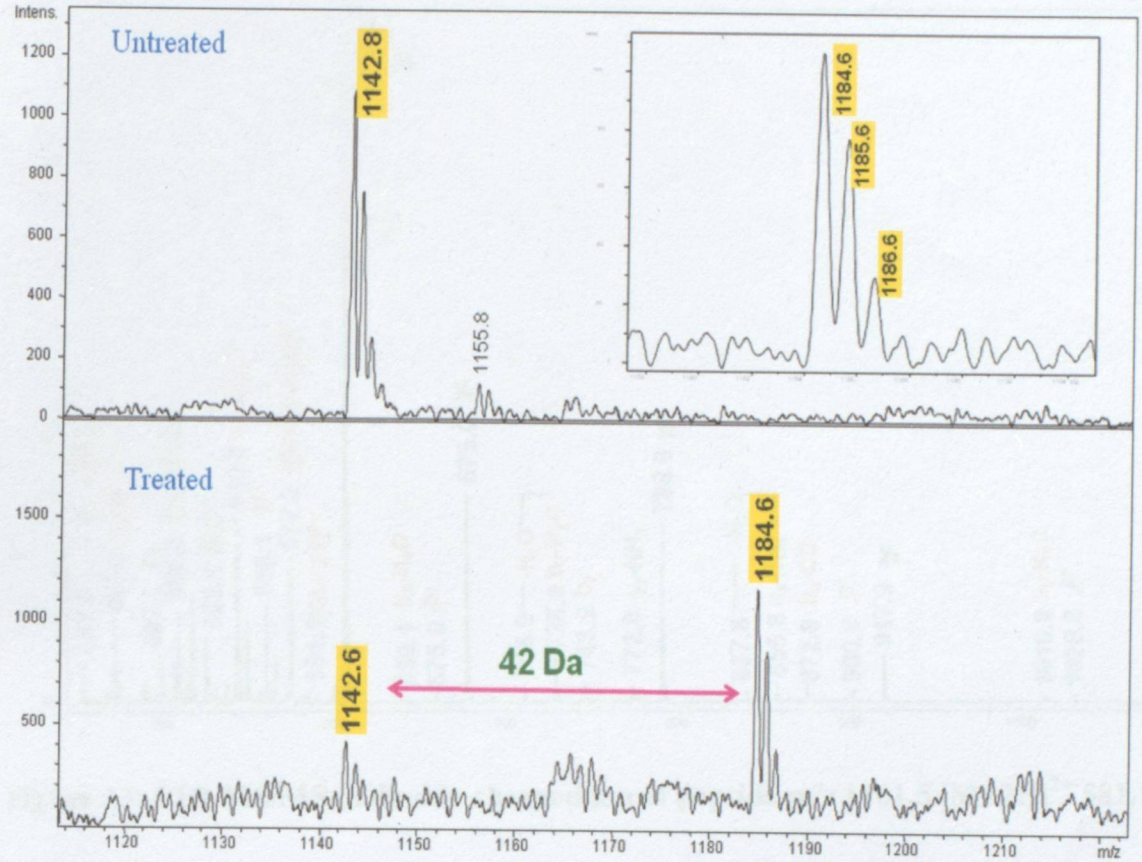


Figure 11: Acetylation of HPLC fraction 12

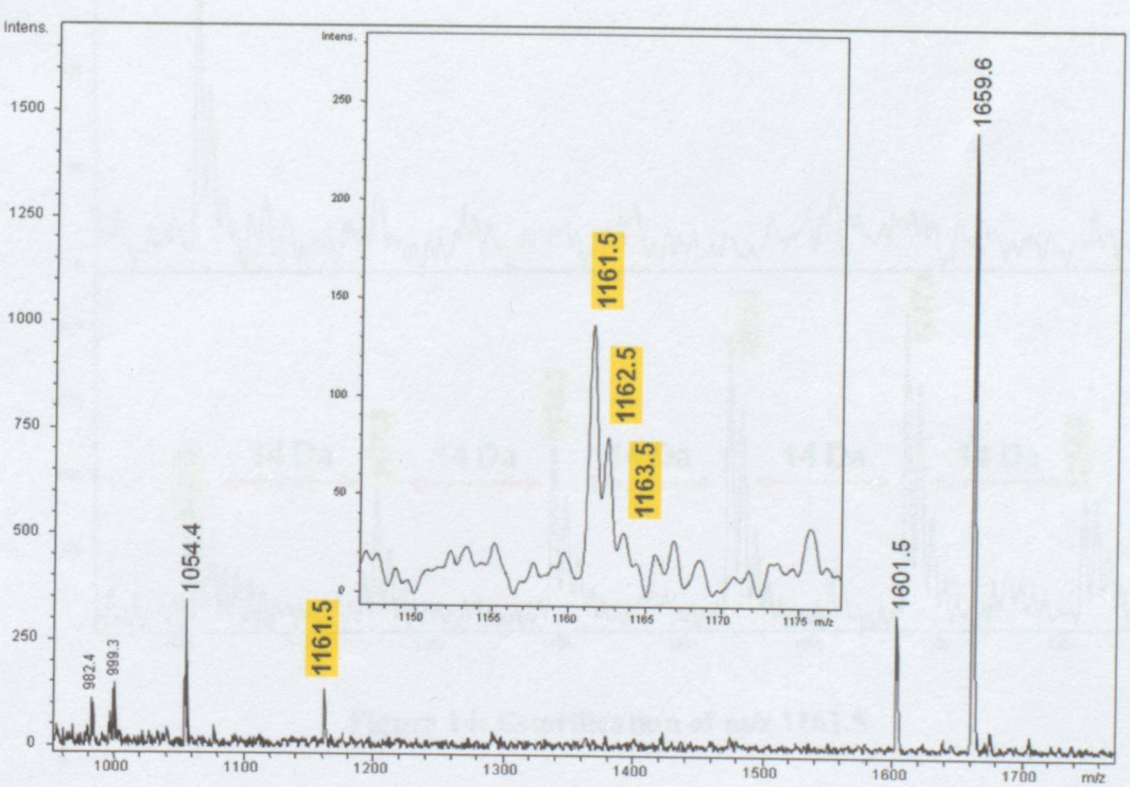


Figure 12: MALDI MS of HPLC fraction 3

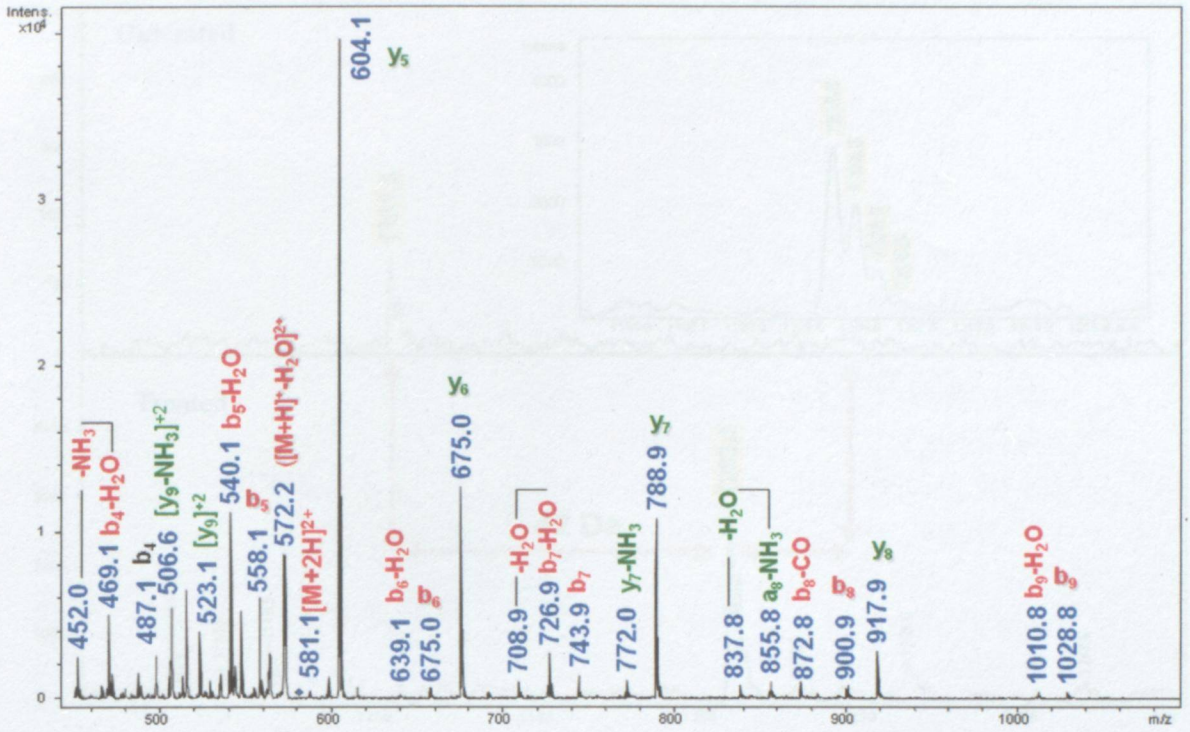


Figure 13: CID-MS/MS of doubly charged ion of peptide m/z 1161.5 $[M+2H]^{2+}$ 581.1 m/z

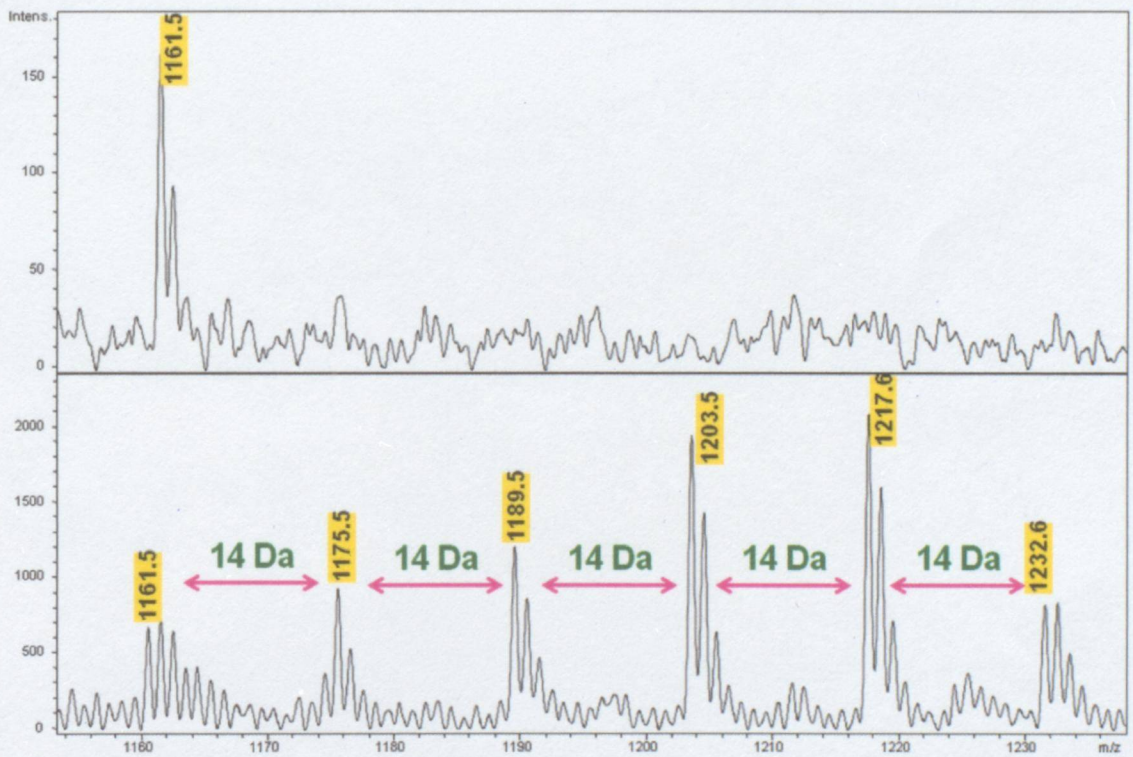


Figure 14: Esterification of m/z 1161.5

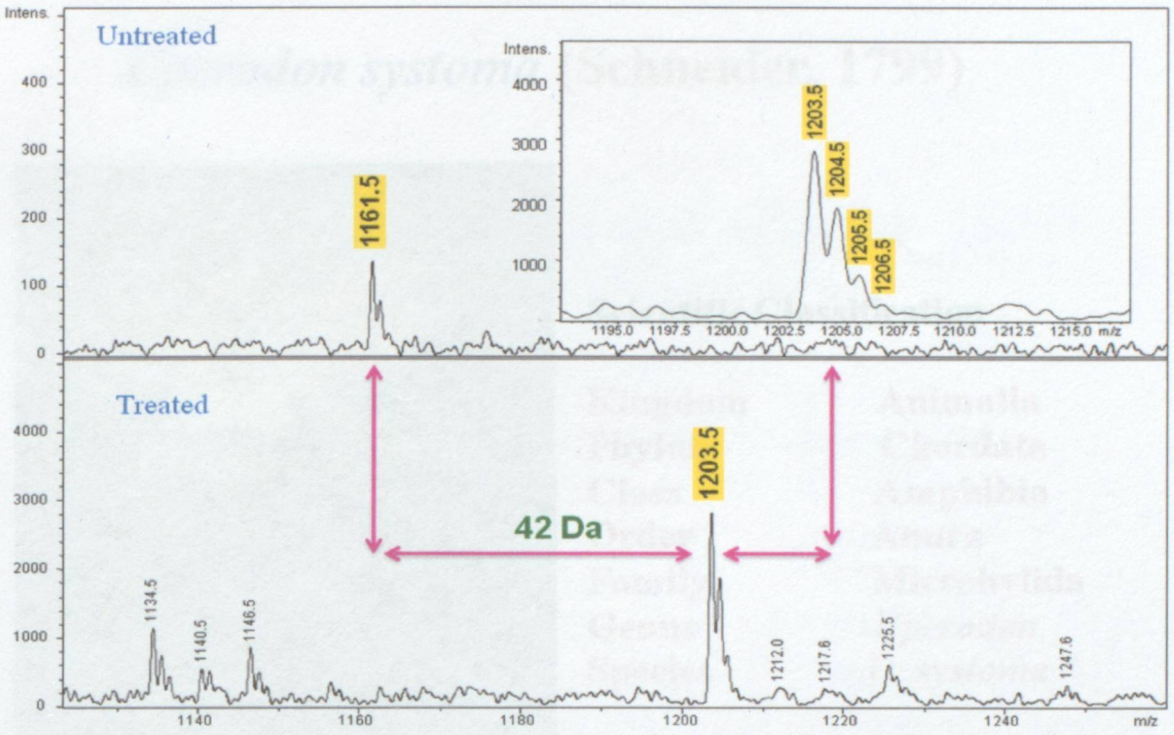


Figure 15: Acetylation of 1161.5 m/z

Uperodon systoma (Schneider, 1799)

Scientific Classification

Kingdom	Animalia
Phylum	Chordata
Class	Amphibia
Order	Anura
Family	Microhylida
Genus	*Uperodon*
Species	*U. systoma*

Collected from Orissa

Figure 16: ***Uperodon systoma***

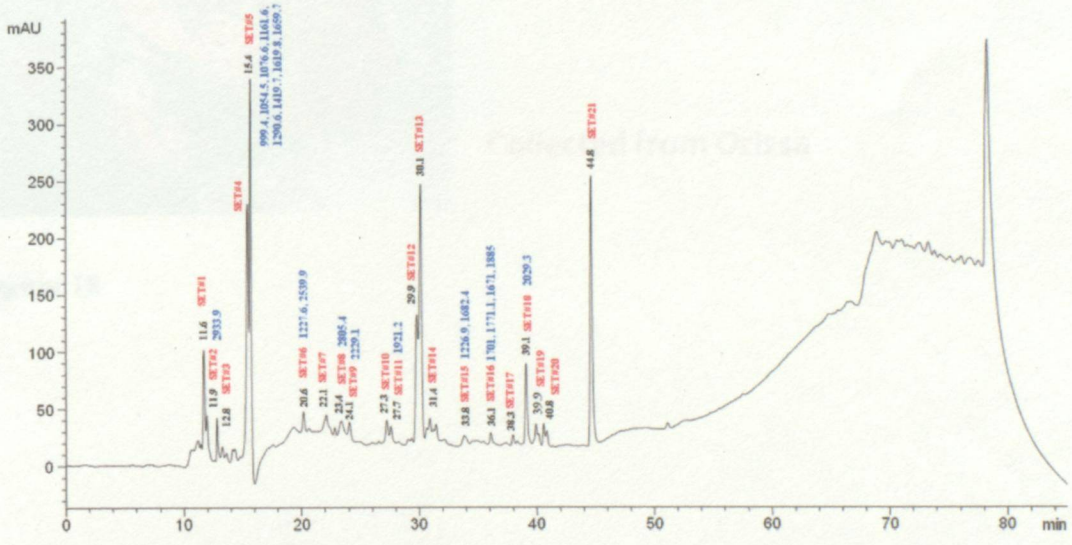
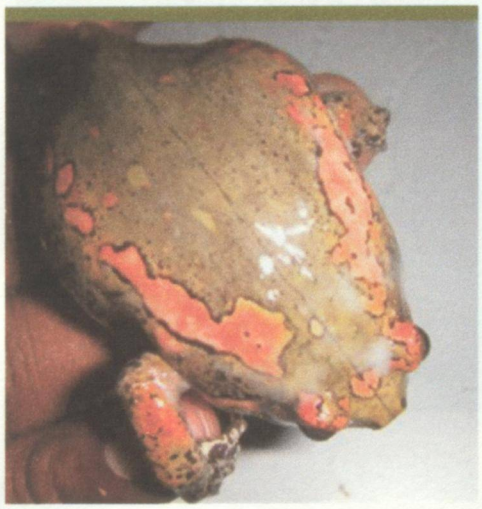


Figure 17: Reverse Phase HPLC Profile of ***Uperodon systoma*** **skin secretion**

Kaloula taprobanica (Parker, 1934)
(*Kaloula pulchra*)



Scientific classification

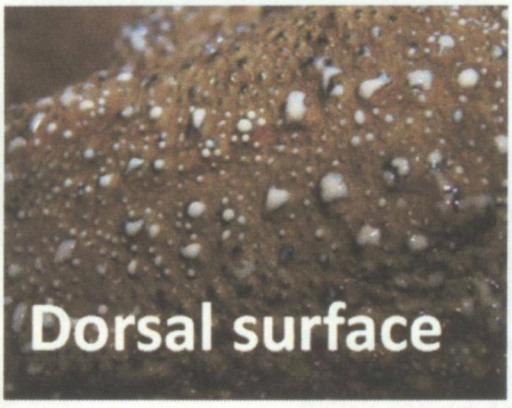
Kingdom	**Animalia**
Phylum	**Chordata**
Class	**Amphibia**
Order	**Anura**
Family	**Microhylidae**
Genus	*Kaloula*
Species	*K. taprobanica*

After 9V electrical stimulation

Collected from Orissa

Figure 18

Duttaphrynus himalayanus (Günther, 1864)



Scientific classification

Kingdom	**Animalia**
Phylum	**Chordata**
Class	**Amphibia**
Family	**Anura**
Order	**Bufonidae**
Genus	***Duttaphrynus***
Species	***D. himalayanus***

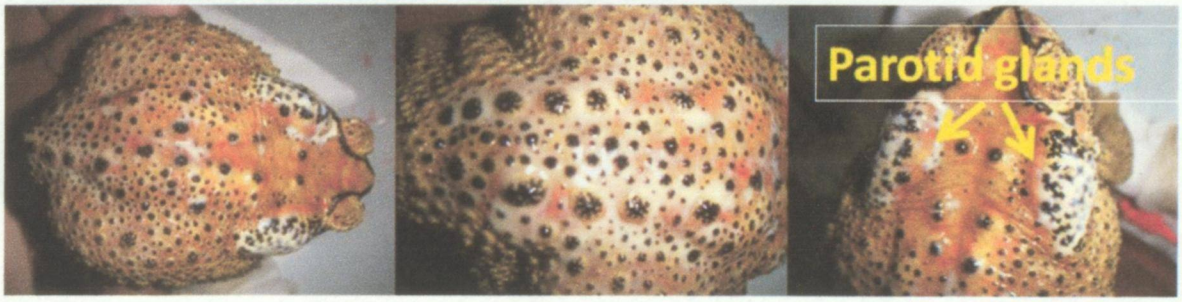
Collected from Dehradun

Figure 19

Duttaphrynus melanostictus (Schneider, 1799)

Scientific classification

Kingdom	Animalia
Phylum	Chordata
Class	Amphibia
Order	Anura
Family	Bufonidae
Genus	*Duttaphrynus*
Species	*D. melanostictus*



Dorsal surface after 9V electrical stimulation

Collected from Andaman

Figure 20

References:

Hancock R. E. W., Falla T. & Brown M. H. (1995) Cationic bactericidal peptides. *Advances in Microbial Physiology* **37** 135–175.

Hancock R. E. W & Lehrer R. (1998) Cationic peptides: a new source of antibiotics. *Trends in Biotechnology* **16** 82–88.

Lazarus L. H. & Atilla, M. (1993) The toad, ugly and venomous, wears yet a precious jewel in his skin. *Progress in Neurobiology*. **41**, 473-507.

Perez, M. Paul and P. Bazerque (1990)Antibiotic assay by agar-well diffusion method, *Acta Biologiae et Medicine Experimentalist* **15**, 13–115.

Simmaco M., Mignogna G. & Barra D. (1998) Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* **47** 435–450.

Sudarslal S., Majumdar S., Ramasamy P., Dhawan R., Pal P. P., Ramaswami M., Lala A. K., Sikdar S. K. , Sarma S. P, Krishnan K. S & Balaram P. Sodium channel modulating activity in a d-conotoxin from an Indian marine snail. *FEBS Letters* 553(1-2): 209-12, 2003.

Tyler M. J, Stone D. J. M, Bowie J. H. (1992) A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. *Journal of Pharmacological and Toxicological Methods* **28**:199–200.