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Analysis of Bromotryptophan and Hydroxyproline Modifications by High-Resolution, High-Accuracy Precursor Ion Scanning Utilizing Fragment Ions with Mass-Deficient Mass Tags

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Protein modifications are often detected by precursor ion scanning. When quadrupole TOF mass spectrometers are used for precursor ion scanning with high-resolution, high-accuracy fragment ion selection, "reporter" ions are required to have a unique mass within ±0.04 Da or less instead of ±0.5 Da on triple quadrupole mass spectrometers, the traditional instrument used for precursor ion scanning. Thus, characteristic fragment ions can be utilized even if other fragment ions have the same nominal mass as long as the characteristic fragment ions are slightly mass deficient as compared to the other fragments, i.e., when they have an inherent mass-deficient mass tag. Here, the immonium ions of bromotryptophan and hydroxyproline are described as two fragment ions characteristic for tryptophan-brominated and proline-hydroxylated peptides, respectively. The "reporter" ion of trytophan-brominated peptides is highly mass deficient due to the presence of bromine, thereby allowing the selective detection of these species and the distinction from other dipeptidic a-, b-, and y-fragment ions by high-resolution, high-accuracy precursor ion scanning. This strategy also enables the differentiation between precursors giving rise to the oxygen-containing immonium ion of hydroxyproline and precursors of the immonium ions of near-ubiquitous leucine/isoleucine. Both immonium ions have the same nominal mass of 86 Da, but the exact masses differ by less than 0.04 Da. High-resolution, high-accuracy precursor ion scanning enabled the identification of proline-hydroxylated and tryptophan-brominated species and the directed analysis of species carrying these modifications in a highly complex *Conus textile* conotoxin mixture. This lead to the characterization of one novel *C. textile* conotoxin containing a bromotryptophan residue and one novel *C. textile* conotoxin carrying two hydroxyproline residues.

After the sequencing of the human genome and having established methods for large-scale proteomics, major research efforts are now directed toward the analysis of protein modifications as they are of paramount importance for protein function.[1,2] Several hundred differently modified amino acid residues have been described so far[3] requiring a vast array of different methods for their analyses. Precursor ion scanning is a valuable tool for the detection of protein modifications as it is specific, sensitive, capable of handling complex sample mixtures, and broadly applicable to several types of modification such as phosphorylation,[4−7] glycosylation,[8,9] acetylation,[10] acylation,[11] 4-hydroxy-2-nonenal adduction,[12] sulfation,[11,13] and arginine dimethylation.[14]

Using precursor ion experiments for the selective detection of modified peptides in complex mixtures such as protein digests, the potential characteristic reporter ion must fulfill one major prerequisite to be useful for this type of experiment: The reporter ions must have unique masses in order to ensure specificity of the particular precursor ion experiments. If the other components present in the mixture give rise to fragment ions with the same nominal mass, nonspecific ion signals in the precursor ion spectrum will result. The definition of the term "unique mass" is determined by the resolution of the mass spectrometer used for the experiments. The traditional instrument used for precursor ion experiments is the triple quadrupole mass spectrometer with unit resolution, such that the masses of the reporter ions have to

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be unique within ±0.5 Da. Fragment ions that differ by less than this value cannot be resolved, resulting in reduced specificity of the precursor ion experiment.

This general lack of resolution of triple quadrupole instruments could in principle be overcome by the use of high-resolution, high-accuracy quadrupole TOF-type tandem mass spectrometers. For well-defined peaks, this type of instrument provides accuracies of <10 ppm and full width at half-maximum (fwhm) resolution exceeding 5000 even in the lower m/z-range.[15,16] However, to use these instruments for precursor ion scanning and to utilize the high resolution and high accuracy for this purpose, i.e., to circumvent quadrupole TOF-specific limitations, software and hardware solutions had to be implemented. The former addresses the problem that the TOF mass analyzer cannot be used as mass filter,[17] whereas two-dimensional ion trapping in combination with so-called Q_2-pulsing is the hardware solution used to compensate for the bias against small ionic species (m/z <250) observed for orthogonal acceleration TOF mass analyzers.[18−20] These hardware changes increased the duty cycle of quadrupole TOF instruments 10−20-fold, such that sensitivities for precursor ion experiments are similar to those performed on triple quadrupoles, the traditional instrument type used for precursor ion experiments.[21]

When quadrupole TOF type instruments are used for precursor ion experiments, the redefined "unique mass" facilitated by the high resolution must be accompanied with concomitant high accuracy in order to ensure unambiguous peak assignment. This means that quadrupole TOF mass spectrometers are the instruments of choice for this new type of precursor ion scanning, allowing the resolution of fragment ions that have the same nominal mass but differ in the exact masses by less than 0.06 Da. As a result of this high resolution and high accuracy, a characteristic reporter fragment ion for precursor ion scanning does not have to be unique within ±0.5 Da but <±0.04 Da thereby broadening the applicability of precursor ion experiments as recently shown: (i) tyrosine-phosphorylated species can selectively be detected based on the resolution of the immonium ion of phosphotyrosine at m/z 216.043 and other peptide-derived fragment ions of m/z 216.098 and larger;[21,22] we successfully applied this method in signaling research leading to the analysis of novel in vivo tyrosine phosphorylation sites and to the cloning of novel signaling molecules;[23,24] (ii) tyrosine-nitrated species can be identified based on the reporter ion at m/z 181.061 which is only 0.037 Da lighter than the interfering peptide-derived fragments.[25] These small mass shifts to lower m/z values are caused by a higher incidence of mass-deficient atoms such as oxygen, phosphorus, or sulfur in the particular reporter fragment ions. This results in an inherent *Ma*ss-*D*eficient *Ma*ss *T*ag (MaDMaT) for certain characteristic "reporter" ions as observed for the immonium ions of phosphotyrosine or nitrotyrosine. However, there are other protein modifications that contain a large fraction of mass-deficient elements and are amenable to precursor ion scanning experiments with high-resolution, high-accuracy fragment ion selection such as oxidation and halogenation because oxygen and also the halogens are mass deficient.

A conotoxin mixture isolated from *Conus textile* was analyzed by mass spectrometry as a test case with the aim to identify bromotryptophan- and hydroxyproline-containing components by high-resolution, high-accuracy precursor ion scanning and to sequence those species in a directed manner. Bromination of tryptophan and hydroxylation of proline residues are two modifications among many others that have been described for conotoxins (C-terminal amidation, glycosylation, sulfation of tyrosine residues, γ-carboxylation of glutamic acid residues, and disulfide bridge formation).[26−29] Conotoxins are highly potent peptide-based neurotoxins generated by the predatory marine snails of the genus *Conus*. The conotoxins are in general short (10−35 amino acid residues) cysteine-rich peptides with a well-defined three-dimensional structure imparted by the numerous disulfide bonds within the molecule. The targets of the conotoxins are in general specific ion channels and neuroreceptors in the central nervous system of the prey where they take effect within seconds, enabling snails to hunt fast-moving marine animals such as fish. Although many of the components are highly potent and lethal by themselves, the cone snails inject a complex mixture guaranteeing quick and broad response, i.e., fast paralysis and death of the prey.

Here we report characteristic fragment ions for both bromotryptophan- and hydroxyproline-containing peptides, which allowed their selective detection by precursor ion experiments on high-resolution, high-accuracy quadrupole TOF tandem mass spectrometers. Even though other peptide-derived fragment ions have the same nominal mass as the two characteristic fragment ions mentioned above, differentiation between the fragment ion of interest and interfering fragment ions is possible since both reporter ions carry a MaDMaT. This led to the directed sequencing of two novel *C. textile* conotoxins, one carrying two hydroxyproline residues whereas the other one is tryptophan-brominated.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Sigma-Aldrich (Vallensbaek Strand, Denmark). High-purity solvents for mass

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spectrometric analysis were purchased from Labscan (Dublin, Ireland).

Sample Preparation. The crude conotoxin mixture, isolated from *C. textile*,[29] was dissolved in 100 μL of tris-buffered saline (TBS) at a concentration of 10 mg/mL. For the reduction of the cysteine residues, 1 μL of 0.5 M tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added to 10 μL of the conotoxin solution prior to incubation for 2 h at room temperature. Subsequently, half of the reduced conotoxin mixture was desalted and concentrated on a Poros R1, Poros R2, and Oligo R3 (Perseptive, Framingham, MA) triple microcolumn prepared in GELoader tips (Eppendorf, Hamburg, Germany) following described procedures.[30,31] Use of a triple microcolumn provided a crude fractionation of the conotoxin mixture and removed a major fraction of the proteins present in the mixture as shown by SDS−PAGE. We discourage from the use of dithiolthreitol (DTT) for reducing disulfide bonds if Poros R1, Poros R2, and Oligo R3 microcolumns are subsequently used because they are not an efficient means for the removal of DTT, which interferes with ionization during nanoelectrospray experiments.

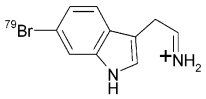
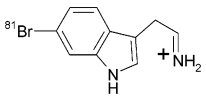
Mass Spectrometry. The conotoxins were desalted on the microcolumns (see above) by washing with 10 μL of 5% formic acid followed by elution with 5% formic acid in 20%, 40%, and 60% methanol/water directly into nanoelectrospray needles (Protana Engineering A/S, Odense, Denmark). All fractions were investigated by precursor ion scanning performed on a QSTAR Pulsar quadrupole time-of-flight mass spectrometer (AB/MDS Sciex, Concord, ON, Canada), equipped with a nanoelectrospray ion source (Protana Engineering). Precursor ion scans were acquired with a dwell time of 50 ms at a step size of 1 Da and with the Q_2-pulsing function turned on. Nitrogen was used as the collision gas at a recorded pressure of 5.3×10^{-5} Torr. The collision energy was ramped over the m/z range proportional to one-tenth of the m/z value of the precursor ion. Having a mass spectrometer in an air-conditioned environment ensures that a normal 2-point calibration is sufficient to provide the accuracy needed for high-resolution, high-accuracy precursor ion experiments.

RESULTS AND DISCUSSION

Bromotryptophan. Brominated amino acid residues are especially common in marine organisms because of the accessibility of bromine in the marine environment. Bromotryptophan-containing natural products from several organisms have also been described.[32] However, bromotryptophan within peptides/proteins encoded by mRNA has only been reported in 1997 and only for conotoxins.[33,34] The exact function of the bromination in conotoxins is currently under investigation.

Brominated compounds are well suited for the MaDMaT precursor ion scanning because the exact masses of both major isotopes of bromine (^{79}Br, ^{81}Br) are more than 0.08 Da below their nominal mass (78.9183 vs 79 and 80.9163 vs 81 Da). As a consequence, the immonium ion of [^{79}Br]bromotryptophan is at least 0.088 Da lighter than other peptide-derived fragment ions with the same nominal mass of 237 Da, whereas the immonium ion containing the ^{81}Br isotope is at least 0.102 Da lighter than interfering peptide fragments at 239 Da (see Table 1; depicted are the structures of the immonium ions of 6-bromotryptophan as this is the most common site of bromination). The number of potentially interfering fragment ions increases 6-fold if the loss of water and ammonia of the a-, b-, and y-ions is also considered. Even though these types of fragment ions are not commonly observed under the CID conditions used for peptide sequencing, they are easily formed with the increased collision energies used in the precursor ion experiments to promote generation of immonium ions. Nevertheless, none of these ions interferes with the mass of the bromotryptophan reporter ion either.

Table 1. Structure of the Immonium Ion of 6-Bromotryptophan and List of Exact Masses of All Possible a-, b- and y-Type Fragment Ions of Unmodified Peptides Giving Rise to Signals at a Nominal Mass of 237 or 239 Da upon Low-Energy CID[a]

Fragment Ion	Exact Mass [Da]	ΔMass [Da]
	237.003	
y_2 (MS); y_2 (AMox)	237.091	0.088
y_2 (AF); a_2 (TY)	237.124	0.121
b_2 (HV)	237.135	0.132
	239.001	
y_2 (GY)	239.103	0.102
b_2 (HT); a_2 (EH)	239.114	0.113

[a]Loss of ammonia or water is not taken into account.

The mass spectrum of the Oligo R3/40% methanol fraction is shown in Figure 1A. The complexity of this sample is evident despite the prefractionation. Precursor ion experiments to identify bromotryptophan-containing precursors giving rise to the characteristic bromotryptophan immonium ion at m/z 237.003 were performed on this particular Oligo R3 fraction. These experiments were carried out with and without high-resolution, high-accuracy fragment ion selection and are displayed in Figure 1B and C, respectively. The nonspecific, low-resolution precursor ion experiment (m/z 237.10 ± 0.25) shows several major ion signals. However, the specific, high-resolution, high-accuracy experiment (m/z 237.003 ± 0.020) revealed that only the ion signal at m/z 698 was attributable to a tryptophan-brominated compound.

An identical set of experiments was performed for the ^{81}Br-containing immonium ion at m/z 239.001 (precursors of (m/z 239.10 ± 0.25) and precursors (m/z 239.001 ± 0.020); see Figure 1D and E). The low-resolution precursor ion experiment at m/z 239.10 ± 0.25 versus the low-resolution experiment at m/z 237.10 ± 0.25 shows less intense nonspecific ion signals and might be reflective of the fact that there are less interfering ions at this

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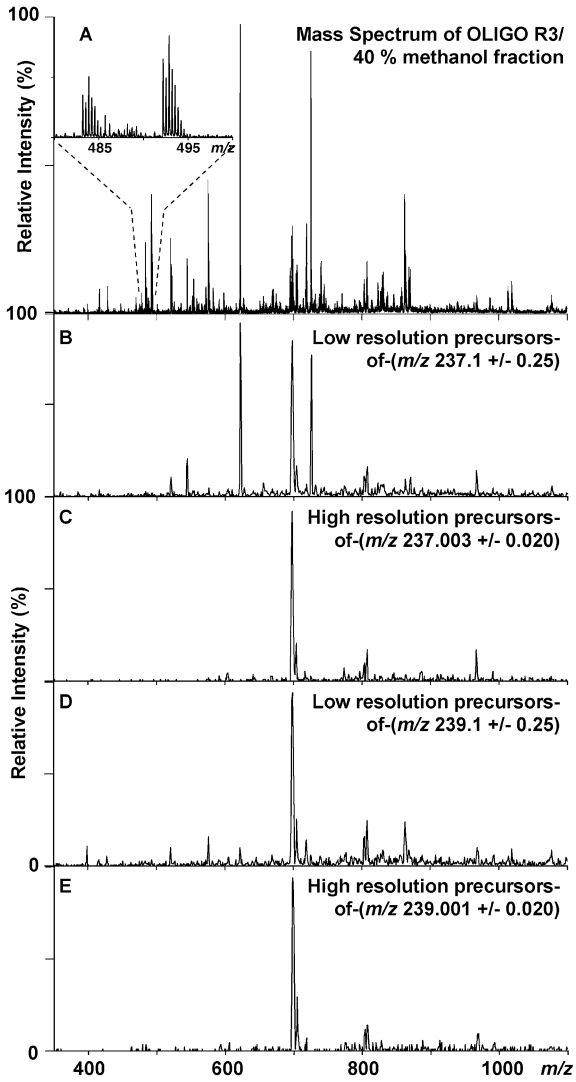

Figure 1. Analysis of the Oligo R3/40% methanol fraction of a crude conotoxin mixture derived from *C. textile*. (A) Nanoelectrospray quadrupole-TOF mass spectrum of the conotoxin mixture. The inset shows two peak pairs with isotope pattern resembling those of brominated species. (B) Precursor ion scan for *m*/*z* 237.1 (±0.25). This spectrum is comparable to a similar experiment on a well-tuned triple quadrupole mass spectrometer. Apart from the [^{79}Br]bromotryptophan-containing conotoxin, signals corresponding to precursors giving rise to interfering fragment ions are observed. Thus, no substantial claim regarding the presence of bromotryptophan in this mixture can be made. (C) Precursor ion scan for *m*/*z* 237.003 (±0.020). The major ion signal corresponds to the main tryptophan-brominated species in this fraction. (D) Precursor ion scan for *m*/*z* 239.1 (±0.25) similar to a precursor ion experiment on a well-tuned triple quadrupole mass spectrometer. Apart from the [^{81}Br]bromotryptophan-containing conotoxin, numerous nonspecific ion signals are observed. (E) Precursor ion scan for *m*/*z* 239.001 (±0.020) showing the same the same major tryptophan-brominated species as Figure 2C.

mass (see Table 1). Nevertheless, the nonspecific ion signals outnumber the specific ion signals in both experiments.

Only one major species in this fraction of the *C. textile* conotoxin mixture was tryptophan-brominated. This doubly charged species at *m*/*z* 698.7 was subjected to further MS/MS analysis in order to determine the peptide sequence. The resulting product ion spectrum is shown in Figure 2. Complete y- and b-fragment ion series were observable, providing the complete sequence of a novel *C. textile* conotoxin. Its primary structure is H_2N-XCCYPNVW(Br)CCD$-CO_2H$ (X = L and I, which cannot normally be differentiated by low-energy CID; high-energy CID or cell culturing with labeled amino acids is required to differentiate leucine and isoleucine by mass spectrometry[35]). The distribution of the cysteine residues (...CC.....CC...) makes this novel conotoxin a member of the T-superfamily.[36] The strong y_1-ion at *m*/*z* 134 allows the unambiguous assignment of an aspartic acid residue with a free carboxyl group at the C-terminus of the conotoxin despite the fact that conotoxins are commonly amidated at the C-termini.[26] A homology search with the sequence of this novel conotoxin did not retrieve any peptides with homologous sequences, indicating the limitations of homology-based de novo sequencing software.[37]

Instead of using precursor ion scanning for the identification of compounds containing brominated tryptophan residues, the mass spectrum can also be scanned for ion signals with an isotope pattern characteristic for bromine-containing compounds, i.e., two monoisotopic peaks of similar intensity 2 Da apart. However, this method can be quite error prone, as there are several possible explanations for a peak pair of similar abundance: (i) pure coincidence, (ii) incomplete reduction of a compound with one disulfide bond yielding only 50% reduced species, and (iii) bromination in general. If the mass spectrum in Figure 1A is scrutinized for this characteristic pattern, two groups of ion signals at *m*/*z* 483.21/483.87 and 492.21/492.88 are found (see inset Figure 1A). However, the precursor ion spectra did not display any ion signal at the *m*/*z* values of these peak pairs such that it can be presumed that the compounds giving rise to these peaks did not contain bromotryptophan residues; nevertheless, it is conceivable that these ion signals originated from bromotryptophan-containing species, but insufficient fragmentation prevented the detection by precursor ion scanning. Further experiments would be required to elucidate which of the above-mentioned reasons caused the isotope pattern observed, but it is clear that the MaDMaT method is far more specific than scanning for isotope patterns.

Hydroxyproline. Proline hydroxylation is a very common protein modification. In plants, it is found in numerous glycoproteins whereas in animals it is associated with some of the most abundant proteins: collagens, the matrix of bone, skin, and other tissues. The hydroxylation of proline residues within collagen has an important influence on its stability.[38] Apart from structural functions, proline hydroxylation also has a crucial role in oxygen sensing and the regulation of gene expression by oxygen as recently shown.[39,40]

Proline hydroxylation has been observed repeatedly as a posttranslation modification in conotoxins.[26] Although hydroxyproline has the same nominal residue mass of 113 Da as the

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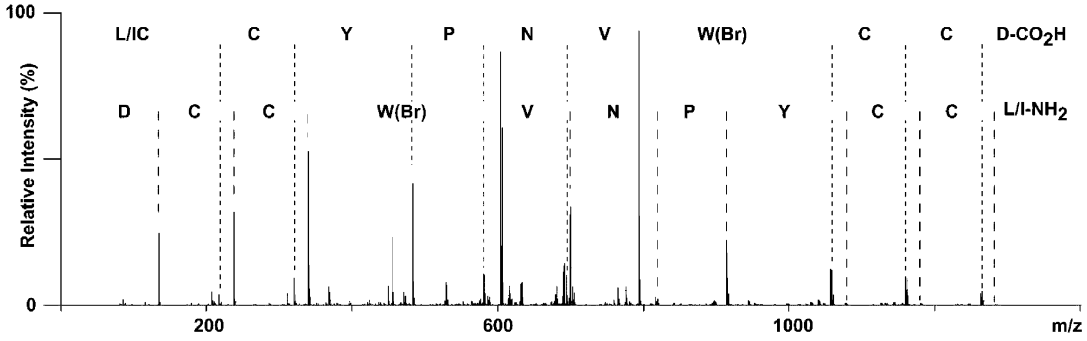
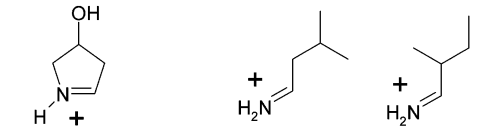


Figure 2. Sequencing of the bromotryptophan-containing conotoxin in the Oligo R3/40% methanol fraction of a crude conotoxin mixture derived from *C. textile*. The doubly charged peptide at *m*/*z* 698.7, detected by precursor ion scanning for *m*/*z* 237.003 and 239.001 (±0.02; Figure 2C and E), was sequenced by tandem MS. Complete y- and b-fragment ion series were observable. The complete sequence could be derived from the MS/MS spectrum.



Hydroxyproline Immonium Ion — **C_4H_8NO: 86.061 Da**

Leucine/Isoleucine Immonium Ion — **$C_5H_{12}N$: 86.097 Da**

Figure 3. Structures of the immonium ions of 4-hydroxyproline and leucine/isoleucine of the same nominal mass.

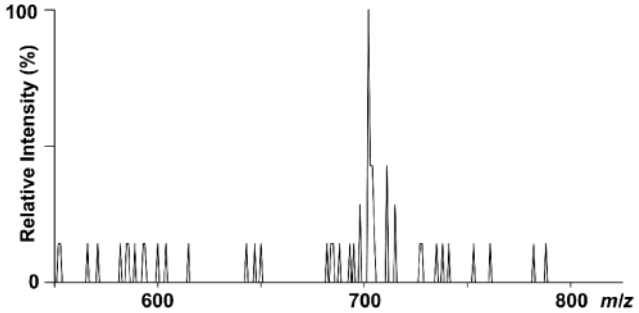


Figure 4. Limit of detection of the precursor ion scan for *m*/*z* 86.063 (±0.020). A purified hydroxyproline-containing conotoxin was sprayed at a concentration of 5 fmol/μL. For the spectrum shown, 24 scans (20 s/scan) were accumulated. The dwell time was set to 50 ms, and the step size was 1 Da.

ubiquitous leucine/isoleucine, the exact masses of these natural residues and the modified residue differ by 0.036 Da due to the presence of mass deficient oxygen in hydroxyproline residues. The immonium ions also show this mass difference of 0.036 Da: 86.061 Da for the immonium ion of hydroxyproline (C_4H_8NO) versus 86.097 Da for the immonium ion of leucine/isoleucine ($C_5H_{12}N$) (see Figure 3; 4-hydroxyproline is depicted as this is the most common site of hydroxylation). Thus, this modification is amenable to MaDMaT precursor ion scanning too.

To investigate the detection limit of this method, a dilution series was prepared using a purified hydroxyproline-containing conotoxin peptide (sequence and characterization will be described elsewhere). As new nanoelectrospray needles were used for every experiment, crossover of the different samples was not an issue. The limit of detection was determined to be in the low-nanomolar range as shown in Figure 4. This figure displays the spectrum of a precursors of m/z 86.06 ± 0.02 experiment using a 5 nM (5 fmol/μL) solution of the model conotoxin, which could be acquired in a reasonable period of time (24 scans, 20 s/scan, 1 Da step size, 50 ms dwell time) compared to the spraying time of the nanoelectrospray experiment. Although the peak at m/z 702 contains only few ion counts, the fair S/N in this spectrum results from the high-accuracy fragment ion selection employed in the precursor ion scan (width ± 0.02 Da) such that the average background signal is less than one event.

The mass spectrum of the Poros R2/20% methanol fraction of the same sample as analyzed above is shown in Figure 5A. Again, numerous ion signals are observable with little overlap between this fraction and the Oligo R3 fraction used for the bromotryptophan analysis as shown in Figure 1A. A conventional, i.e., low-resolution precursors of m/z 86.1 ± 0.25 experiment is shown in Figure 5B displaying a large number of ion signals. Hardly any reduction in complexity was achieved as leucine or isoleucine is found in almost every peptide. Clearly, a precursor ion experiment is of little utility in the detection of proline-hydroxylated peptide species using a low-resolution, low-accuracy tandem mass spectrometer.

A precursor ion scanning experiment with high-resolution, high-accuracy fragment ion selection for the detection of precursors of m/z 86.06 ± 0.02 is shown in Figure 5C. This experiment, which is selective for hydroxyproline-containing peptide species, resulted in only one significant ion signal attributable to one major hydroxyproline-containing peptide species. This peptide only gave rise to a minor ion signal of less than 15% abundance (indicated by an arrow in Figure 5B) in the corresponding conventional, i.e., low-resolution, low-accuracy precursor ion experiment.

Tandem mass spectrometric experiments were performed on this double-charged proline-hydroxylated species at m/z 681.27. The product ion spectrum is shown in Figure 6. A hydroxyproline residue within a peptide sequence shows a similar effect on the fragmentation behavior to unmodified proline residues; that is, the major fragmentation pathway is the cleavage of the peptide bond N-terminal to the modified proline residue. As a result, one peptide bond was preferably cleaved, significantly suppressing the cleavage of all other peptide bonds. However, it was possible to derive from the MS/MS data the sequence of all amino acid

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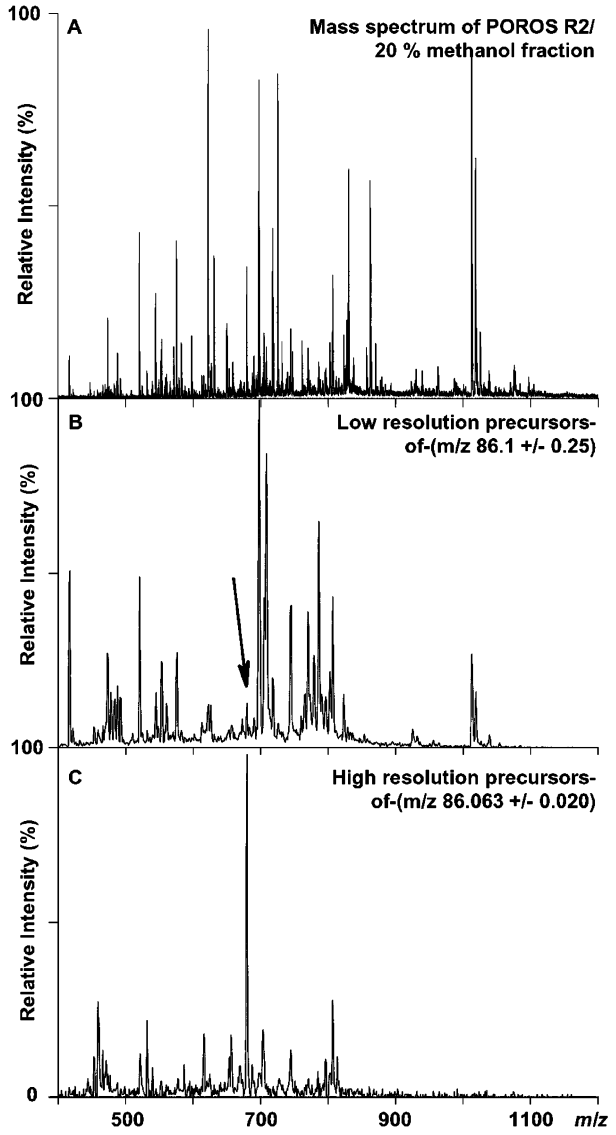

Figure 5. Analysis of the Poros R2/20% methanol fraction of a crude conotoxin mixture derived from *C. textile*. (A) Nanoelectrospray quadrupole-TOF mass spectrum of this particular Poros R2 fraction. (B) Precursor ion scan for *m*/*z* 86.1 (±0.25). This spectrum is comparable to a similar experiment on a well-tuned triple quadrupole mass spectrometer. Numerous ion signals attributable to leucine or iosoleucine-containing conotoxins are observable. The ion signal of the proline-hydroxylated conotoxin species is of only minor abundance. Thus, this type of precursor ion experiment is not suitable for the selective detection of hydroxyproline-containing peptides. (C) Precursor ion scan for *m*/*z* 86.063 (±0.020). The major ion signal corresponds to the main tryptophan-brominated species in this fraction.

residues with the exception of the first two. The following primary structure could be determined for the hydroxyproline-containing peptide: $H_2N-(P(OH)/C)VCGYRMCVP(OH)C-CONH_2$ with the C-terminus being amidated. The C-terminal sequence could be unambiguously identified based on the double charged b-ions (indicated by arrows in Figure 6), but the order of the two N-terminal amino acid residues remained ambiguous. The quadrupole TOF mass spectrometer provided a mass accuracy that allowed the determination of the two N-terminal amino acids as being hydroxyproline (P(OH)) and cysteine (C) based on the b_2 fragment ion (217.064 Da). Hence, the other options (CL) (217.101 Da), (DT), and (ES) (both 217.082 Da) could be rejected. The absence of any y_{11}-ion might indicate that a hydroxyproline is found in the N-terminal position, thereby suppressing the cleavage of the peptide bond C-terminal to the hydroxyproline residue. Further evidence for an N-terminal position of the hydroxyproline residue was provided by fragmentation studies on this conotoxin prior to reduction, i.e., in its disulfide bridged state. We observed a fragment ion corresponding to $[M + H - 113]^+$, which could be attributable to the y_{11}-ion (data not shown). This product ion corresponds to the only fragment that can be generated by a single peptide bond cleavage, presuming disulfide bridges span the N- and the C-terminal region such that all other single peptide bond cleavages lead to fragment ions indistinguishable from the precursor ion. Nevertheless, additional experiments are required to confirm this notion. Database searching retrieved a highly homologous conotoxin from *Conus pennaceus*. However, the sequence similarity was confined to the C-terminal region of the peptide, leaving the N-terminal region ambiguous ((P/C)VCGYRMCVPC vs ...KSTCCGYRMCVPCG).

CONCLUSION AND PERSPECTIVE

Many protein modifications give rise to characteristic fragment ions with an inherent MaDMaT, i.e., fragment ions that differ in mass by 400 ppm or less from other peptide-derived fragment ions with the same nominal mass. This mass shift to lower *m*/*z* values is caused by the high incidence of mass-deficient atoms such as oxygen, phosphorus, sulfur, or halogens. These species can be selectively detected in complex mixtures by precursor ion scanning with high-resolution, high-accuracy fragment ion selection without interferences from nominally isobaric fragment ions, thereby broadening the applicability of precursor ion scanning for the analysis of protein modifications.

Bromotryptophan is a modified amino acid residue that gives rise to fragment ions detectable by MaDMaT as shown for the selective detection of tryptophan-brominated conotoxins in a *C. textile* conotoxin mixture. No interferences from other peptide-derived fragment ions were observed. Subsequent sequencing of this bromotryptophan-containing peptide led to the characterization of a novel *C. textile* conotoxin.

A MaDMaT fragment ion also enabled the differentiation between leucine/isoleucine-containing peptides and those containing hydroxyproline even though the mass difference between the immonium ions of these species is less than 0.04 Da. This allowed the directed analysis of the major proline-hydroxylated species within the fraction analyzed, leading to the discovery of another novel *C. textile* conotoxin. Testing the detection limits of this method provided fair signal-to noise ratios in the low-nanomolar range: even a 5 nM solution (5 fmol/μL) gave a S/N of ~7.

It is conceivable that comprehensive LC/MS and LC/MS/MS analysis with appropriate data mining will become the method of choice for many modification studies on known proteins. However, this approach requires prior knowledge of the protein sequence and cannot be applied to heavily modified species because the number of possible sequences increases in a combinatorial fashion with the number of allowed modifications, thereby excluding successful data mining, at least by currently employed methods. This warrants the need for alternative methods for the analysis of protein modifications such as high-resolution, high-accuracy

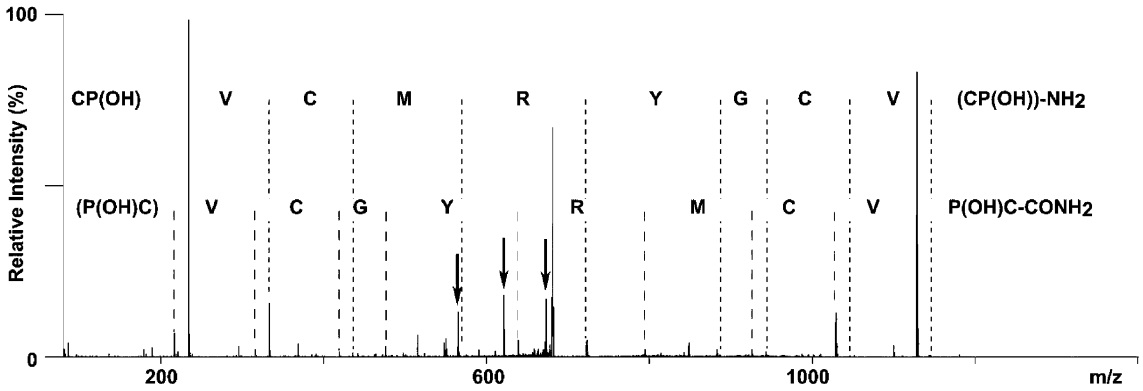

Figure 6. Sequencing of the hydroxyproline-containing conotoxin in the Poros R2/20% methanol fraction of a crude conotoxin mixture derived from *C. textile*. The doubly charged peptide at *m*/*z* 681.27, detected by precursor ion scanning for *m*/*z* 86.063 (±0.02; Figure 3C), was sequenced by tandem MS. Almost complete fragment b- and y-ion series corresponding to different charge states are observe. The sequence of 11 out of 12 amino acids including two hydroxyproline residues could be derived from the MS/MS spectrum. Arrows indicate doubly charged b-type fragment ions.

precursor ion scanning as demonstrated here. In the conotoxin example shown, the peptide sequences were not known and the peptides were extensively modified, which would have made the problem exceedingly difficult to analyze by methods other than the one introduced here.

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