FT-ICR MS-based structural characterization of a novel conotoxin from *Conus monile*: Determination of bromotryptophan by use of electron capture dissociation

Sudarslal Sadasivan Nair[1], Mark R. Emmett[1], Carol L. Nilsson[1], K.S. Krishnan[2], Padmanabhan Balaram[3], Alan G. Marshall[1]

[1]National High Magnetic Field Laboratory, Florida State University, FL 32310, USA

[2]Tata Institute of Fundamental Research, Mumbai 400005, India

[3]Indian Institute of Science, Bangalore 560065, India

INTRODUCTION

Primary structure, disulfide connectivity and post-translational modifications of biomolecules such as proteins and peptides can be determined by the application of fragmentation techniques unique to FT-ICR (1). We have performed electron capture dissociation (ECD) analysis of a peptide toxin (Mo1274) isolated from a vermivorous snail, *Conus monile*, to identify and localize bromination of tryptophan, a rare post-translational modification. The complete sequence, GNWCCSARVCC, in which W denotes bromotryptophan, was derived from MS-based *de novo* sequencing, employing electron capture dissociation, infrared multiphoton dissociation (IRMPD) and collision-induced dissociation (CID). Disulfide connectivity of Mo1274, between cysteine pairs 1-3 and 2-4, was determined by mass spectrometry in combination with chemical derivatization with tris(2-carboxyethyl) phosphine (TCEP), followed by differential alkylation with N-ethylmaleimide (NEM) and iodoacetamide.

RESULTS

Determination of Primary Structure

The mass spectrum of one of the HPLC fractions had an unusual isotopic distribution pattern, presumptive of containing one bromine atom. (Figure 1). The doubly-charged ion species, upon deconvolution yielded a neutral monoisotopic molecular mass of 1274.32 Da and is thus labeled Mo1274.

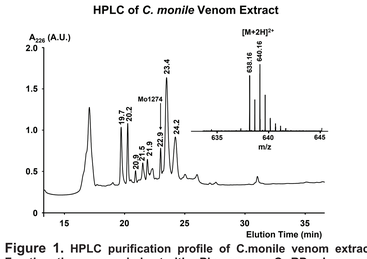

Figure 1. HPLC purification profile of C.monile venom extract. Fractionation was carried out with a Phenomenex C_{18} RP column and eluted at 1 mL min^{-1} with a linear gradient of acetonitrile, containing 0.1% TFA. Inset shows a 9.4 T ESI FT-ICR mass spectral segment for the LC peak at 22.9 min (denoted by an arrow in the chromatogram). The doubly charged peak at m/z 638.16 after deconvolution yielded a monoisotopic neutral mass of 1274.32 Da, subsequently referred to as Mo1274.

Following reduction with TCEP and alkylation with NEM, the mass of Mo1274 increased to 1778.54 Da, indicating the presence of two disulfide bonds. From CID and IRMPD data (Figures 2 & 3), a partial sequence was derived through manual analysis, including assignment of a bromotryptophan near the N-terminus.

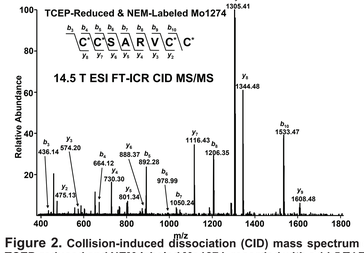

Figure 2. Collision-induced dissociation (CID) mass spectrum of TCEP-reduced and NEM-labeled Mo1274, recorded with a 14.5 T LTQ FT-ICR mass spectrometer. The doubly-charged ion at m/z 890.30 was selected as the precursor ion. Inset shows the sequence derived from this MS/MS spectrum. C* denotes alkylated cysteine.

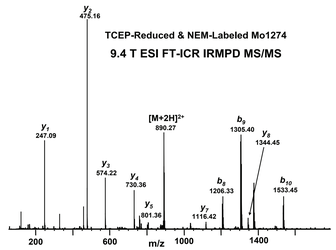

Figure 3. Infrared multiphoton dissociation (IRMPD) mass spectrum of TCEP-reduced and NEM-labeled Mo1274, recorded with a 9.4 T FT-ICR mass spectrometer. The doubly-charged ion at m/z 890.27 was selected as the precursor ion.

Complete amino acid sequence was obtained from ECD of the modified peptide, which produced distinct *z*- ions that retained the tryptophan bromination (Figure 4). The sequence was validated after digesting the NEM labeled peptide with trypsin, followed by MS/MS analysis (Figure 5).

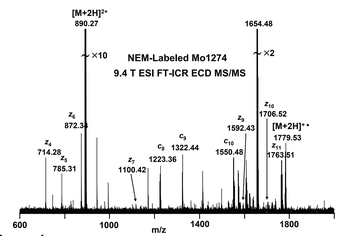

Figure 4. Electron capture dissociation (ECD) mass spectrum of doubly protonated, fully NEM-labeled Mo1274. The difference in mass of 57 Da between z_{10} and z_{11} ions identifies a glycine residue at position 1 of Mo1274. Similarly, the mass difference of 114 Da obtained from the ion pairs z_8 and z_{10} may be assigned to an asparagine residue at position 2. Note the peak at m/z 1654.48, corresponding to the previously unreported loss of one NEM moiety from the $[M+2H]^+$ ion species.

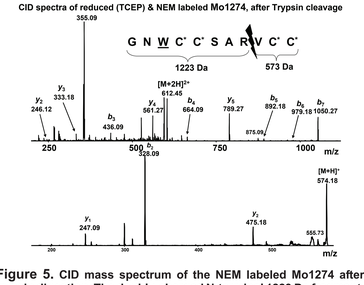

Figure 5. CID mass spectrum of the NEM labeled Mo1274 after trypsin digestion. The doubly-charged N-terminal 1223 Da fragment, and the C-terminal 573 Da fragments were isolated in a linear ion trap and subjected to CID MS/MS fragmentation.

Unique losses induced by ECD

When ECD was performed on the doubly protonated species of the *native* peptide, a loss of Br (m = 78.91) was observed (Figure 6). The loss of bromine is demonstrated not only by the mass difference but also in the change in the isotopic distribution. To probe the role of disulfide bonds among these different modes of fragmentation by ECD, we generated a partially modified peptide fraction (in which one of the two disulfides of Mo1274 was alkylated with NEM) followed by ECD fragmentation. In addition to Br loss, a prominent peak with a mass difference of 125.05 Da, from the charge reduced species was observed (Figure7). A similar peak was observed by ECD when all cysteines of Mo1274 were modified by NEM (Figure 4). These data indicate loss of NEM from the modified cysteine, given that the exact mass of NEM is 125.047 Da.

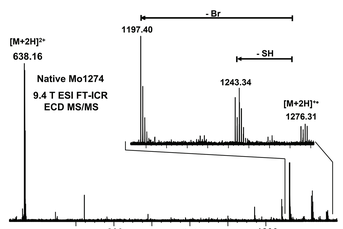

Figure 6. ECD mass spectrum of native Mo1274. The doubly protonated ion at m/z 638.16 was selected as the precursor ion. The ion of m/z 1276.31 $[M+2H]^+$ is formed from the precursor ion $[M+2H]^{2+}$ by charge neutralization. The prominent ions at m/z 1243.34 and 1197.40 originate from m/z 1276.31 through the loss of SH and Br moieties, respectively.

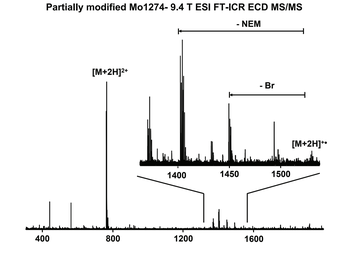

Figure 7. ECD mass spectrum of partially modified Mo1274, in which one pair of cysteines was labeled by NEM. Loss of Br and NEM represent the major peaks.

Disulfide linkage map

IRMPD analysis of the reduced and differentially labeled Mo1274 (sequence: GNWCCSARVCC, in which W is bromotryptophan) indicate that cysteine pairs 1-3 and 2-4 are disulfide-bonded (Figure 8).

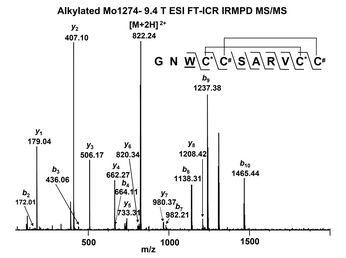

Figure 8. Infrared multiphoton dissociation mass spectrum of differentially alkylated Mo1274. The doubly-charged ion at m/z 822.24 is the precursor ion. Direct sequence analysis shows that the cysteines (labeled as C^*) at amino acid positions 4 and 10 are alkylated by NEM and the remaining cysteines (labeled as C^*) at positions 5 and 11 are alkylated by iodoacetamide.

METHOD

Isolation and purification of venom toxin

Snail venom preserved in ethanol was concentrated and fractionated with an HP 1100 series HPLC system. Initial purification was performed with a semi- preparative reversed-phase C18 column (Jupiter, Phenomenex, 10×250 mm,) with a 50 min 20% to 95% gradient elution of solution A (0.1% trifluoroacetic acid [TFA] [v/v] in water) and solution B (0.1% TFA [v/v] in acetonitrile). Final purification was done through an analytical column (Zorbax, 4.6×250 mm).

Chemical derivatization

Partial reduction and alkylation

For partial reduction, approximately 250 pmol of Mo1274 was dissolved in 50: 50 (v/v) water, 0.1% formic acid / acetonitrile and mixed with stock TCEP prepared in 200 mM sodium citrate buffer, pH 3.0, to obtain a final TCEP concentration of 5 mM. The solution was incubated at 37 °C for 5 min. Subsequent alkylation was performed by the addition of N-ethyl maleimide stock solution to a final concentration of 75 mM, followed by incubation at room temperature for 1 hour. Mixtures of partially and fully modified fractions were separated by the use of reversed-phase HPLC.

Complete reduction and alkylation

The remaining disulfide bond in the partially reduced and alkylated Mo1274 was completely reduced by treatment with 10 mM TCEP in 100 mM Tris-HCl buffer, pH 8.0 at 37 °C for 1 hour, followed by alkylation with 25 mM iodoacetamide at room temperature in the dark for 1 hour. The resulting peptide was purified by reversed-phase HPLC and analyzed by FT-ICR MS.

Mass spectrometry

All experiments were conducted with a home-built, unshielded 7.0 T (2), passively shielded 9.4 T FT-ICR mass spectrometer (3) or a linear ion trap LTQ system (Thermo Electron Corporation) coupled to a home-built 14.5 T ICR (4). Samples were infused by use of an automated nanoelectrospray system (Nanomate, Advion Biosciences, Ithaca, NY). For IRMPD, quadrupole-isolated precursor ions were photon irradiated with a 40 W, 10.6 μm CO_2 laser (Synrad, Mukilteo, WA). During ECD, the isolated ions were collided with low energy electrons emitted from an indirectly heated dispenser cathode (Heat Wave, Watsonville, CA). CID was performed in the linear ion trap mass spectrometer with He as the collision gas.

CONCLUSIONS

The primary structure of Mo1274, GNWCCSARVCC, in which W denotes bromotryptophan, was determined through mass spectrometry-based *de novo* sequencing. The disulfide connectivity between cysteine pairs 1-3 and 2-4 were established through chemical derivatization, followed by FT-ICR MS. Determination of the sequence, cysteine framework and assignment of disulfide linkage map classifies Mo1274 in the T-1 family of conotoxins.

In this study, ECD of the reduced and alkylated Mo1274 retains bromine; but if the disulfide bonds are still intact, ECD results in the loss of bromine. The loss of bromine under different conditions could prove useful for future conotoxin and similar analyses. It is possible that the presence of a disulfide bond favors an alternative fragmentation pathway that destabilizes the bromine linkage and releases it from the tryptophan residue.

ECD fragmentation of the fully or partially modified peptide generated loss of NEM. We were not able to observe that loss by IRMPD or CID. To the best of our knowledge, this result is the first observation of ECD-induced NEM loss.

ACKNOWLEDGMENTS

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Beauty and Beast: Collection of Cone snails from the Indian coast.