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RESEARCH ARTICLE

Unusual Fragmentation of β-Linked Peptides by ExD Tandem Mass Spectrometry

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Abstract
Ion-electron reaction based fragmentation methods (ExD) in tandem mass spectrometry (MS), such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) represent a powerful tool for biological analysis. ExD methods have been used to differentiate the presence of the isoaspartate (isoAsp) from the aspartate (Asp) in peptides and proteins. IsoAsp is a β_3-type amino acid that has an additional methylene group in the backbone, forming a C_α–C_β bond within the polypeptide chain. Cleavage of this bond provides specific fragments that allow differentiation of the isomers. The presence of a C_α–C_β bond within the backbone is unique to β-amino acids, suggesting a similar application of ExD toward the analysis of peptides containing other β-type amino acids. In the current study, ECD and ETD analysis of several β-amino acid containing peptides was performed. It was found that N–C_β and C_α–C_β bond cleavages were rare, providing few *c* and *z•* type fragments, which was attributed to the instability of the C_β radical. Instead, the electron capture resulted primarily in the formation of *a•* and *y* fragments, representing an alternative fragmentation pathway, likely initiated by the electron capture at a backbone amide nitrogen protonation site within the β amino acid residues.

Key words: Ion molecule reactions, Odd electron fragmentation, Electron capture dissociation, Electron transfer dissociation

Introduction

Electron capture dissociation (ECD) [1–3] and other ion-electron reaction methods such as electron ionization/impact dissociation (EID) [4], and electron transfer dissociation (ETD) [5] (collectively known as ExD) belong to a group of odd-electron (OE) fragmentation techniques, which also include ultraviolet photodissociation (UVPD) [6], and metastable atom-activated dissociation (MAD) [7]. These methods can generate fragments often unobservable in conventional slow-heating fragmentation techniques such as low energy collisionally activated dissociation (CAD) [8] and infrared multiphoton dissociation (IRMPD) [9]. ECD and ETD are perhaps the most widely used among these OE fragmentation methods. In ECD of polypeptides, a low energy electron is captured by the multiply charged molecular ion either at a protonated backbone carbonyl site according to the Cornell mechanism [1, 10], or in a π orbital of an amide group in the presence of a remote charge according to the Utah-Washington mechanism [11], producing a charge reduced cation radical, with the subsequent backbone N–C_α bond cleavage leading to the formation of c and z• fragments. The radical on z• can induce further rearrangements within the molecule, a free radical cascade [12], producing additional backbone and side chain cleavages both in the proximity of and remote from the initial radical site [13]. The fragmentation pattern generated by

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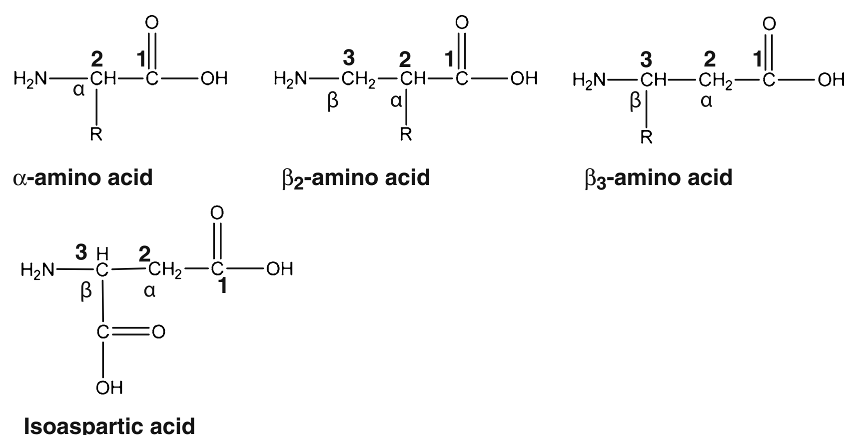
ECD may vary dramatically depending on the number and location of certain amino acids, modifications, and charges [14–18]. A number of experimental and computational studies have been carried out since ECD was first introduced [1,19], yet the mechanisms remain under discussion, probably because multiple competing pathways are involved [20–31]. Nevertheless, the capability of ECD to produce unique fragment ions not obtainable by conventional methods has led to a rapid optimization of ECD and the development of other ion-electron reaction based tandem MS techniques [4, 5, 21]. ExD has been broadly applied towards the structural analysis of various types of biomolecules, including proteins, oligosaccharides, oligonucleotides, and others [32–35]. Further, ECD and ETD were found to be particularly useful for the characterization of post translational modifications (PTMs) in proteins as they cleave the backbone while preserving the labile groups and non-covalent interactions upon fragmentation [2, 36].

Deamidation of asparagine (Asn) and glutamine (Gln) residues and, similarly, isomerization of aspartic (Asp) and glutamic (Glu) acid residues, are two of the most common PTMs found in all proteins that have been studied recently. These PTMs accumulate with age in long lived proteins and are frequently associated with age related diseases, such as amyloid diseases and cataract formation in the eyes [37–39]. Both reactions are nonenzymatic and proceed spontaneously in physiologic conditions via formation of a succinimide or glutarimide ring intermediate, followed by rapid hydrolysis. Deamidation introduces an approximately 1 Da mass shift to the protein molecular mass (+0.984 Da) and can be easily identified using mass spectrometry (MS) [40]. Isomerization does not change the molecular mass of the protein and thus cannot be identified as easily. However, the application of tandem MS methods, in particular, ExD techniques, has shown successful results: isomers of aspartic acid can be unambiguously identified using ECD, ETD, and EID methods [37, 41–48]; likewise, identification of γ-glutamic acid by ECD seems to be promising [49].

Isoaspartic acid is a β-type amino acid that has one extra CH_2 group in the polypeptide backbone, and one fewer on the side chain compared to Asp. ExD of isoAsp containing peptides generates additional signature fragment ions (c+57 and z•−57) at the positions of the isomerized residues, allowing differentiation from the non-modified residues (the fragmentation scheme can be found in Supplemental Material, Figure S1.). These ions are formed by the C_α–C_β backbone bond cleavage. In peptides consisting solely of α-amino acid residues, there is no such bond within the backbone. Fragments produced upon C_α–C_β bond rupture are unique to β-amino acid residues and could be used to locate the position of the β-amino acid if observed. The result from the isoAsp experiment suggested a possible extension of the method to characterize peptides containing other β-type amino acids [50–52].

Similar to the isoAsp, a β-amino acid has an extra methylene group incorporated between its amino and carboxylate groups compared to its α-analogues. There are two types of β-amino acids: β_2 and β_3, with the side chain attached to the α and β carbon respectively (Scheme 1). β-Amino acids do not normally occur in nature except for β-alanine and β-aspartate (isoAsp); neither do β-peptides, but those can be synthesized [53, 54]. It should be noted here that naturally occurring β-alanine and β-aspartic acid have the same total number of carbons as their α analogues—one more in the backbone and one fewer on the side chain; however, β-amino acids normally used for β-peptide synthesis often have an extra carbon within the backbone, but contain the same side chain as those in α-amino acids and, thus, are called β-homo-amino acids (in this study all β-amino acids in synthetic peptides are β-homo-amino acids).

β-Peptides have been a subject of intense studies that investigated their structural, biological properties, and their interactions involved in peptide folding. β-Peptides were found to have richer conformational energy surface with more stable secondary structures [55]. They also fold into helices or hairpin-type structures with larger variety than α-peptide secondary structures. β-Peptides are very stable against proteolytic degradation and other enzymes in human and various living organisms [56]. These features provide great potential for β-peptides in biomedical application as



Scheme 1. β-Amino acid nomenclature

proteolytically stable therapeutics. A fast and accurate MS based method would be of a great utility for analysis of β-peptide structure. In this study, the potential of the ExD based tandem MS methods to differentiate β-amino acid containing peptides from their α-analogues, as well as β_2 from β_3-type amino acids was investigated. In general, our results are in good agreement with the findings of recent ECD/ETD studies of small β-peptides [57] and ε-peptides [58]. In the present study, in addition to simple model peptides such as Q_{06} and Substance P, a more complicated system of Puma BH_3 peptide analogues of 26 amino residues was investigated. Furthermore, charge state dependence of the fragment appearance was studied. An alternative mechanism of ion-electron reaction induced dissociation of peptides within β-amino acid residues is discussed.

Experimental

Materials

The Q_{06} β-peptide (β_2Vβ_2Aβ_2Lβ_3Vβ_3Aβ_3L) was kindly provided by Professor D. Seebach, and Dr. J. Gardiner, ETH Zurich, Switzerland. C-terminally amidated Substance P with two amino acids modified to β_3-type amino acids (RPKPβQQFFGβLM) was custom synthesized by AnaSpec (San Jose, CA, USA). Puma BH_3 pro-apoptotic protein analogue I (βEEQβWAREβIGAβQLRRβMADβDLNAβ-QYEβRR) and analogue II (βEEQWβAREβIGAQβLRRβ-MADDβLNAβQYERβR) were kindly provided by the group of Professor S. Gellman at the University of Wisconsin (Madison, WI, USA), where β indicates β_3-type amino acids. Other reagents: non-modified Substance P, (2-aminoethyl)-trimethylammonium chloride hydrochloride (cholamine), triethylamine (TEA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA); 2-(1 H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) from Novabiochem (La Jolla, CA, USA); and 1-hydroxybenzotriazole (HOBt) from AK Scientific, Inc. (Palo Alto, CA, USA).

Cholamine Reaction

Covalent attachment of cholamine to the Q_{06} β-peptide was performed as previously described [59]. Briefly, 0.1 μmol of Q_{06} β-peptide was treated sequentially with 10 μL of 200 mM HOBt in DMSO, 200 μL of 100 mM cholamine in DMSO containing 200 mM TEA, and 10 μL of 200 mM HBTU in DMSO. The sample was left to react overnight at room temperature, and purified using a ZipTip C_{18} solid phase micropipette extraction column before MS analysis.

Mass Spectrometry

Most ECD experiments were performed on a custom built qQq-FTICR MS with a nanospray source and a 7 T actively shielded magnet [60, 61]. Samples were nanosprayed (50 nL/min, room temperature) at 5 μM concentration in 50:50 MeOH:H_2O with 1% formic acid. Ions were isolated in the first quadrupole Q_1, accumulated in the second quadrupole Q_2, and transmitted into the ICR cell where they were irradiated with electrons emitted from an indirectly heated dispenser cathode (Heatwave; Watsonville, CA, USA) for ion fragmentation. The following ECD and EID parameters were employed: electron irradiation time 35–100 ms, cathode potential −0.2 to 1.2 V (ECD), −18 V (EID). Acquired spectra were zerofilled twice, internally calibrated, and analyzed manually using BUDA (Boston University Data Analysis, ver. 1.4, © 2000 by Peter B. O'Connor). ECD experiments of triply charged Substance P ions were performed on solariX FTICR instrument (Bruker Daltonics, Billerica, MA, USA) with 12 T actively shielded magnet. Electrospray was applied for enhanced production of triply charged ions.

ETD spectra with supplemental activation were acquired on an amaZon Ion Trap instrument (Bruker Daltonics, Billerica, MA, USA) using fluoranthene as the ETD reagent. Peptides were electrosprayed (2 μL/min, glass capillary temperature 220 °C) at 1 μM concentration in 50:50 MeOH: H_2O with 1% formic acid using an Apollo II ion source. Data acquired on solariX and amaZon were analyzed using Bruker's ESI Compass DataAnalysis 4.0 software.

Results and Discussion

Q_{06} β Peptide $_{\beta 2}V_{\beta 2}A_{\beta 2}L_{\beta 3}V_{\beta 3}A_{\beta 3}L$

Although the Q_{06} β-peptide only contains six β-amino acid residues, it can form helical secondary structures [55, 62]. This small and relatively simple peptide contains both β_2 and β_3 type amino acids, making it potentially an ideal system to study for the differentiation of β_2 and β_3 amino acids. However, only singly charged ions $[M+H]^+$ were detected in all ESI/ECD experiments, either with the nanospray or with the electrospray ionization sources (Supplemental Figure S2a), which is likely due to the lack of basic amino acid residues within the peptide sequence. Since ECD and ETD are accompanied by charge neutralization upon the electron capture or transfer, they can not be performed on singly charged ions as the products would be neutral and undetectable. In this case, other fragmentation techniques could be applied that do produce fragments from singly charged ions such as CAD [8] or IRMPD [9]. Expectedly, IRMPD of the Q_{06} β peptide resulted in *b* and *y* fragments with no information on the position of the modifications (Supplemental Figure S2b) [50]. Additionally, electron ionization/impact dissociation (EID) can generate ECD type fragments from singly charged molecular ions [4], as well as C_α-C_β cleavage for isoaspartic acid [45]. However, only *b* and *y* fragments were detected in EID mass spectra of the Q_{06} β-peptide (Supplemental Figure S2c) [51]. The lack of *c* and $z^\bullet$ fragments could be due to the low fragmentation efficiency of EID in this particular experiment. Thus, the well established ECD method would seem to be a better approach, but it requires an increase in the number of charges on the peptide.

Charge Increase

Three different approaches were applied in order to enhance the formation of higher charged molecular ions. These include the addition of nitrobenzyl alcohol or calcium salt into the ESI solution, and the covalent attachment of the cholamine tag to the peptide [50, 59, 63–65]. Although doubly charged ions were observed in all three cases, only the cholamine reaction produced sufficient ions for ECD analysis. Inefficiency of other methods could be due to the lack of a preferred calcium ion binding site and potentially low charge stabilization in the peptide. Cholamine reacts with the carboxylic acid and was attached to the peptide at its C-terminus. The second charge could be provided by the protonation of the N-terminal amine. Doubly charged species $[M_{Ch}^{+} + H]^{2+}$ were readily observed in the ESI MS, isolated, and further subjected to ECD (Figure 1).

Similar experiments of C-terminal charge tag attachment to α-peptides were done by Hunt's group [66]. Their result demonstrated increased formation of *z* ions. In the current study, no *z* fragments and only one ECD-type fragment, c_5, was observed for the Q_{06} β-peptide. The electron capture at the quaternary ammonium provides an abundant loss of the neutral trimethylamine 59.0735=$N(CH_3)_3$, leaving the radical on the C-terminal methylene group (Figure 1). This is expected to further induce radical initiated reactions and cleavages, such as the loss of ethylene. Although charge neutralization on the quaternary ammonium may not lead to backbone cleavages [67], electron capture at the protonated N-terminal amine of the doubly charged Q_{06} peptide should result in backbone fragmentations. Further, the recombination energy of the protonated N-terminal amino group is higher than that of the quaternary ammonium as calculated for the singly charged model cations by Jensen et al. [4.3 eV ($MeNH_3^{+}$) versus 3.1 eV (NMe_4^{+})] [68], making it the preferred electron capture site. However, ECD type fragments were lacking in the ECD spectrum of the tagged Q_{06} peptide, which was dominated by b_5 *and y* fragments which could be merely the result of the residual vibrational excitation [10]. Alternatively, the formation of *y* fragments could be facilitated by the backbone nitrogen protonation or a rearrangement that transfers a hydrogen atom to the backbone nitrogen [2, 15, 58]. Upon charge neutralization at the tag site and the subsequent tag loss, the excessive vibrational energy could induce the formation of the b_5 ion, similar to that suggested by Cooper et al. in their study of *b*-ion formation in ECD [14]. Nevertheless, it is hard to make definitive conclusions why this unusual fragmentation pattern was observed as two variables were introduced at the same time in the studied system: a cholamine tag and the incorporation of β-amino acids.

ETD of Q_{06} β Peptide

Electron transfer dissociation was further used to analyze the Q_{06} β peptide. As opposed to the first ESI experiment, a small peak corresponding to the doubly charged molecular ions, $[M_{Q06} + 2H]^{2+}$, was observed, which could be due to the difference in the ionization sources between two instruments. In particular, the glass capillary in the electrospray source used in the ETD study was heated to 220 °C which could possibly increase the desolvation efficiency and production of higher charged ions compared to the unheated source used in the previous ECD experiment.

ETD of the Q_{06} β peptide produced an unusual fragmentation pattern with abundant *y* and *a*• fragments, and only two *c* fragments in low abundance (Figure 2). The assignment of a_5• ion was ambiguous due to the interference from x_4 ion [x_4 (*m/z*=497.33), and a_5• (*m/z* = 497.39)], which could not be resolved using the ion trap. The assignment is probably correct, because *x* ions are not

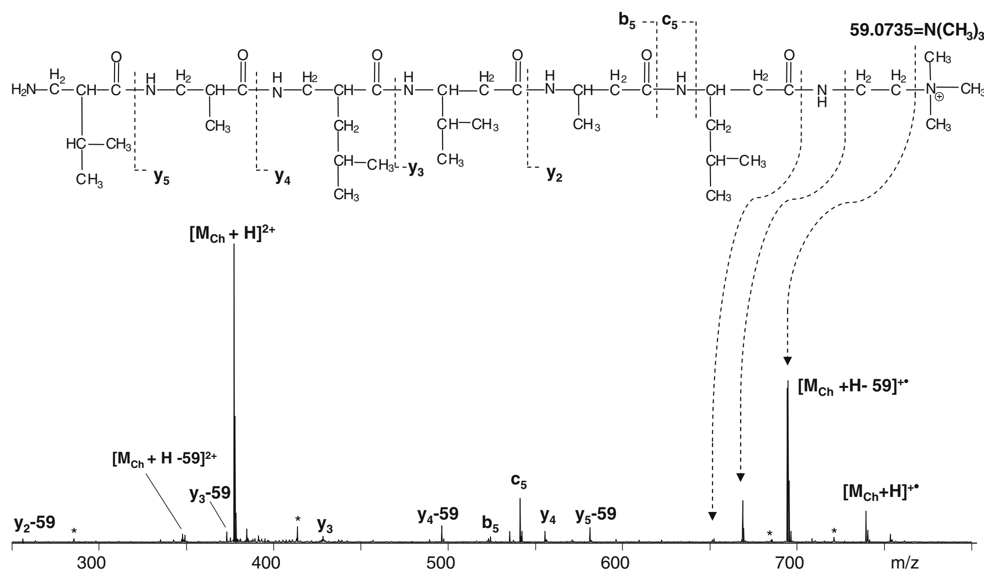


Figure 1. ECD of Q_{06}•cholamine

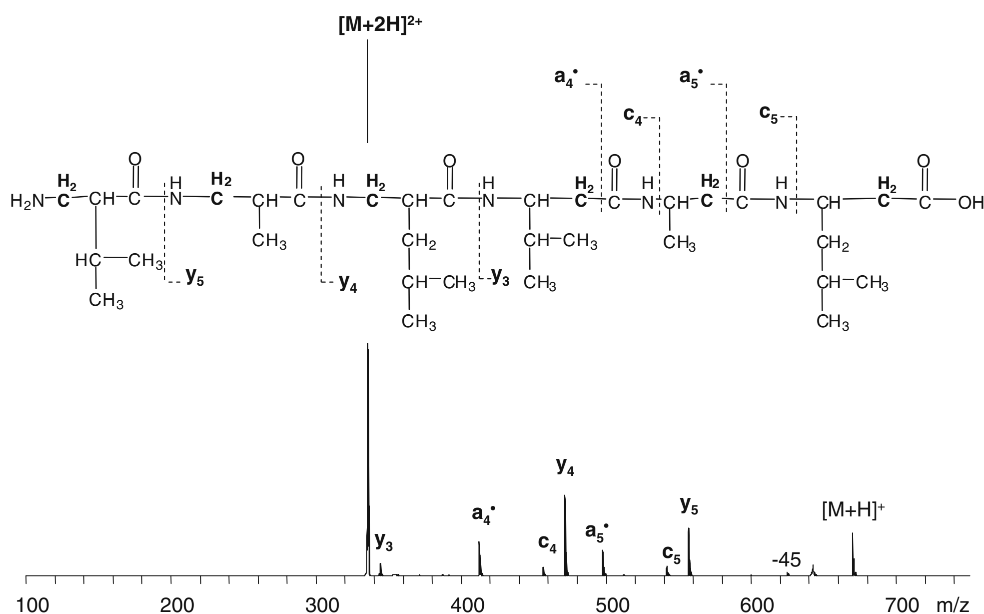


Figure 2. ETD of Q_{06} β_2/β_3-β peptide

commonly observed in ECD and ETD, and they were not detected for this peptide in ECD experiments. It is not clear how such abundant *y* fragments could be formed without a C-terminal charge carrier. It might be due to the backbone nitrogen protonation, similar to the cholamine attached Q_{06} β-peptide ECD results. Likewise, the alternative dissociation pathway seems to be enhanced. Meanwhile, low vibrational energy applied to the charge reduced species to increase dissociation efficiency upon electron transfer (smart decomposition) may also be a reason for enhanced *y* fragment formation. However, this cannot explain the formation of *a•* fragment ions as those are radicals species and more likely to be a product of ion-electron reaction. In general, the observed fragmentation pattern for this peptide is not following the usual ETD behavior. ExD of this very simple β-peptide demonstrates a big difference for fragment formation in β-peptides compared to their α-analogues.

Modified Substance P

In order to better understand the unusual ExD behavior of β-peptides, ECD experiments were carried out on a well studied system, the Substance P peptide and its variant, which was modified at two positions: glutamine 5 and leucine 10 to β_3-homo amino acids. Q_5 and L_{10} were specifically selected for modification as they normally provide abundant c_4 and c_9 fragments (Figure 3a), and the expected fragments resulted from

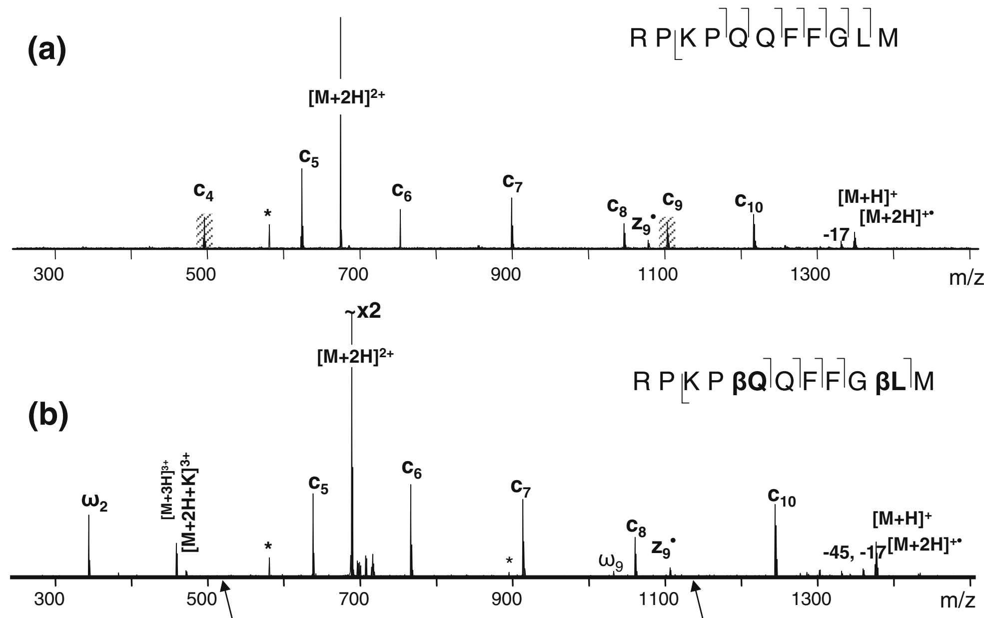


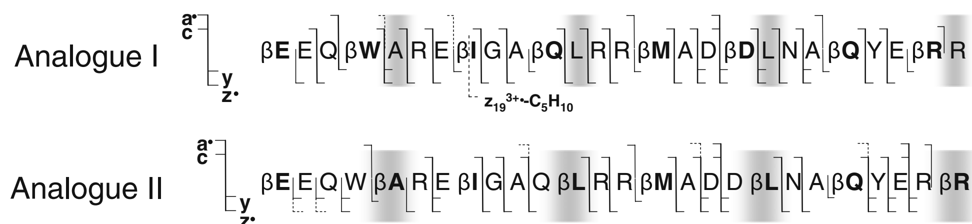
Figure 3. ECD of Substance P: (**a**) unmodified, (**b**) modified at Q_5 and L_{10} to β_3 -type amino acids

the C_α–C_β bond cleavage (discussed below) would not interfere with other peaks in the spectrum. The introduction of the two extra methylene groups in the backbone increased the molecular mass of the peptide by 28 Da. Fragment ion mass shift at 14 Da per additional methylene group was also observed correspondingly. As was discussed in the introduction, the extra methylene group present in isoAsp leads to a C_α–C_β backbone bond cleavage and the formation of signature diagnostic fragments. Similar cleavages were expected for the current modifications which would result in formation of c_4+85 and c_9+70 fragment ions. However, not only were these ions absent, the c_4 and c_9 fragments also disappeared from the ECD spectrum entirely (Figure 3). One may speculate that introduction of the CH_2 group could lead to such a conformational change that would hinder the formation of c_4 and c_9 fragments; or they may still be formed, but not separated due to the new, possibly tighter hydrogen bonding, although the incorporation of only one extra methylene group is unlikely to create such a big difference in intramolecular hydrogen bonds. As proposed previously [51,52], it is more likely that the absence of C_α–C_β bond cleavages resulted from the lack of radical stabilization effect by a side-chain carbonyl group as in isoAsp containing peptides. Further, in α-amino acid residues, the α-carbon radical formed by N–C_α bond cleavage can be resonantly stabilized by the neighboring carbonyl (Scheme 2); in β-amino acid residues, the carbonyl is located further away providing no such stabilization, making the N–C_β bond cleavage not energetically favorable. Within the isoAsp, however, a carboxylic acid on a side chain is located at the close proximity to the backbone β-carbon atom, which can play a stabilization role for the β-carbon radical and thus ensure both the C_α-C_β and N-C_β bond cleavages (supplemental material S1). In agreement with this explanation, in a previous ECD study of γ-glutamic acid containing peptides, N–C_γ, C_α–C_β, and C_β–C_γ bond cleavages were all observed [49]. Similar stabilizing effect can be provided by the aromatic structure as well, such as that observed in the β-phenylalanine containing peptides, which was recently reported by Hamidane et al. [57] supporting the radical stability hypothesis.

Puma BH_3 Protein Analogues

Further ECD analysis was performed on a set of bigger peptides, originally designed to mimic foldamer ligands for the BH_3 recognition cleft of the protein Bcl-x_L [69]. The primary sequence of the two 26-residue α/β-peptide analogues corresponds to a Puma BH_3 domain (EEQWAREIGAQLRRMADDLNAQYERR). Both peptide analogues have amino acid residues modified to a β_3-homo amino acid after each second or third residue, but not all at the same positions. Such a backbone repeat ααβαααβ allows formation of an α-helix like conformation that helps mimic the original binding behavior of an α-helical domain. For the purpose of the current study, the two peptide analogues provide an excellent system for the direct comparison of fragmentation within α- and β-type amino acids. The ECD spectrum of the 3.3 kDa Puma BH_3 protein analogue I (**βE**EQ**βW**ARE**βI**GA**βQ**LRR**βM**AD**βD**LNA**βQ**YE**βR**R) is shown in Figure 4; the ECD spectrum of the analogue II (**βE**EQW**βA**RE**βI**GAQ**βL**RR**βM**ADD**βL**NA**βQ**YER**βR**) can be seen in Supplemental Material (S3). The spectra show nearly complete sequence coverage with various types of fragments observed, including *a•*, *y*, *c*, and *z•* fragments. In agreement with the modified Substance P result, no N–C_β bond cleavages were observed for the β-type amino acid residues, but the corresponding α-amino acid residues all provided such cleavages. For example, *c* and *z•* fragments are observed at the α-Ala_5, α-$Leu_{12,19}$, and α-Arg_{26} positions (analogue I) but not in the β-Ala_5, β-$Leu_{12,19}$, and β-Arg_{26} positions (analogue II) (Scheme 3, relevant residues are highlighted). The only N–C_β bond rupture was detected for β-Asp provided by the z_9^{2+} and c_{17}^{2+} fragments (Figure 4). Interestingly, this is a β-homo aspartic acid; i.e., the side-chain carboxylic acid is separated from the backbone β-carbon by one methylene group. Hence, β-Asp side chain carbonyl cannot provide the same stabilization for the C_β radical as in the native isoAsp, and the z_9^{2+} and c_{17}^{2+} fragments were only present at low abundance.

As was noted earlier, the Puma BH_3 peptides studied here have α-helix like conformations. It is possible that the N–C_β bonds can be cleaved but protected from dissociation due to strong hydrogen bonding within the α-helix as was suggested previously [70] and consistent with the results from ECD mechanistic studies [24, 26]. Yet, there were many *c* and $z^\bullet$ fragments present except for those missing within β-residues suggesting that the peptide was relatively unfolded, and thus, the hydrogen bonds should not interfere with the fragment separation. Furthermore, dissociation of the C_α–C_β bond now seems to be an exception rather than the rule for β-amino acid residues as only one peak representing the C_α–C_β cleavage of isoleucine was identified provided by the $z_{19}^{3+\bullet}$-C_5H_{10} fragment ion (Figure 4).



Scheme **2.** ECD cleavage scheme of Puma BH_3 protein analogues I and II

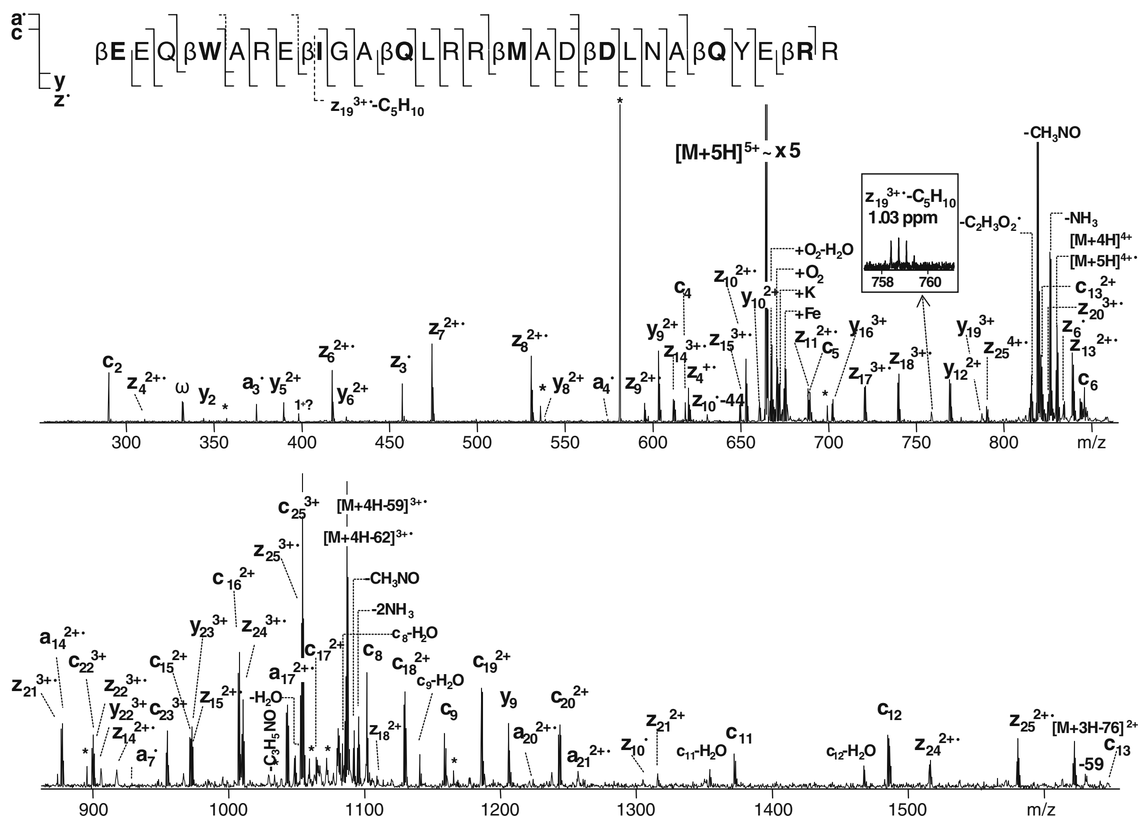
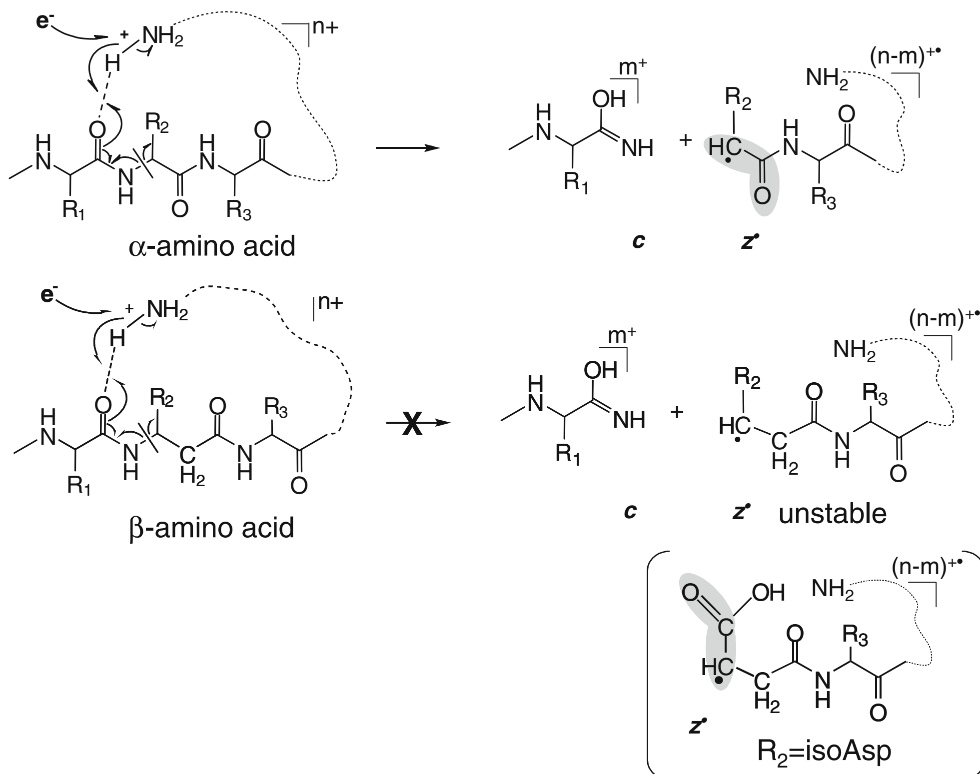


Figure 4. ECD of Puma BH_3 protein analogue I

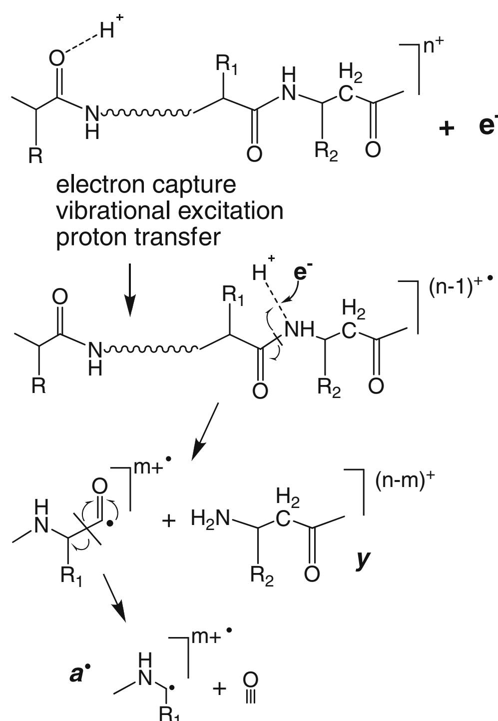


Scheme **3.** ECD mechanism within α-versus β-amino acid residues

Indeed, according to Turecek and coworkers [71] who studied the β-alanine N-methyl amide model system, dissociation of the C_α–C_β bond was slow and in competition with other dissociations from the most stable composition with a radical located on the C-terminal amide carbonyl. To conclude, the N-C_β and C_α–C_β backbone cleavages are still possible, but do not represent the dominant channel of fragmentation within β-amino acid residues.

Proposed Mechanism

The incorporation of an extra methylene group within the polypeptide backbone increases the flexibility of the molecule. The conformational change is probably not that dramatic, but an increase in internal rotations is expected, resulting in decreased steric hindrance. Thus, the backbone nitrogen may become more exposed for hydrogen bonding. In addition, elongation of the backbone within the β-residue moves the amide nitrogen and the following amide carbonyl apart removing the captodative stabilization effect at the C_β. Therefore, the N–C_β bond dissociation becomes a less favorable process, thus shifting dissociation to other fragmentation channels. One such channel may proceed via backbone nitrogen protonation, leading to increased $a^\bullet$ and y fragment ion formation (Scheme 4) as was proposed in an early ECD paper [3]. The N–C_β bond rupture may still occur, creating c fragment and unstable intermediate $z\bullet$ fragment that, if formed, will probably undergo further rapid dissociation, and is thus not observed. However, it is more likely, that upon electron capture the radical at the backbone amide hydrogen will induce the homolytic cleavage of the peptide bond and further loss of a CO molecule to produce the more stable $a\bullet$ and y fragment ions. It is interesting to note that, unlike the doubly charged Q_{06} peptide, the $a\bullet/y$ fragmentation channel was not observed in the ECD spectrum of the doubly charged β-Substance P variant.



Scheme **4.** ECD mechanism within β-amino acid residues

A closer look at the Q_{06} β-peptide and the modified Substance P revealed that, besides the nature of the amino acids, the clear difference between the peptides is the presence of basic amino acid residues, which could dictate the sites of protonation and thus the sites of electron capture dissociation. The two charges carried by the Substance P peptide would preferably reside at the Arg and Lys side chains, and are solvated by the carbonyl groups of the peptide. According to the Cornell ECD mechanism [10], upon electron capture at the protonated site, hydrogen migration to various carbonyl groups would result in N–C_α bond cleavages. Due to arginine being a poor hydrogen donor, backbone fragmentations are most likely initiated by electron capture at the protonated N-terminal or lysine side chain amino group [19,28,72]. In the case of the β-Substance P, this process will result in the formation of unstable C_β radicals within the βQ and βL residues, which correlates with their disappearance. On the other hand, in the Q_{06} β-peptide, there are no basic amino acids. As was discussed earlier, the first protonation site would be the N-terminal amine, and the second proton would be mobile within the polypeptide backbone. Thus electron capture would occur at the N-terminus or on the backbone rather than on the side chain and the electron induced fragmentation of the Q_{06} peptide would occur via a different mechanism to that of Substance P. Introducing the third charge to the Substance P peptide should lead to the protonation of an additional site within the molecule, which is likely one of the backbone amide nitrogen or carbonyls (see below), since N-terminal amine protonation seems unlikely because of the strong columbic repulsions by the nearby protonated Lys and Arg residues. To test this hypothesis, triply charged β-Substance P was subject to the ECD analysis (Figure 5). Indeed, many $a\bullet$ and y fragments now appeared in the spectra. Interestingly, they often were slightly higher in abundance in the modified substance P variant as shown in the inset (Figure 5). Nonetheless, all of the $a\bullet$ and y fragments were observed in both peptides, except for y_2 and $a_9\bullet$, which were exclusively present in the modified Substance P variant. The last two fragments are formed due to the cleavages within the β-Leu_{10} residue. Note that the c_9 fragment was dramatically reduced in modified variant. This is consistent with the previous results and supports the proposed hypothesis for the fragmentation within the β-amino acid residues.

The role of backbone nitrogen protonation in ECD has been previously discussed in the literature. In the original ECD study, the $a\bullet$ and y ions were proposed to be formed from the backbone nitrogen protonated species [3]. Backbone nitrogen protonation was also suggested to play a role

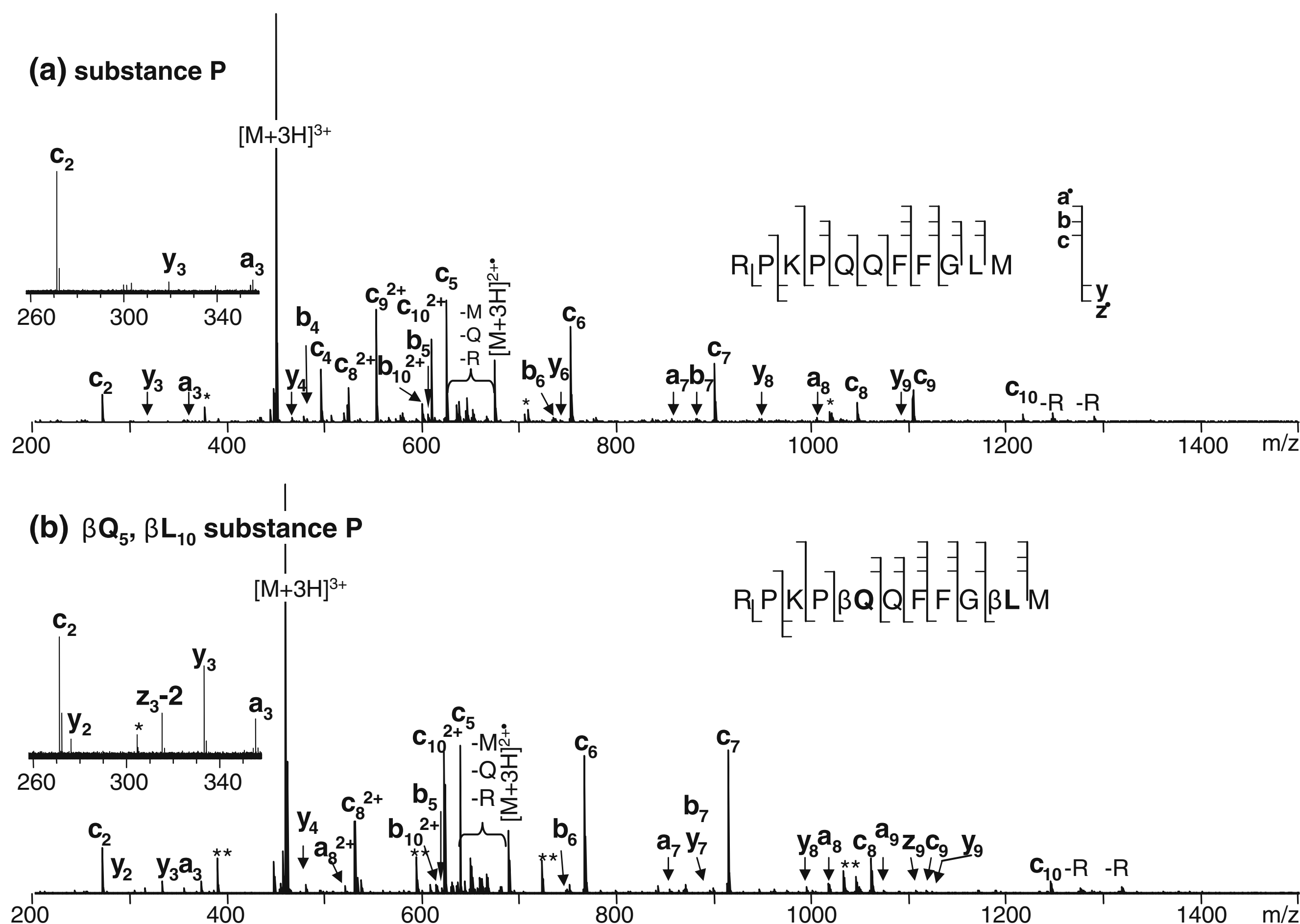


Figure 5. ECD of triply charged substance P $[M+3\ H]^{3+}$: (**a**) unmodified, (**b**) modified at Q_5 and L_{10} to β_3-type amino acids

in the formation of *b* ions in ECD. Theoretical investigation by the Uggerud group showed that electron capture by nitrogen protonated N-methyl-acetamide resulted in rapid amide bond dissociation and production of CH_3CO and NH_2CH_2, corresponding to the *b* and *y* ions in peptides [73]. Concomitantly, *b*• ions can further lose CO to form *a*• ions. In addition, *b* ions were present in ECD of peptides without basic amino acid residues [16]. It was suggested that upon backbone nitrogen protonation, the peptide bond could be cleaved to form a *b*•/*y* ion pair, and the subsequent intra-complex hydrogen atom transfer within this long-lived ion pair could lead to the formation of *b* and $y^{\bullet}$ ions. Backbone amide nitrogen protonation was also invoked to explain the *a*•, *b*, and *b*• ion formation in nitrated peptides that were either acetylated at the N-terminus, or lacking basic amino acid residues [74]. In the current study, however, neither *b* nor *y* ions observed were radicals. Thus, these *b* ions were most likely formed via the energetic fragmentation of vibrationally excited even-electron charged reduced species [14].

It should be noted that protonation on the backbone nitrogen is not thermodynamically favorable [75–77]. For instance, for the model system of N-methylacetamide, the proton affinity of the carbonyl oxygen was calculated to be ~60 kJ/mol higher than that of the backbone nitrogen [76]. This is in agreement with the study of the dipeptide Lys-Gly, where the carbonyl oxygen protonated species was calculated to be ~45 kJ/mol more stable than the backbone nitrogen protonated species [77]. Based on these results, peptide fragmentation via protonation of the backbone nitrogen would seem unlikely. However, theoretical investigations have usually been done on very small model systems, which often do not possess extensive intramolecular interactions such as hydrogen bonding and salt bridges that are expected to play a more important role in the fragmentation of larger peptides. Charge solvation by nearby backbone and side chain groups could appreciably change the relative stabilities of different protonated species. In β-peptides, the elongation of the backbone may make it better positioned (due to less steric hindrance by the adjacent side chain) for hydrogen bond formation. The addition of an extra methylene group in the backbone can also slightly increases the gas-phase basicity of the amide nitrogen, as the electron withdrawing carbonyl group is replaced by the electron donating alkyl group. Further, electron capture by the precursor ion could increase its internal energy considerably (by several eVs), and less favored protonation sites may become significantly populated as the system relaxes from the initial Rydberg state to low lying electronic states. In other words, the proton may initially reside on the carbonyl oxygen, but could migrate to the backbone amide

nitrogen upon excitation. This argument is similar to the one used in the mobile proton model to explain the low energy CAD fragmentation behavior of peptide ions [75–78], where it was proposed that the proton initially resides on the thermodynamically more stable sites, such as the lysine side chain or backbone oxygen, and later migrates to the less favorable sites, including the backbone amide nitrogen upon collisional activation to facilitate fragmentations. Finally, for the β-linked peptides studied here, electron capture at the protonated oxygen site cannot lead to "normal" *c/z* fragmentation due to the radical instability, which may further drive the migration of the proton to the less preferable backbone nitrogen site that could lead to the formation of $a^{\bullet}$ and *y* ions upon electron capture. In general, the abundance of these unusual ECD fragments was fairly low, as expected from the low population of nitrogen protonated species (Fig. 5), but they were nonetheless present in competitive abundance when the primary ECD fragmentation channel was blocked within β-amino acid residues.

Further experiments are needed to test the proposed mechanism. In addition, basicity measurements and theoretical investigations specifically for β-amino acid containing peptides could provide a better understanding on how these *a*• and *y* type fragments are formed.

Conclusions

Various peptides containing β-amino acid residues were analyzed in this study. Remarkably, N–C_β bond cleavages were rare within the β-residues, and C_α–C_β cleavages were seldom observed providing no evidence of the β-residues in spite of previous results of the isoaspartic acid. Furthermore, no distinct difference was found for the fragmentation within β_2 versus β_3-type amino acid residues. Meanwhile, *a*• and *y* fragments were often produced at β-residues, particularly for the bigger peptides with α-helical like structures. The lack of *z*• and *c* fragments and increased *a*• and *y* fragment formation could imply the presence of β-residues in the peptide; however, this is a poor signature, because of the normal appearance of *a*• and *y* fragments in ExD spectra of α-peptides, and various other reasons that can contribute to the disappearance of *z*• and *c* fragments. Thus, currently, ExD methods cannot be used to reliably differentiate α- from β- or β_2 from β_3 type amino acids.

The introduction of one extra methylene group into the polypeptide chain destabilizes the C_β radical formed by the N–C_β bond rupture making this channel of fragmentation less favorable. Thus, the fragmentation occurs via alternative channels. The dominant products appear to be the *a*• and *y* fragments, with the exception when the side chain of the β-residue can provide radical stabilization for the formation of the *z*• and *c* fragments. It is suggested that appearance of such fragments may require protonation on the backbone amide nitrogen, which is further supported by the charge state-dependent study of modified Substance P peptide. The fragmentation mechanism for β-peptides has been proposed via backbone nitrogen protonation similarly to what was originally proposed for the ECD of α-peptides.

The minor ECD pathway of *a*• and *y* fragment formations was little studied. Future studies of this fragmentation pathway would help with our understanding of the fragmentation of the β-amino acid containing peptides. Further ExD studies as well as computational studies and kinetic analysis specifically for the β-amino acids are needed for better characterization of β-peptides.

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