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Combined Electron Transfer Dissociation–Collision-Induced Dissociation Fragmentation in the Mass Spectrometric Distinction of Leucine, Isoleucine, and Hydroxyproline Residues in Peptide Natural Products

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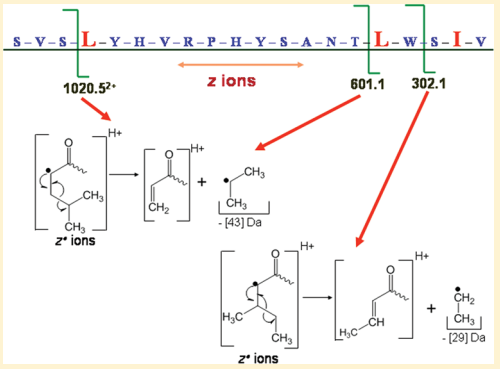
Supporting Information

ABSTRACT: Distinctions between isobaric residues have been a major challenge in mass spectrometric peptide sequencing. Here, we propose a methodology for distinction among isobaric leucine, isoleucine, and hydroxyproline, a commonly found post-translationally modified amino acid with a nominal mass of 113 Da, through a combined electron transfer dissociation–collision-induced dissociation approach. While the absence of *c* and $z^{\bullet}$ ions, corresponding to the Yyy-Xxx (Xxx = Leu, Ile, or Hyp) segment, is indicative of the presence of hydroxyproline, loss of isopropyl (Δm = 43 Da) or ethyl radicals (Δm = 29 Da), through collisional activation of *z* radical ions, are characteristic of leucine or isoleucine, respectively. Radical migration processes permit distinctions even in cases where the specific $z^{\bullet}$ ions, corresponding to the Yyy–Leu or –Ile segments, are absent or of low intensity. This tandem mass spectrometric (MS^n) method has been successfully implemented in a liquid chromatography–MS^n platform to determine the identity of 23 different isobaric residues from a mixture of five different peptides. The approach is convenient for distinction of isobaric residues from any crude peptide mixture, typically encountered in natural peptide libraries or proteomic analysis.



KEYWORDS: *isobaric amino acids, electron transfer dissociation, $z^{\bullet}$ ions, collision-induced dissociation, peptaibols, Conus peptides, wasp venom peptides*

INTRODUCTION

Rapid advances in gas phase fragmentation methodologies, such as collision-induced dissociation (CID), electron capture dissociation (ECD), and electron transfer dissociation (ETD), have made mass spectrometry the method of choice for determining peptide sequences.[1−6] Online liquid chromatography–tandem mass spectrometry (LC–MS/MS) procedures are particularly attractive for determining sequences of individual components in complex peptide mixtures, most frequently encountered in proteomic analysis.[7−10] These methods have also been extremely valuable in the characterization of peptide mixtures from natural sources.[11,12] While the conventional CID technique largely yields *b* and *y* ions through C(O)NH amide bond cleavage, fragmentation initiated through electron capture (ECD/ETD) of the peptide polycation leads to the dissociation of the N–C^{α} bond, typically generating even electron *c* and odd electron $z^{\bullet}$ ions.[13−22] A frequently encountered problem in mass spectral peptide sequencing is the need to distinguish between the isobaric residue leucine and isoleucine (residue mass of 113.16 Da).[12,23] Further, in many peptide natural products, post-translational modification of proline residues by hydroxylation leads to the presence of hydroxyproline (Hyp, O), having a residue mass of 113.12 Da.[24] The distinctions among Leu, Ile, and Hyp are often not routine. Hydroxyproline may be distinguished from Leu and Ile by high-resolution mass determination, which requires a mass accuracy of 0.04 Da.[23] Xxx–Hyp bonds are often more susceptible to cleavage under CID conditions, giving rise to intense fragment ions that may prove to be useful only in specific cases. The distinctions between Leu and Ile residues have usually been achieved via high-energy CID (HE-CID) with characteristic neutral losses involving cleavage of the C^{β}–C^{γ} bonds.[25−28] Under HE-CID conditions, fragmentation of different side chains as well as backbone peptide bonds typically gives rise to a complex MS/MS spectrum. The diagnostic

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fragment ions are often found to be of low intensity, making assignments ambiguous. Also, under HE-CID conditions, facile loss of any acid labile post-translational modification makes the process of assignment even more difficult. Marshall and co-workers have shown extensive characteristic side chain losses of different amino acids in ECD spectra, near the precursor m/z region.[29] Further, side chain losses from specific radical $z^{\bullet}$ ions have been shown by Zubarev and co-workers in the hot electron capture dissociation (HECD) spectra of peptide polycations.[30] The processes of radical migration and radical-mediated secondary fragmentation have been probed by O'Connor and co-workers through ECD of diprotonated cyclic peptides.[31] Observation of extensive side chain losses and neutral amino acid residues has been explained in terms of a "cascade of free radical reaction" that is initiated from the precursor where one radical center has been created through the transfer of one electron. Further studies with peptides containing α-deuterated glycine residues provide experimental evidence of α-hydrogen abstraction during the process of radical migration along the peptide backbone.[32] A similar radical nature of the $z^{\bullet}$ ions, formed during ETD fragmentation, has been established by McLuckey and co-workers through the observation of an oxygen adduct and more recently through collisional activation of the $z^{\bullet}$ ions.[33,34] Using hot electrons during electron capture dissociation (HECD), radical-mediated backbone cleavages have been shown to be useful in effecting the distinction of Leu from Ile,[35,36] but the technical difficulties in implementing ECD in mass spectrometers other than FT-ICR MS limit the application of this method. McLuckey and co-workers have established that fragment ions obtained under ETD conditions may then be subjected to further CID (ETD−CID) fragmentation for the observation of side chain specific losses through radical-induced cleavages, in a QTRAP instrument, using synthetic model sequences.[34] Under conditions of electron transfer dissociation/electron capture dissociation (ETD/ECD) fragmentation, cleavage of the N−C$^{\alpha}$ bond at the Pro residue does not lead to the formation of characteristic c and $z^{\bullet}$ ions due to the constraints of side chain backbone cyclization in the pyrrolidine ring.[17] The absence of such diagnostic ions can be used to signal the presence of Pro or Hyp residues. Herein, we present an extension of these approaches to several peptides of natural origin containing multiple Leu, Ile, and Hyp residues and further demonstrate the applicability of this method in effective distinction among these isobaric amino acids from a peptide mixture.

EXPERIMENTAL SECTION

The bee venom peptide melittin was a commercial sample from Sigma (St. Lous, MO). The fungal peptaibols, antiamoebin II, and zervamicin IIB were obtained as described previously.[37,38] A novel peptide, Bt2328, was obtained from the venom of the marine cone snail *Conus betulinus* by reverse phase high-performance liquid chromatography (HPLC) fractionation of the mixture of venom peptides. Similarly, the wasp peptide Vt1512 was obtained from the venom of social wasp species *Vespa tropica*. The synthetic Bt2328 sample was synthesized via a typical solid phase peptide synthesis protocol using Fmoc chemistry.

The ETD−CID experiments were conducted using a HCT Ultra ETD II ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The purified peptide samples (8 μM), dissolved in a 50:50 H_2O/acetonirile mixture, containing 0.1% formic acid (zervamicin IIB and antiameobin II were dissolved in 100% MeOH, containing 0.1% formic acid, and the solutions were directly infused into the mass spectrometer), were injected into the mass spectrometer typically at a flow rate of 2 μL/min using an injector pump. The tandem mass spectrometric (MS^n) experiments in the direct infusion study were performed manually. After observation of the desired multiply charged precursor in MS, the precursor ion was manually chosen for ETD fragmentation. The ETD reaction time was set at 75 ms for zervamicin and antiamoebin, 85 ms for Bt2328 and Vt1512, and 100 ms for melittin. All the ETD MS^2 experiments were performed using "smart decomposition" (supplemental activation) that provides a low-background collision gas to increase the product ion abundances through probable dissociation of the noncovalent bonds (see Discussion). From the MS^2 ETD spectra, desired precursor ions were subsequently chosen for CID MS^3 fragmentation inside the ion trap through collision with He gas. The fragmentation amplitude (V_{p-p}) was kept between 1 and 3. All the experiments were conducted at a scan speed of 8100 m/z s^{-1}. The spectra were averaged over 6−10 scans. For LC−MS^n analysis, these peptides (5 μL each) were mixed together. The LC−MS analysis was performed by coupling the mentioned ion trap mass spectrometer with an Agilent 1100 HPLC system and the peptide samples were separated on a reverse phase C18 analytical column (Porshell 120, SB-C18, particle size of 2.7 μm, dimensions of 4.6 mm × 150 mm) using a H_2O/acetonitrile mixture (0.1% formic acid) as the solvent system at a flow rate of 0.2 mL/min. The gradient was set from 90% H_2O to 100% ACN over 45 min in a linear gradient. The LC−MS^n experiments were performed in a data-independent manner. Before the start of the LC run, the total run time was divided into different segments as per the retention times of the five peptides used. In these individual segments, the m/z values of the precursor ions to be chosen for MS^2/MS^3 experiments were manually inserted into the acquisition software. During the LC−MS^n study, the scan speed was set to 26000 m/z s^{-1}, and typically, the spectra were averaged over three to five scans. The ETD reaction time was set at 75 ms for zervamicin and antiamoebin, 85 ms for Bt2328 and Vt1512, and 100 ms for melittin. In this case also, smart decomposition (supplemental activation) was used while acquiring the ETD MS^2 spectra. Subsequent CID fragmentation was conducted inside the ion trap through the collision of He gas with the ions of interest. The HE-CID experiments were performed on an Ultra Flextreme MALDI system (Bruker Daltonics) equipped with a 337 nm laser operated at 50 Hz. Ar was used as the collision gas at ~4 × 10^{-6} to 1 × 10^{-5} mbar of pressure.

RESULTS

The application of the combined ETD−CID procedure, for distinction among isoleucine (I), leucine (L), and hydroxyproline (O), is illustrated using examples of diverse, naturally occurring peptides. This approach is based on the fate of the initially formed aminoketyl radical under ETD conditions and subsequent side chain fragmentations from $z^{\bullet}$ ions under CID conditions, as illustrated in Scheme 1. The $z^{\bullet}$ ions, formed with a radical at the C$^{\alpha}$ position of the Ile or Leu residue, can undergo radical-induced cleavage of the C$^{\beta}$−C$^{\gamma}$ bond. In the case of Leu, this results in a neutral loss of 43 Da, while for Ile, a loss of 29 Da is observed.[32,34] If Hyp residues are present in the sequence, the formation of aminoketyl radicals does not lead to further fragmentation of the Xxx−Hyp linkage. It should

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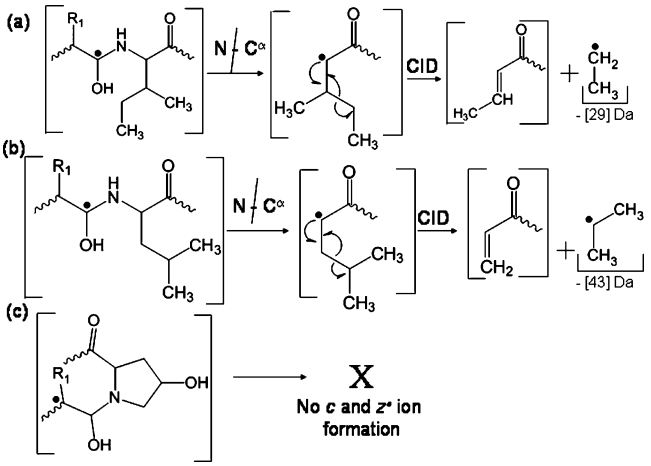
Scheme 1. Mechanistic Pathways for Characteristic Side Chain Losses for (a) Isoleucine and (b) Leucine from the Nascent Aminoketyl Radicals Leading to the Formation of $z^{\bullet}$ Ions That Are Subsequently Collisionally Activated and (c) Hydroxyproline, for Which the Initially Formed Aminoketyl Radical Cannot Lead to N−C$^{\alpha}$ Bond Cleavage



be noted that the absence of fragment ions corresponding to N−C$^{\alpha}$ bond cleavage in ETD spectra provides support for the presence of a Hyp residue but does not rule out the presence of Xxx−Ile or −Leu fragments, which may be resistant to fragmentation. However, unlike CID experiments in which backbone cleavage is strongly sequence-dependent, in ETD MS/MS spectra of peptides, N−C$^{\alpha}$ bond fragmentation does not appear to be strongly influenced by the amino acid sequence. Indeed, in our study with diverse peptide sequences, of 20 Xxx−Ile or −Leu bonds, we have not encountered any example of any Xxx−Ile or −Leu bond that is recalcitrant to cleavage under electron transfer conditions. In specific cases, any further ambiguity can also be resolved through ETD fragmentation of other possible charge states. A consistent absence of *c* and *z* ions, corresponding to the Xxx−Ile, −Leu, or −Hyp segment, in the ETD MS/MS spectra of all the charge states would strongly signal a presence of the Hyp residue.

Peptaibol Antibiotics

The peptaibols make up a microheterogeneous group of polypeptides of fungal origin that have been widely investigated.[37,38] They are characterized by a high proportion of the nonprotein amino acid α-aminoisobutyric acid (Aib, U) and the presence of a C-terminal aminoalcohol residue. Several classes of peptaibols also contain Hyp residues. Figure 1 shows the ETD spectrum of antiamoebin II [Ac-FUUUJGLUUOQJOUPF*, where Ac is acetyl, U is α-aminoisobutyric acid (Aib), J is isovaline (Iva), O is hydroxyproline (Hyp), and F* is phenylalaninol (Phol)] by directly infusing the peptide and fragmenting the $[M + H + K]^{2+}$ species at m/z 855.0. Some of the low-intensity fragment ions are shown as insets. The variation in the isotopic patterns for some of the very low intensity peaks may be attributed to their extremely low signal intensity. Under these conditions, all observed *c* and $z^{\bullet}$ ions corresponded to their potassiated species. This indicates a selective capture of a proton by the reduced carbonyl groups as compared to potassium. This feature has also been noted in earlier studies and substantiated by the calculation of recombination energy at the two positively charged sites.[39] All the observed m/z values of these *c* and $z^{\bullet}$ ions are found to

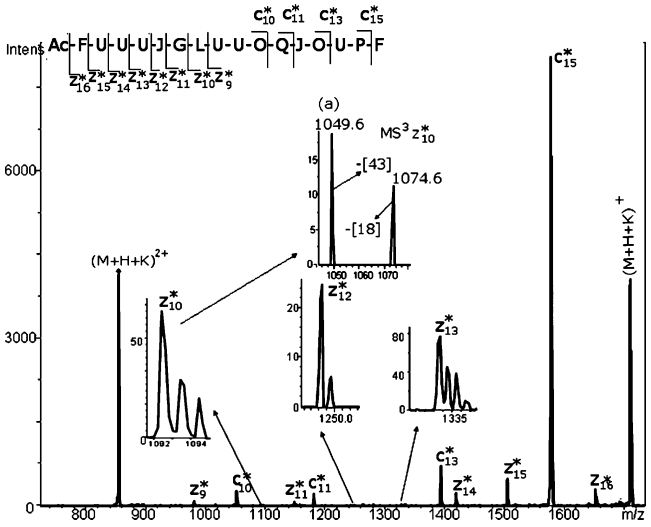


Figure 1. ETD MS/MS spectrum of $(M + H + K)^{2+}$ species (m/z 855.0) of antiamoebin II. Inset a shows the CID fragmentation spectrum of the potassiated $z^{\bullet}_{10}$ ion. The schematic summarizes the observed *c* and *z* ions. The symbol *c** stands for the potassiated even electron *c* ion and *z** the potassiated odd electron *z* ion.

be greater than that of the doubly charged precursor. The observed fragment ions are consistent with the previously determined sequence, with three residues having a residue mass of 113 Da. Of these, two are Hyp residues, at positions 10 and 13, while Leu is present at position 7. Here, the potassiated doubly charged species was chosen because of its higher abundance. Typically, for these classes of extremely hydrophobic peptides, a very high intensity sodiated and potassiated species is observed under electrospray conditions. In this case, similar results were obtained for the $[M + H + Na]^{2+}$ species (data not shown). Inspection of the ETD fragmentation pattern establishes the absence of $c_n/z^{\bullet}_n$ ions corresponding to cleavages at Aib_9−Hyp_{10} and Iva $(isovaline)_{12}$−Hyp_{13} bonds. Figure 1a shows the CID fragmentation of the $z^{\bullet}$ ion at m/z 1092.6. The appearance of the product ion at m/z 1049.6, corresponding to a neutral loss of 43 Da, confirms the presence of a Leu residue at position 7. Here the ion at m/z 1074.6 corresponds to a loss of H_2O, most feasibly from the side chain of Hyp residues. The related 16-residue peptaibol zervamicin IIB (Ac-WIQJITULUOQUOUPF*) possesses five residues having a residue mass of 113 Da: Hyp residues at positions 10 and 13, Ile residues at positions 2 and 5, and Leu at position 8. Figure 2 shows the distribution of product ions obtained through ETD fragmentation of the $(M + H + K)^{2+}$ species, by directly infusing the peptide. The distribution of *c* and $z^{\bullet}$ ions is completely in agreement with the previously determined sequence. As anticipated, fragment ions corresponding to cleavage at the Xxx−Pro or −Hyp segments are not observed. The insets to Figure 2 show the key product ions obtained by subjecting the specifically selected $z^{\bullet}$ ions to further fragmentation under CID conditions. To confirm the presence of Leu_8, the choice of the $z^{\bullet}_9$ ion at m/z 993.6 would have been desirable. However, the low intensity of this ion precluded further CID fragmentation. Similarly, the $z^{\bullet}_{10}$ ion at m/z 1078.6 was also of low intensity. Consequently, an intense $z^{\bullet}_{11}$ ion at m/z 1179.6 was further fragmented under CID conditions. As one can see in Figure 2a, a neutral loss of 43 Da is observed, confirming the presence of Leu at position 8. As noted by the groups of McLuckey and O'Connor, radical migration along the

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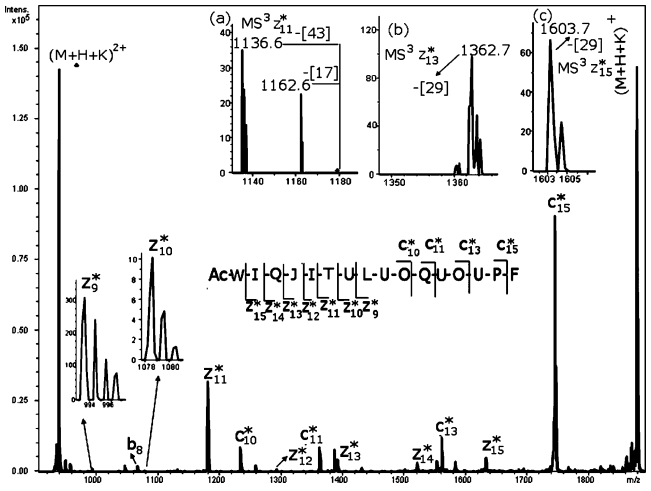

Figure 2. ETD MS/MS spectrum of $(M + H + K)^{2+}$ species (m/z 939.1) of zervamicin IIB. Insets show the CID fragmentation spectra of (a) $z^{\bullet}_{11}$, (b) $z^{\bullet}_{13}$, and (c) $z^{\bullet}_{15}$ ions. The schematic summarizes the observed c and z ions. The symbol c^* stands for the potassiated even electron c ion and z^* the potassiated odd electron z ion.

backbone can induce cleavages, at positions remote from the N-terminus of the fragment ion.[31,34] In our study, this phenomenon has been judiciously used to distinguish between Leu and Ile, in cases where the specific $z^{\bullet}$ ions were of not sufficient intensity. In Figure 2a, a neutral loss of 17 Da is consistent with the elimination of a hydroxyl radical ($^{\bullet}$OH) from the N-terminal threonine residue. Similarly in Figure 2b, observation of a neutral loss of 29 Da, upon selection and subsequent CID fragmentation of the $z^{\bullet}_{13}$ ion, confirms the presence of Ile at position 5. In this case, too, the $z^{\bullet}_{12}$ ion, having Ile at the N-terminus, could not be fragmented because of its low intensity. While the migration of the radical from the initial site of generation is possible, the observed side chain cleavages appear to occur preferentially at proximal Leu or Ile residues. An intense loss of 29 Da, as shown in Figure 2c, upon CID fragmentation of the $z^{\bullet}_{15}$ ion at m/z 1632.7, confirms the presence of Ile at position 2. It is to be noted that $z^{\bullet}_{15}$ contains a Leu residue in addition to an Ile. The absence of the product ions corresponding to the Leu side chain fragment may be attributed to the remoteness of the Leu residue from the initially formed radical centers. Interestingly, McLuckey and co-workers have shown that when both Ile and Leu are remote from the initially formed radical center, radical migration processes appear to favor the side chain fragmentation of Leu,[34] presumably as a consequence of the higher stability of the eliminated tertiary radical of Leu than the secondary radical of Ile. A similar result has also been observed by us. It should be stressed that when the radical center is initially generated at either Ile or Leu, a very high abundance of the corresponding side chain fragmentation is exclusively observed. The CID MS^3 spectra of $z^{\bullet}_{15}$ of zervamicin IIb is an example of such behavior.

Bt2328

The venom of marine cone snails has been extensively studied, and a large number of disulfide-bonded peptides (conotoxins) have been characterized.[40] Cone snail venoms also contain acyclic sequences that have been less studied.[41,42] During the course of our work, aimed at sequencing *conus* peptide libraries, we encountered a linear peptide with a molecular mass of 2328 Da (Bt2328), produced by *C. betulinus*. The sequence of this peptide was determined by mass spectrometry, using CID and ETD fragmentation. Figures S1a and S2 of the Supporting Information show CID and ETD MS^2 spectra of Bt2328. *De novo* sequencing from mass spectral data yielded a 20-residue sequence, SVSXYHVRPHYSANTXWSXV (X is Leu, Ile, or Hyp) with residues having a mass of 113 Da at position 4, 16, and 19, which could be assigned to leucine, isoleucine, or hydroxyproline. The presence of $z^{\bullet}_5$, $z^{\bullet}_{17}$, and c_{18} ions negates the possibility of Hyp at these positions. Initial HE-CID experiments, performed in MALDI, yielded w_2, w_5, and d_4 ions that were indicative of Ile at position 19 and Leu at positions 4 and 16 (data not shown). Subsequently, these peptides were subjected to ETD−CID experiments. Figure S2 of the Supporting Information shows the ETD spectrum obtained for the $(M + 4H)^{4+}$ species at m/z 583.1 through direct infusion of the peptide of interest. Here also, the low intensity of the $z^{\bullet}_2$ ion precluded a direct assignment of the residue at position 19. Hence, the assignment of Ile at position 19 was made, exploiting the process of radical migration, through the selection and subsequent CID fragmentation of the $z^{\bullet}_3$ ion at m/z 302.1, which results in a neutral loss of 29 Da (Figure 3).

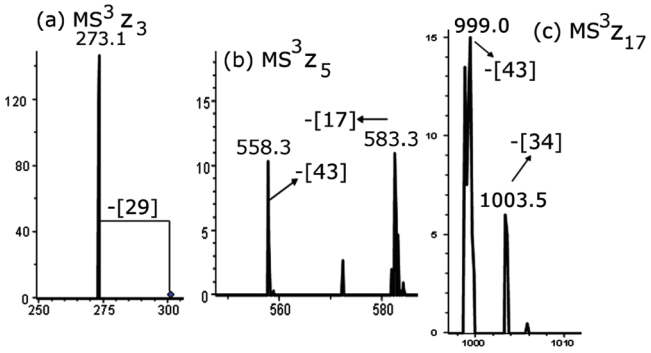

Figure 3. CID fragmentation spectra of (a) $z^{\bullet}_3$, (b) $z^{\bullet}_5$, and (c) $z^{\bullet}_{17}$ ions of Bt2328.

The $z^{\bullet}_5$ ion at m/z 601.3 upon further CID fragmentation yields a product ion at m/z 558.3, which corresponds to a neutral loss of 43 Da, establishing Leu at position 16. The neutral loss of 17 Da leading to a product ion at m/z 583.3 may be assigned as a loss of hydroxyl radical ($^{\bullet}$OH) from Ser at position 18. Selection of the doubly charged $z^{\bullet}_{17}$ species at m/z 1020.5 yielded the doubly charged product ion at m/z 999.0, corresponding to a neutral loss of 43 Da. This confirms the presence of Leu at position 4. A low-intensity ion at m/z 1003.5 may be assigned to a doubly charged species arising from a loss of 34 Da, corresponding to an elimination of a hydroxyl radical and ammonia. Indeed, the sequence contains hydroxylated residues at positions 12 (Ser), 15 (Thr), and 18 (Ser) and an Arg at position 8 that is capable of losing NH_3. The correctness of the sequence was confirmed by comparison with a chemically synthesized peptide. Panels a and b of Figure S1 of the Supporting Information show the comparative CID MS^2 spectra of the natural and synthetic peptide. The examples of zervamicin IIB and Bt2328 illustrate, in spite of the absence of specific z ions, how the distinction between the isobaric residues can be made by fragmenting the larger $z^{\bullet}$ ions containing the ambiguous residue, exploiting the radical migration phenomenon.

Vt1512

The venom of social wasps also contains a complex mixture of linear peptides. During the course of investigation directed

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towards characterization of the natural peptide library from wasp venom, we encountered sequences rich in Leu and Ile residues. In such cases, conventional *de novo* sequencing using CID methods yielded ambiguous results at several sequence positions that were assigned residue masses of 113 Da. Figure S3 of the Supporting Information shows the ETD spectrum of one of the wasp venom peptides, Vt1512 (XNXKAXAAX-AKKXF*, where X is Leu or Ile and the asterisk denotes a C-terminal amidation), isolated from the crude venom of *V. tropica*, obtained by the transfer of electron to $(M + 3H)^{3+}$ species through a direct infusion experiment. Figure 4 illustrates

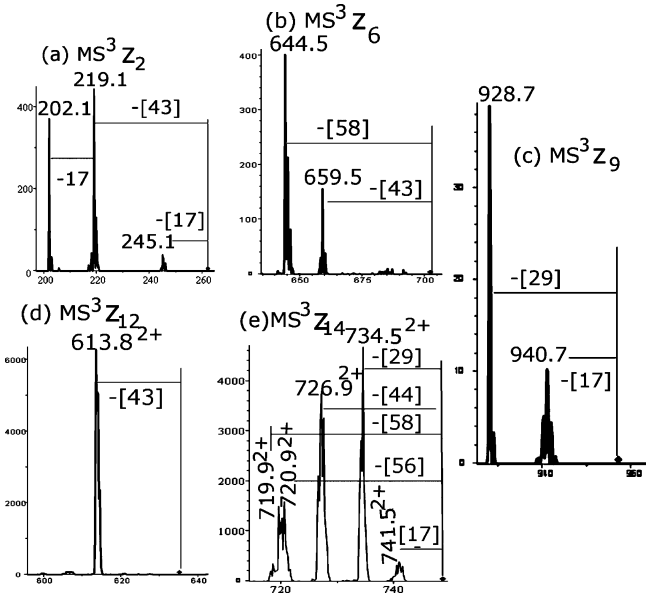

Figure 4. CID fragmentation spectra of (a) $z^{\bullet}_2$, (b) $z^{\bullet}_6$, (c) $z^{\bullet}_9$, (d) $z^{\bullet}_{12}$, and (e) $z^{\bullet}_{14}$ ions of Vt1512.

the observed neutral losses upon fragmentation of the $z^{\bullet}$ ions at m/z 262.1, 702.5, 957.7, 635.3^{2+}, and 748.7^{2+}. The loss of 43 Da from $z^{\bullet}_2$ (m/z 262.1) and $z^{\bullet}_6$ (m/z 702.5) establishes the presence of a Leu residue at positions 13 and 9, respectively. Interestingly, the neutral loss of 17 Da is observed from both the parent and product ion at m/z 262.1 and 219.1, respectively. This $z^{\bullet}_2$ ion corresponds to the C-terminal dipeptide Leu-Phe amide, with 17 Da losses being attributable to elimination of NH_3. In addition, the neutral loss of 58 Da from the ion at m/z 702.5 to yield the product ion at m/z 644.1 may be attributed to the Lys side chain. The $z^{\bullet}_9$ ion at m/z 957.7 upon CID shows a neutral loss of 29 Da, establishing Ile at position 6. Doubly charged ETD MS^2 ions at m/z 635.3 and 749.0 are assigned to fragment ions $z^{\bullet}_{12}$ and $z^{\bullet}_{14}$, respectively. The observed product ion at m/z 613.8^{2+}, shown in Figure 4, upon CID of the $z^{\bullet}_{12}$ ion at m/z 635.3^{2+}, is consistent with a Leu at position 3. In the case of the $z^{\bullet}_{14}$ ion at m/z 749.0^{2+}, neutral losses of 29 Da (Ile), 44 Da (Asn/C-terminal $CONH_2$), and 58 Da (Lys) are consistent with the determined sequence. In addition, the 56 Da loss may be assigned to the even electron loss of a four-carbon unit (CH_3−CH═CH−CH_3) from Ile, generated via C^{α}−C^{β} bond cleavage. In the case of Vt1512, the use of conventional CID MS/MS, together with ETD−CID MS^3 experiments, permitted determination of the sequence of a previously uncharacterized peptide.

Melittin

The 26-residue highly basic peptide melittin (molecular mass of 2846 Da; GIGAVLKVLTTGLPALISWIKRKRQQ*, where the asterisk denotes a C-terminal amidation), isolated from bee venom, is used to illustrate a situation that may lead to ambiguity in application of the ETD−CID method. Figure S4 of the Supporting Information shows the fragmentation pattern obtained by the transfer of electron to the $(M + 5H)^{5+}$ species through a direct infusion experiment. Figure 5 shows the

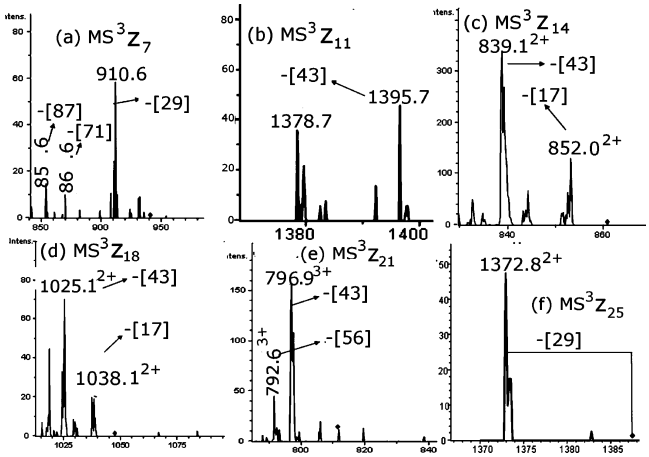

Figure 5. CID fragmentation spectra of (a) $z^{\bullet}_7$, (b) $z^{\bullet}_{11}$, (c) $z^{\bullet}_{14}$, (d) $z^{\bullet}_{18}$, (e) $z^{\bullet}_{21}$, and (f) $z^{\bullet}_{11}$ ions of melittin.

neutral losses obtained upon subjecting specific $z^{\bullet}$ ions to CID conditions. The sequence contains six residues with a residue mass of 113 Da, assigned to Leu or Ile. Selection and fragmentation of the $z^{\bullet}_7$ ion at m/z 939.6 yields a neutral loss of 29 Da (product ion at m/z 910.6), confirming an Ile at position 20. The loss of 87 and 71 Da can be attributed to the loss of $CH_3CH_2NHC(NH)NH_2$ and H_2C═$CH(CH_2)_2NH_2$ groups from the side chains of Arg and Lys residues, respectively. Fragmentation of the $z^{\bullet}_{11}$ ion at m/z 1438.9 reveals a neutral loss of 43 Da consistent with Leu at position 16, which is the N-terminal end of the $z^{\bullet}$ ion. Fragmentation of the doubly charged $z^{\bullet}_{14}$ ion (m/z 860.6) yielded the anticipated neutral loss of 43 Da, confirming Leu at position 13. Similarly, fragmentation of the doubly charged $z^{\bullet}_{18}$ and triply charged $z^{\bullet}_{21}$ ion at m/z 1046.6 and 811.2 yielded a neutral loss of 43 Da, establishing the presence of Leu at positions 9 and 6, respectively. The doubly charged $z^{\bullet}_{25}$ ion at m/z 1387.3 upon further fragmentation reveals a neutral loss of 29 Da, consistent with an Ile at position 2. In our case, the low intensity of the $z^{\bullet}_{10}$ ion at m/z 1326.8 precluded confirmation of Ile at position 17. Similar problems, encountered in the previous sequences, were resolved utilizing the phenomenon of radical migration, where larger $z^{\bullet}$ ions, containing the ambiguous residue, were chosen for fragmentation. However, in this case, the presence of an Ile at position 6 restricts the use of such an approach, as $z^{\bullet}$ ions containing Ile_{17} would also contain Ile_6. Hence, upon CID fragmentation of these z ions, any observed neutral loss of 29 Da cannot be unambiguously assigned to one of these Ile residues. The results obtained with melittin suggest that in proteomic identification of unknown sequences, ambiguity may arise because of the low intensity of specifically useful $z^{\bullet}$ ions, especially in cases where successive multiple Leu or Ile residues occur in the sequence. This

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problem may be overcome by the collection of ETD−CID data from a different charge state or by turning to shorter peptide sequences generated by selective proteolysis. Alternatively, one may use the proton transfer strategy of McLuckey and co-workers or "supercharging" reagents, as shown by Williams and co-workers,[43,44] to increase the abundance of lower or higher charge states, permitting their analysis.

Direct Analysis of the Peptide Mixture

To evaluate whether the ETD−CID protocol could be conveniently extended to peptide mixtures, we examined a system consisting of the five components described above; namely, antiamoebin II, zervamicin IIB, Bt2328, V1512, and melittin. All five components separate readily under reverse phase HPLC conditions, permitting LC−MS^n analysis. Further fragmentation of the selective $z^\bullet$ ions, of the components of the mixture, yielded results identical to those obtained with the pure peptides (Figures S5−S9 of the Supporting Information). The compatibility of this approach with LC−MS methods ensures that the combination of CID and ETD methods can be effective in the convenient characterization of individual components of peptide mixtures, typically encountered in natural peptide libraries and in proteomic analysis. It should be stressed that the focus of this work has been on analysis of peptides whose sequences are not related to those in genome or proteome databases. Such mixtures are frequently encountered in the secretions and venoms of a large number of organisms. They are often the products of nonribosomal peptide synthesis or gene products that have been subsequently heavily post-translationally modified. Interestingly, in a recent report by Chait and co-workers, a methodology for rapid sequencing of venom peptides, involving chemical modification to provide good ETD MS^2 spectra, has been described.[12] In this case, Leu versus Ile ambiguities were not resolved.

DISCUSSION

A further point that emerges from this study is that while C^β−C^γ bond cleavages are facile, leading to formation of product ions that are diagnostic for specific side chains, other fragmentation modes are also detected. For example, product ions produced by C^α−C^β bond cleavage are also observed. The losses of 71 Da from the Lys side chain and 56 Da from Leu and Ile side chains are generally observed. The loss of hydroxyl radical from the Thr side chain is also observed in Bt2328 and melittin. Interestingly, no such loss of hydroxyl radical (M = 17 Da) is observed in the case of γ-hydroxyproline, where N−C^α bond cleavage must result in a charge-reduced species that has the same mass as that of the parent ion. This is presumably because of the distance between the C^α and C^γ atoms. It should also be noted that the ETD spectra were obtained under conditions of supplemental activation (termed smart decomposition) that provides a low-energy background collision to separate $[c + z]$ pairs, increasing product ion abundances.[45] Although the work of Coon and co-workers shows increasing $c^\bullet/c'$ ratios and decreasing $z^\bullet/z'$ ratios under supplemental activation conditions,[45] the extensive radical-mediated secondary fragmentations observed upon CID activation of all the studied $z^\bullet$ ions suggest that the use of supplemental activation, at least in this case, does not lead to complete electron parity reversal of the $z^\bullet$ ions. Interestingly, a greater abundance of the radical c and even electron z ions was observed upon CID fragmentation of the charge-reduced precursors (CRCID).[46] In ECD experiments, with an increase in the ion internal energy through vibrational activation, a decreasing $c^\bullet/c'$ ratio and an increasing $z^\bullet/z'$ ratio were detected.[47] In a separate study, using oxygen along with He as a buffer gas, McLuckey and co-workers observed extensive O_2 adducts with the $z^\bullet$ ions, proving its radical character.[33] No such adduct was found with any c ions. In several cases, secondary fragmentation has been shown to occur at a site remote from the initially formed radical center. This process of migration of the radical along the peptide backbone may proceed through the transfer of an H atom between two centers that are in spatial proximity, as previously described by O'Connor and co-workers.[31] Detailed studies of the sequence dependence of radical migration in $z^\bullet$ ions, generated by electron transfer dissociation, may provide further mechanistic insights. It is also noteworthy that prior to the MS^3 collisional activation of the $z^\bullet$ ions, there is a probability that the target ion may lose its radical nature during the isolation step. Figure S10 of the Supporting Information shows the isolation spectra of all the $z^\bullet$ ions of Vt1512. It is evident from the figure that, at least in this case, all the isolated $z^\bullet$ ions retained their radical character during the isolation step. Marshall and co-workers have previously shown the presence of characteristic neutral losses from the side chains of different amino acids in ECD spectra, near the m/z region of the precursor or charge-reduced precursors.[29] Very recently, Coon and co-workers have shown similar results produced in ETD spectra of several proteolytic peptides.[48] Although such neutral losses enhance the confidence in amino acid assignments, for peptides with multiple isobaric residues, such as those used here, the observation of a characteristic side chain loss from the precursor ion cannot alone assign the position of the residue in the peptide chain. The definitive precursor−product ion relationship established in the MS^3 experiments makes it the method of choice.

ASSOCIATED CONTENT

Supporting Information

Figures S1−S10 showing mass spectrometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

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