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Identification of Tyrosine Sulfation in *Conus pennaceus* Conotoxins α-PnIA and α-PnIB: Further Investigation of Labile Sulfo- and Phosphopeptides by Electrospray, Matrix-assisted Laser Desorption/Ionization (MALDI) and Atmospheric Pressure MALDI Mass Spectrometry†

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Liquid chromatography/electrospray ionization mass spectrometry was used to investigate the peptide composition of the venom of *Conus pennaceus*, a molluscivorous cone shell from the Red Sea. Based on observed M_rs, this venom contained all known conotoxins previously isolated and identified from this species. Interestingly, the doubly protonated species of only two of these conotoxins, α-PnIA and α-PnIB, showed additional related ions at +40 *m/z* (+80 Da), indicating the presence of either sulfation or phosphorylation in both components. High-performance liquid chromatographic (HPLC) fractions containing these two conotoxins were examined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry in both positive and negative ion modes, as well as by MALDI high-energy collision-induced dissociation. These experiments established the presence of a single sulfated tyrosine residue within both α-PnIA and α-PnIB. Hence their post-translationally modified sequences are GCCSLPPCAANNPDY(S)C-NH_2 (α-PnIA) and GCCSLPPCALSNPDY(S)C-NH_2 (α-PnIB). This assignment was supported by comparison of their mass spectral behavior with that of known sulfated and phosphorylated peptides. This data clarified further the distinguishing features of the ionization and fragmentation of such modified peptides. Selective disulfide folding of synthetic α-PnIB demonstrated that both sulfated and non-sulfated toxins co-elute on reversed-phase HPLC and that α-PnIB possesses the same disulfide connectivity as other 'classical' α-conotoxins reported previously. **Copyright © 1999 John Wiley & Sons, Ltd.**

KEYWORDS: sulfotyrosine; conotoxin; electrospray ionization; matrix-assisted laser desorption/ionization; atmospheric pressure matrix-assisted laser desorption/ionization

INTRODUCTION

Conotoxins are derived from venomous marine gastropods belonging to the Toxglossa family (Latin: poisonous tongued; genus *Conus*), which are widely distributed throughout the tropical and sub-tropical waters of the world. The venoms comprise a cocktail of short peptides (10–40 amino acids) that are highly constrained structurally by conserved patterns of multiple disulfide bridges. These venoms are used in conjunction with a unique harpoon-like radular tooth to immobilize prey rapidly.[1,2] Investigations of biologically active components of these venoms have yielded a wide variety of novel constituents which act selectively at a number of physiological sites, including the sodium and calcium channels, the nicotinic acetylcholine receptor and the sodium inactivation channel.[1] Pharmacologically these peptides have been classified into four main groups: μ-, ω-, α- and δ-conotoxins according to their respective targets.[3] Many of the peptides identified to date contain post-translational modifications such as γ-carboxylation of glutamic acid,[4,5] hydroxylation,[6,7] bromination,[8,9] D-amino acids[10] and *C*-terminal amidation.[1] Recently, sulfation was reported for an α-conotoxin from *Conus episcopateus.*[11]

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Conotoxins are of inherent interest as highly selective neurophysiological research tools and lead compounds for drug design. This latter feature is well illustrated by an ω-conotoxin, MVIIA (also known as SNX-111), isolated from *C. magus*. It has been shown to possess powerful analgesic properties against severe pain in AIDS and terminal cancer patients without the development of addiction or tolerance.[12] The venom of the molluscivorous snail *C. pennaceus* has been the centre of several investigations and different α-, μ-, γ- and ω-conotoxins have been characterized.[13–21] In the first report on *C. pennaceus*, two molluscan specific neurotoxic peptides, α-PnIA and α-PnIB, were found selectively to block the acetylcholine receptors of cultured *Aplysia* neurones.[13] The sequences of these highly conserved 16-residue peptides were established as GCCSLPPCAANNPDYC-NH_2 (α-PnIA) and GCCSLPPCALSNPDYC-NH_2 (α-PnIB).[13] Based on mass spectrometric evidence in this study, an additional component was detected that could represent the corresponding phosphorylated or sulfated analog.[13] Subsequently, chemical synthesis, non-selective folding and X-ray diffraction studies of the major synthetic products resulted in assignment of their disulfide connectivity,[17,19] although the identity of these synthetic conformers with the native conotoxins was not established. Here we report further analysis of the constituents of *C. pennaceus* venom and establish the post-translational modification of tyrosine by sulfation in both α-PnIA and α-PnIB by mass spectrometric methods, consistent with our earlier findings.[13]

EXPERIMENTAL

Materials

The Vt fraction of the venom was isolated as described previously.[13] The phosphorylated peptide EDYEY(P)TARF-NH_2 (M_r 1271.6) was provided by the ABRF peptide synthesis research group study. The sulfopeptide caerulein (qQDY(S)TGWMDF (M_r 1352.4)), was supplied by Sigma.

Synthetic α-PnIB was prepared on Rink Amide MBHA resin using an Applied Biosystems 433A synthesizer, using single coupling fluorenyloxycarbonyl (Fmoc) protocols with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and hydroxybenzotriazole (HOBt) activation, capping with *N*-(2-chlorobenzyloxycarbonyloxy)succinimide.[22] Deprotection and cleavage from the resin were carried out at room temperature for 2 h, with a 95% trifluoroacetic acid (TFA) solution (5 ml) containing thioanisole (0.25 ml) and ethanedithiol (0.125 ml) as scavengers. Subsequently it was found by mass spectrometry that these cleavage conditions caused loss of sulfate from sulfotyrosine. However, further experiments with lower concentrations of TFA (50%) established that the sulfopeptide could be cleaved successfully without the loss occurring. The crude peptide was purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) (C-18 Vydac, 10 μm, 250 × 10 mm i.d.), equilibrated at 50 °C.

Synthetic α-PnIB was folded by selective deprotection and oxidation of the cysteines. The trityl (trt) groups had been removed from Cys-2 and -8 during cleavage from the resin. The first disulfide bond was formed in 25 mM NH_4HCO_3 (pH 8.3) with continuous stirring at room temperature for 96 h. The folding solution was acidified with 10% TFA, filtered on a 0.45 μm filter and the peptide was isolated by RP-HPLC. The acetamidomethyl groups were removed from Cys-3 and -16 by rapid stirring in a freshly prepared solution of 0.04 M iodine in 50% acetic acid–0.03 M HCl. Excess iodine was quenched after 10 min by addition of thiosulfate. The final product was purified by RP-HPLC.

HPLC co-elution experiments were carried out on a Beckman System Gold instrument equipped with a 35 nl capillary flow cell and a C-18 Vydac column (5 μm, 150 × 1.0 mm i.d.): buffer A, H_2O (0.08% TFA); buffer B, acetonitrile (0.065% TFA); gradient, 5% B for 10 min and 5–100% B over 110 min; flow-rate, 50 μl min^{-1}; detection wavelength, 210 nm.

Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was carried out using a PE Biosystems Mariner orthogonal acceleration–time-of-flight (oa-TOF) mass spectrometer with external mass calibration. For use in the LC-MS mode of operation, the HPLC system consisted of an Applied Biosystems 140B syringe pump solvent delivery system connected to a Model 759A UV detector with a 35 nl capillary flow cell. The eluate at 50 μl min^{-1} was split to allow 4% to enter the electrospray source (2 μl/min), the remainder being diverted for manual fraction collection. A sample of the Vt fraction (equivalent to 80 μg of crude venom) was injected on to a microbore C-18 Vydac column (5 μm, 150 × 1.0 mm i.d.): buffer A, H_2O–0.1% formic acid; buffer B, ethanol–*n*-propanol (5:2)–0.05% formic acid;[23] gradient, 5% B for 10 min and 5–50% B over 110 min.

Positive and negative ion matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained on a PE Biosystems Voyager Elite TOF mass spectrometer in linear and reflectron mode using an extraction delay time of 185 ns. Spectra were accumulated from 100–200 laser shots. Analyte solutions were mixed in a 1:1 ratio with the standard matrix solution of α-cyano-4-hydroxycinnamic acid from Hewlett-Packard or a 'cooler' matrix of 2,6-dihydroxyacetophenone (DHAP)–diammonium hydrogencitrate (DAHC) (10:1, v/v) from Aldrich.[24] A standard mixture of known peptides (Bio-Rad CZE mixture plus ACTH clip peptide) was used for external calibration. Fragment ion spectra obtained by post-source decay (PSD) were smoothed to yield average mass values.[25] Air was used as the collision gas for fragment ions below *m/z* 200.[26] For atmospheric pressure (AP) MALDI measurements, the ESI source of the Mariner oa-TOF instrument was replaced by a laboratory-built AP-MALDI source using a 337 nm nitrogen laser (V. Laiko and A. L. Burlingame, US Patent pending and unpublished work). AP-MALDI mass spectra were recorded with a nozzle–skimmer voltage of 75 V.

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High-energy CID mass spectra were acquired on a Micromass Autospec SE hybrid sector-oa-TOF mass spectrometer,[27,28] calibrated in the negative ion mode using caerulein. Ionization was effected by MALDI using a dihydroxybenzoic acid matrix (saturated solution in 98% acetone, 2% H_2O–TFA (0.1%)). Only the ^{12}C isobars of the precursor ions of interest were selected in MS1 for CID and fragment ion analysis.

RESULTS AND DISCUSSION

Separation and LC/ESI-MS

The venom of *Conus pennaceus* was separated on a Sephadex G50 column to give two major fractions. As reported by Fainzilber *et al.*,[13] the mollusc-paralyzing activity is contained in the low molecular mass fraction containing peptides which elute close to the total volume (Vt) of the column. An aliquot of the Vt fraction corresponding to 80 μg of the crude venom was injected onto a C-18 microbore column. The small proportion of the LC flow diverted into the ESI-oa-TOF mass spectrometer gave the total ion current (TIC) trace shown in Fig. 1. Individual mass spectra yielded mainly doubly charged ions for each peptide but in some cases triply or singly charged species were also observed (see Table 1). Using the oa-TOF analyser, the mass resolution is sufficient to identify easily the charge states of the different ions from the spacing observed for the stable isotope profiles, as shown in the insets to the reconstructed single ion chromatograms in Fig. 1. Analysis of the molecular masses recorded allowed the assignment of all previously isolated conotoxins from the venom: α-PnIA,[13] α-PnIB,[13] μ-PnIVA,[14] μ-PnIVB,[14] ω-PnVIA,[18] ω-PnVIB[18] and γ-PnVIIA.[21]

The M_rs of α-PnIA and α-PnIB derived from the doubly charged ions (*m/z* 811.86 and 819.37) corresponded to those reported previously[13] but other ions higher by 40 *m/z* (80 Da) were also observed (*m/z* 851.86 and 859.36) (Fig. 1 and Table 1). The elution profiles for the higher mass peaks (not shown) demonstrated that these components were indeed due to α-PnIA and α-PnIB and could not be attributed to any other closely eluting compounds in the extract. The data in Fig. 1 were obtained with a nozzle–skimmer voltage (cone voltage) of 125 V. Reducing this to 75 V caused an increase in the relative abundance of the $[M + 2H]^{2+}$ species compared with $[M + 2H - 80]^{2+}$ to about 1:1. Further voltage reductions caused an overall loss of sensitivity without any further change in the peak ratio. A difference of 80 *m/z* was also observed for the corresponding weaker singly charged ions. These results

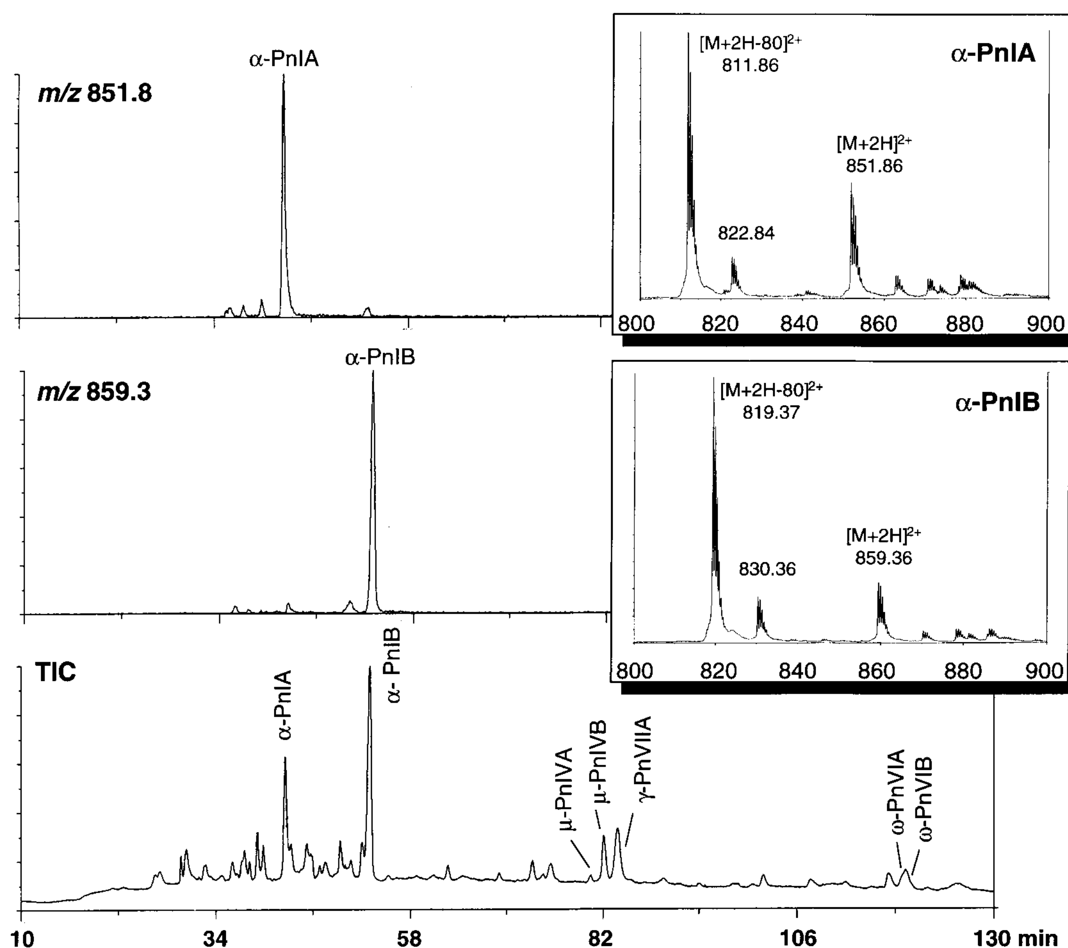

Figure 1. Bottom panel: TIC trace for the LC/ESI-oa-TOF analysis of the Vt fraction of conotoxins from *Conus pennaceus* venom. Top two panels: reconstructed single ion chromatograms for *m/z* 851.86 and 859.36, showing the elution of α-PnIA and α-PnIB. The insets show doubly charged ions in the mass spectra obtained during the elution of α-PnIA at 42.5 min and α-PnIB at 53.0 min.

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Table 1. ESI data obtained from LC/ESI-oa-TOF analysis of the Vt fraction of conotoxins from *Conus pennaceus* venom[a]

Conotoxin	Retention time (min)	$[M+3H]^{3+}$	*m/z* $[M+2H]^{2+}$	$[M+H]^{+}$	Experimental M_r	Theoretical M_r
α-PnIA	42.5		811.86	1622.69	1621.71	1621.59
α-PnIA(S)	42.5		851.86	1702.72	1701.71	1701.65
α-PnIB	53.0		819.37	1637.79	1636.74	1636.62
α-PnIB(S)	53.0		859.36	1717.85	1716.71	1716.68
μ-PnIVB	80.5		932.38		1862.76	1862.65
μ-PnIVA	82.1		895.89		1789.77	1789.65
γ-PnVIIA	83.9	1240.18	1859.78		3717.56	3718.00
ω-PnVIB	119.2	1089.83	1634.34		3266.68	3266.31
ω-PnVIA	119.8	1059.73	1589.22		3176.42	3176.18

[a] The conotoxins listed have been identified previously. The table gives the observed monoisotopic *m/z* for triply, doubly and singly charged ions, the charge states of which were determined by the spacing of the resolved isotopic clusters. The experimental M_r values were derived only from the doubly and/or triply charged ions as the singly charged ions were less well resolved.

support probable phosphorylation or sulfation of these two conotoxins.

MALDI/MS

Further mass spectrometric experiments were performed on the separated fractions containing the natural conotoxins α-PnIA and α-PnIB. A 1 μl aliquot of each was analyzed by UV–MALDI/MS using α-cyano-4-hydroxycinnamic acid as the matrix. The MALDI positive ion spectra for both α-PnIA and α-PnIB in both linear and reflectron mode revealed only the ions at *m/z* 1622.4 and 1637.5 together with corresponding sodium and potassium adduct signals, but no signals of 80 Da higher [Fig. 2(a)]. In addition, no intact phospho- or sulfo-containing molecular ions were observed employing either low laser irradiance in the linear mode or a 'cooler' matrix 2,6-dihydroxyacetophenone (DHAP)–diammonium hydrogencitrate (DAHC).[24] Furthermore, since there is evidence that IR–MALDI is a soft ionization method that permits the detection of labile phosphorylated species not seen by UV–MALDI,[29] we carried out further investigations using this technique. However, no intact molecular ions for the modified conotoxins or the sulfopeptide standard (data not shown) were detected. Finally, further

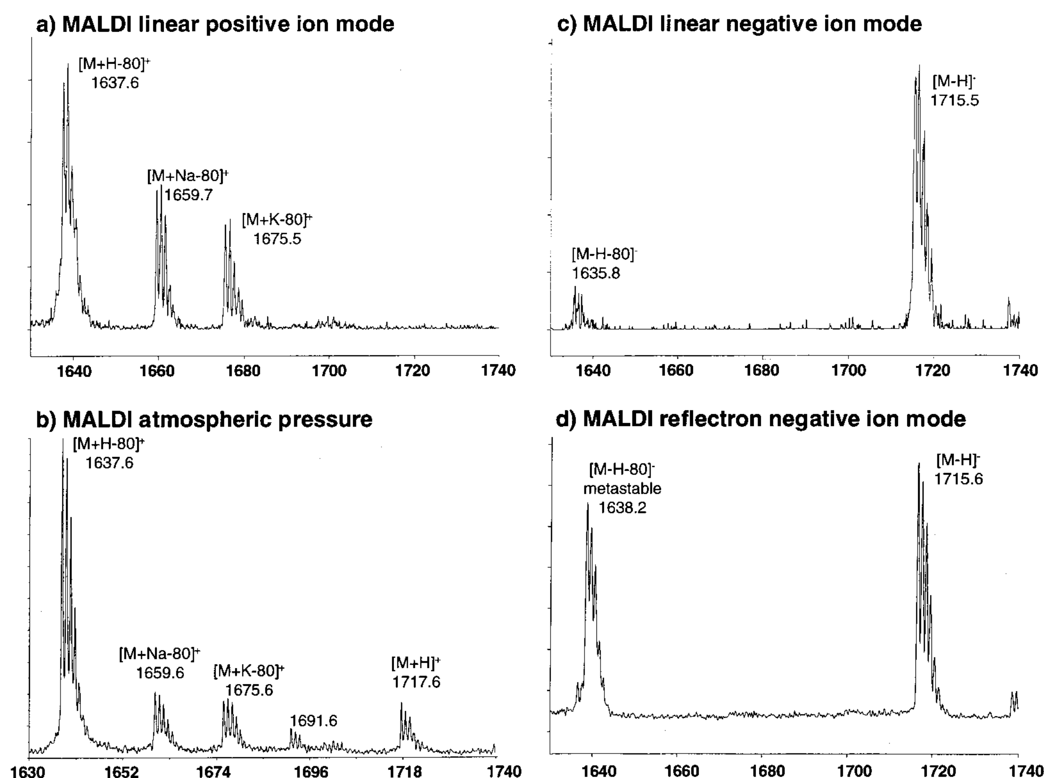

Figure 2. Representative MALDI-mass spectra for α-PnIB obtained under the following conditions: (a) conventional positive ion MALDI in linear mode; (b) positive ion AP-MALDI, also in linear mode; (c) the negative ion spectrum in linear mode; and (d) the negative ion spectrum in reflectron mode.

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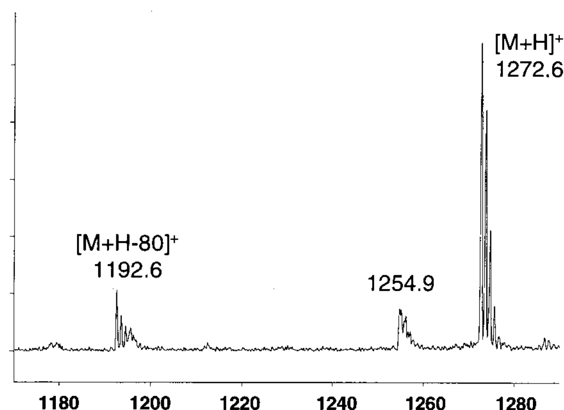
studies in the positive ion mode were carried out employing a new ionization method, atmospheric pressure (AP) MALDI-MS (V. Laiko and A. L. Burlingame, US Patent pending). The AP-MALDI mass spectra were recorded using a prototype source recently developed in this laboratory for the Mariner oa-TOF mass spectrometer.[30] As can be seen in Fig. 2(b), the spectra of all modified peptides clearly revealed the $[M + H]^+$ molecular ions, although the $[M + H - 80]^+$ species were more abundant. This is consistent with other evidence that the internal energy deposition during ionization/desorption by MALDI carried out at atmospheric pressure (V. Laiko and A. L. Burlingame, US Patent pending) is modulated by collisional damping, which preserves a significant fraction of pseudomolecular ions intact for acceleration into the oa-TOF analyser[30] (V. Laiko and A. L. Burlingame, unpublished work).

In earlier studies of sulfopeptides using liquid secondary ion MS (LSIMS), there is evidence that labile $[M + H]^+$ ions of sulfated peptides may lose SO_3 entirely, suggesting erroneously that such modified peptides are in fact not sulfated,[31] as well as contradictory evidence that intact sulfopeptide species may be detected with low level signals.[32] In the negative ion mode, the deprotonated anion was reported earlier for a sulfated peptide from blood coagulation factor III in linear MALDI using ferulic acid as the matrix,[33] while in the case of multiply sulfated peptides varying degrees of loss of 80 Da were observed using LSIMS in glycerol–thioglycerol.[32] Hence the degree of sulfotyrosine lability may depend on the peptide sequence in addition to the ionization conditions employed.

Unlike positive ion MALDI/MS as noted above, the negative ion spectra yielded deprotonated molecular ions for the modified species at m/z 1700.5 (α-PnIA) and 1715.6 (α-PnIB), in both the linear mode [Fig. 2(c)] and reflectron mode [Fig. 2(d)]. The approximate 78 m/z difference between the negative $[M - H]^-$ and the positive $[M + H - 80]^+$ ions observed in the current study indicates an actual difference in M_r of 80 Da, which corroborates the LC/ESI-MS results. Comparable data were obtained for the sulfated peptide standard caerulein, although this also exhibited a very weak but discernible $[M + H]^+$ ion using the DHAP–CHCA matrix (M. Person, unpublished results). In the negative ion linear mode a relatively weak ion at $[M - H - 80]^-$ was observable for all the native peptides plus the sulfated standard, suggesting a 'prompt' in source fragmentation [e.g. Fig. 2(c)]. In the corresponding negative reflectron spectra, more abundant unresolved metastable ions were recorded at slightly higher mass than anticipated, indicating that fragmentation also occurred in flight [e.g. Fig. 2(d)]. Some sulfated peptides have been observed to give $[M - H]^-$ ions in the linear mode but not in the reflectron mode (M. F. Medzihradszky, unpublished results).

The phosphopeptide standard analyzed under the same conditions displayed the phosphorylated molecular ion in both the positive and negative ion reflectron mode with little or no fragmentation (Fig. 3), confirming that the phosphate moiety is less labile than sulfate in the MALDI/MS ionization of peptides containing post-translationally modified tyrosine. Hence these results



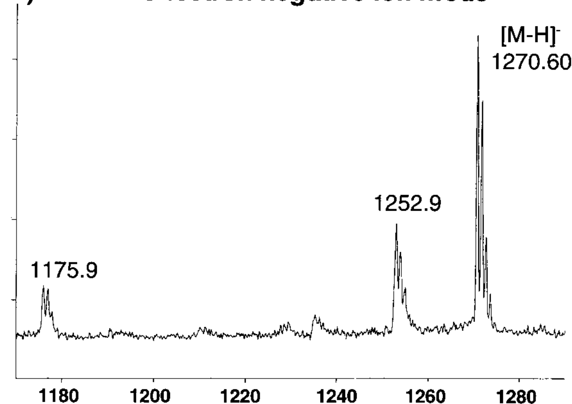


Figure 3. MALDI mass spectra of the phosphorylated standard EDYEY(P)TARF-NH_2 recorded in reflectron mode: (a) positive ion spectrum; (b) negative ion spectrum.

provide evidence that α-PnIA and α-PnIB are most probably sulfated rather than phosphorylated.

MALDI/CID-MS/MS

Since it has been established previously that these isobaric post-translational modifications can be distinguished unambiguously by LSIMS, high-energy CID in the negative ion mode giving the sulfur trioxide radical anion $SO_3^{-\bullet}$ (80 Da) or the PO_3^- (79 Da) anion, respectively,[31] and that the singly charged pseudomolecular ions formed by MALDI fragment in a fashion analogous to ions produced by LSIMS,[27,28] MALDI high-energy CID spectra were recorded employing a hybrid sector magnetic oa-TOF instrument in the negative ion mode. Using 1,5-dihydroxybenzoic acid (DHB) as the matrix, characteristic fragments for the natural conotoxins α-PnIA and α-PnIB in the HPLC fractions and also for the sulfated and phosphorylated standards were observed. In the case of α-PnIA, α-PnIB and the sulfated standard, m/z 80 was detected exclusively (e.g. Fig. 4), whereas for the phosphorylated standard only m/z 79 was observed (data not shown). All peptides gave a related peak at m/z 97 attributable to HSO_4^- in the case of sulfation and to $H_2PO_4^-$ in the phosphorylated case, m/z 96 ($SO_4^{-\bullet}$) ions were also present for all sulfated peptides. As expected, limited sequence-related fragmentation was observed in both the positive and

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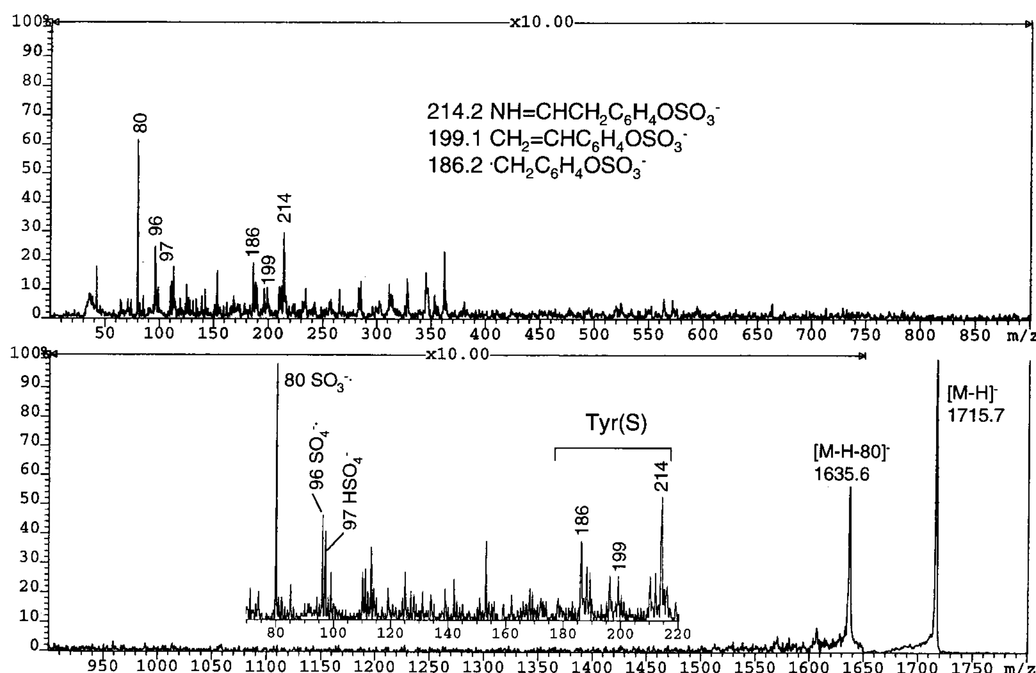

Figure 4. High-energy CID tandem mass spectrum in the negative ion mode of the deprotonated molecular ion of α-PnIB (*m*/*z* 1715.7). The expansion shows low-mass peaks that characterize the post-translational modification as sulfotyrosine.

negative ion CID spectra due to the internally cross-linked nature of the toxin imposed by the presence of the intact disulfide bonds. In contrast, no significant low-mass ions were observed in negative ion MALDI/MS using post-source decay (PSD) that would have permitted analogous differentiation between sulfation and phosphorylation.

In the amino acid sequences of α-PnIA and α-PnIB, sulfation could occur only at the single tyrosine residue present, Tyr-15. Evidence of this was in fact detected by the observation of the deprotonated tyrosine immonium ions at *m*/*z* 214, $NH{=}CHCH_2C_6H_4OSO_3^-$, in the MALDI high-energy negative ion CID spectra together with the hydrogen rearranged product $CH_2{=}CHC_6H_4OSO_3^-$ at *m*/*z* 199 and the radical anion side-chain cleavage product $^{\cdot}CH_2C_6H_4OSO_3^-$ at *m*/*z* 186 (Fig. 4). These observations are in excellent agreement with those previously reported on high-energy CID results for other peptides containing sulfated tyrosine using an LSIMS ion source.[31] This is an important practical consideration since MALDI/TOF/TOF instruments have been shown to generate analogous high-energy CID spectra.[34]

ESI/CID-MS/MS

In addition, when low-energy CID analysis of negative ions formed by ESI was employed, *m*/*z* 79 was in fact observed for the phosphorylated peptide as reported previously by Bean *et al.*[35] However, the sulfopeptides failed to give *m*/*z* 80 under analogous conditions using the small quantities of sample available in the case of the natural toxin. Therefore ESI/CID-MS/MS appears unsuitable for distinguishing unambiguously between these two modifications at picomole levels. For the detection of sulfo-peptides in a mixture this poor sensitivity could present difficulties in using the elegant SIM methodology pioneered by Carr and co-workers that works so well for the detection of phosphorylation.[36]

Synthetic peptides

The chemical synthesis of α-PnIB incorporating sulfated Tyr-15 with trityl (trt) protected Cys-2 and -8 and acetamidomethyl (acm) protected Cys-3 and -16 was attempted, to give the desired product GC(trt)C(acm)SLPPC(trt)AANNPDY(S)C(acm). This strategy would allow selective refolding via two discrete oxidation steps to give the previously identified disulfide-bridged isomer. The trityl groups were removed during general deprotection and cleavage from the resin, allowing the formation of the first disulfide loop (2–8) by slow oxidation in an ammonium hydrogen carbonate buffer. Formation of the second disulfide bond (3–16) took place upon iodine-promoted deprotection of the acm groups.[37] Unfortunately, mass spectrometry of the purified peptide showed loss of the sulfate, probably occurring during cleavage from the resin, consistent with the known acid lability of sulfate.[31] Subsequent cleavage of another sulfotyrosine-containing peptide under milder conditions confirmed this assumption.

Despite the loss of the sulfate from the synthetic material, HPLC analysis showed identical retention times for the synthetic non-sulfated peptide and the α-PnIB peak in the crude venom, even though the LC mass spectra of both constituents confirmed the difference in sulfation (Fig. 5). Furthermore, in an HPLC analysis of the synthetic peptide mixed with the crude Vt fraction of the venom, the sulfated and non-sulfated peptides co-eluted without any peak broadening or distortion. Thus, contrary to earlier suggestions.[11] these results indicate that this type of post-translational

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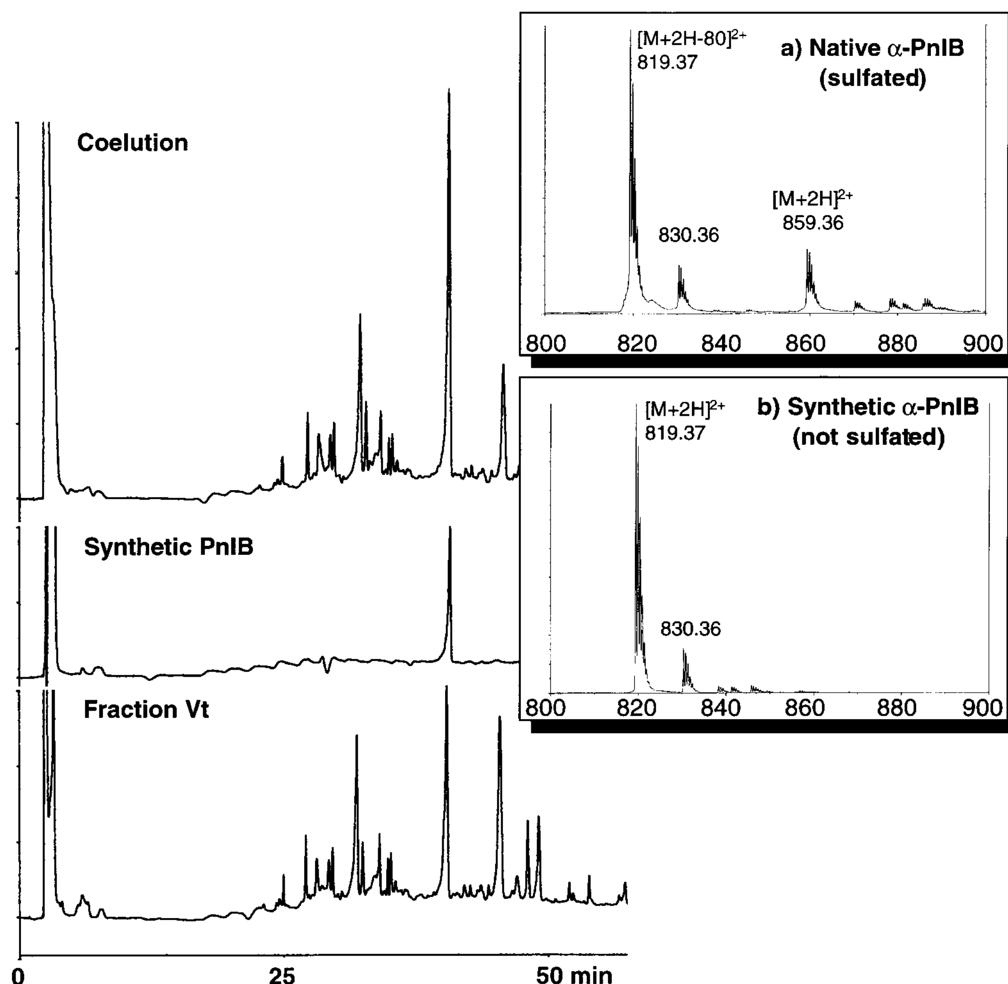

Figure 5. LC/UV traces (210 nm) of the Vt fraction of conotoxins from *Conus pennaceus* venom (bottom), the refolded oxidized synthetic peptide analogous to α-PnIB (middle) and a mixture of the synthetic peptide and the Vt fraction (top). The insets show doubly charged ions in the LC/ESI-oa-TOF mass spectra obtained during elution of the major peak at 40.6 min.

modification may not be detected by comparison of RP-HPLC retention times alone.

CONCLUSION

The LC/ESI-MS analysis of the Vt fraction of venom from *Conus pennaceus* has permitted comparative molecular mass analysis of all the published conotoxins reported previously. It also confirmed the earlier suggestion of additional post-translational modification of two-conotoxins, α-PnIA and α-PnIB. By using negative ion MALDI high-energy CID MS, this study has established the sulfation of Tyr-15 in both toxins. Thus the revised sequences are GCCSLPPCAANNPDY(S)C-NH_2 (α-PnIA) and GCCSLPPCALSNPDY(S)C-NH_2 (α-PnIB). Since the presence of a sulfotyrosine residue has already been reported recently for an α-conotoxin from another moluscivorous species (α-EpI),[11] our results suggest that this novel post-translational modification may prove to be a common feature in tyrosine-containing conotoxins from moluscivorous species. It remains to be determined whether the presence or absence of this modification has an influence on the biological activity. Interestingly, the other known conotoxins in this extract, μ-PnIVA,[14] μ-PnIVB,[14] ω-PnVIA[18] and γ-PnVIIA,[21] also contain tyrosine residues but they showed no evidence of sulfation by LC/ESI-MC. Furthermore, the absence of this modification was confirmed by negative ion MALDI. This suggests that this post-translational modification may occur preferentially in class I (loop 4–7) peptides with α-conotoxin type activity. The sequence identity in the C terminal loop region (NPDY(S)C-NH_2) within α-PnIA, α-PnIB and α-EpI, the other sulfated peptide identified from *C. episcopatus*,[11] may indicate a sequence specific site for a putative sulfotransferase enzyme[38] which carries out this novel post-translational modification. At present there have been no reports of the occurrence of sulfotyrosine in any conotoxins from piscivorous or vermivorous species.

Selective disulfide folding of synthetic α-PnIB demonstrated that both native sulfated and synthetic non-sulfated toxins co-elute. This indicates that this particular post-translational modification cannot be readily detected based on RP-HPLC retention time comparison alone. Furthermore, we have demonstrated that the analysis and identification of conotoxins by positive ion MALDI-MS alone using either UV or IR radiation is unlikely to reveal the presence of sulfotyrosine. However, sulfation is readily revealed in the

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negative ion mode. The combination of ESI-MS and positive and negative ion MALDI has also been revealed to be essential to ascertain other post-translational modification such as γ-carboxglutamic acids in the sequence of TxVIIA a conotoxin from *C. textile*.[4]

Comparison of the UV–MALDI mass spectra in both positive and negative ion modes provides a rapid means of discriminating between peptides containing phospho- and sulfotyrosine. Although both post-translational modifications provide 80 Da differences in M_r, sulfotyrosine-containing peptides give an intact molecular ion only in the negative ion mode, whereas phosphopeptides gives the intact molecular ion in both ionization modes. High-energy CID-MS/MS in the negative ion mode will provide unambiguous confirmation of the assignment.

In summary, we have established that an effective means of identifying new sulfated conotoxins is as follows. First an LC/ESI-MS separation and analysis of the crude venom may reveal ions with +80 Da adducts. Second, MALDI/MS analysis in the negative ion mode will show 80 Da mass differences compared with the corresponding positive ion spectra. Finally, high-energy CID of the $[M - H]^-$ ion will confirm the modification to be sulfation of tyrosine rather than phosphorylation. This approach, which has general utility for other sulfopeptides, is based entirely on mass spectrometry and eliminates the need for all of the chemical and enzymatic procedures[39] involved in more traditional approaches to the detection of sulfation of peptides and proteins.

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