CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200902480

Radical Stability Directs Electron Capture and Transfer Dissociation of β-Amino Acids in Peptides

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Abstract: We report on the characteristics of the radical-ion-driven dissociation of a diverse array of β-amino acids incorporated into α-peptides, as probed by tandem electron-capture and electron-transfer dissociation (ECD/ETD) mass spectrometry. The reported results demonstrate a stronger ECD/ETD dependence on the nature of the amino acid side chain for β-amino acids than for their α-form counterparts. In particular, only aromatic (e.g., β-Phe), and to a substantially lower extent, carbonyl-containing (e.g., β-Glu and β-Gln) amino acid side chains, lead to $N-C_\beta$ bond cleavage in the corresponding β-amino acids. We conclude that radical stabilization must be provided by the side chain to enable the radical-driven fragmentation from the nearby backbone carbonyl carbon to proceed. In contrast with the cleavage of backbones derived from α-amino acids, ECD of peptides composed mainly of β-amino acids reveals a shift in cleavage priority from the $N-C_\beta$ to the $C_\alpha-C$ bond. The incorporation of CH_2 groups into the peptide backbone may thus drastically influence the backbone charge solvation preference. The characteristics of radical-driven β-amino acid dissociation described herein are of particular importance to methods development, applications in peptide sequencing, and peptide and protein modification (e.g., deamidation and isomerization) analysis in life science research.

Keywords: amino acids · electron capture · electron transfer · mass spectrometry · radical ions · radical stability

Introduction

Deciphering the relationship between the structure and activity of peptides and proteins is a topic of primary importance in life science research.[1] To develop more potent drugs more quickly and with better rationale, insights into the biomolecular conformational landscape and an improved understanding of inter- and intramolecular energy relaxation pathways are needed. Introduced several decades ago, β-amino acids and β-peptides[2] were intended to generate a new class of drugs that would be small but structurally rich, functionally active, and more resistant to degradation in the blood stream than α-amino acids and peptides.[3–5] Diverse forms of β-amino acids have been synthesized,[6–8] as shown in Scheme 1, for use as building blocks to produce β-peptides ranging in size from short but structured peptides[4] to imitations of the highly-structured large peptides and small proteins based on α-amino acids.[9] Despite substantial progress in understanding the structure/activity relationship of various β-peptides, their practical use as drugs has not yet been achieved. Scheme 1 implies that β^2-Ala and β^3-Ala residues should have the same structure, and thus will be noted below as β-Ala.

Although β-peptides rarely occur naturally, β-amino acids are known to be formed as reaction products in biological systems.[10] An important example is the formation of an isoaspartyl residue by isomerization of an aspartyl residue in vivo. The resulting isoaspartic acid belongs to a class of β^3-amino acids in which the side-chain β-carbon is incorpo-

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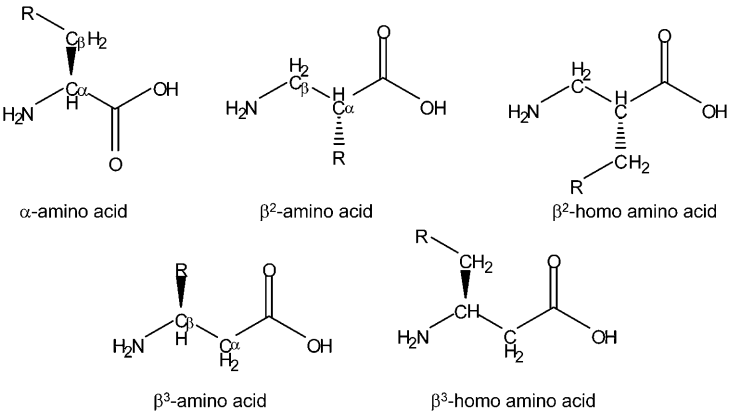
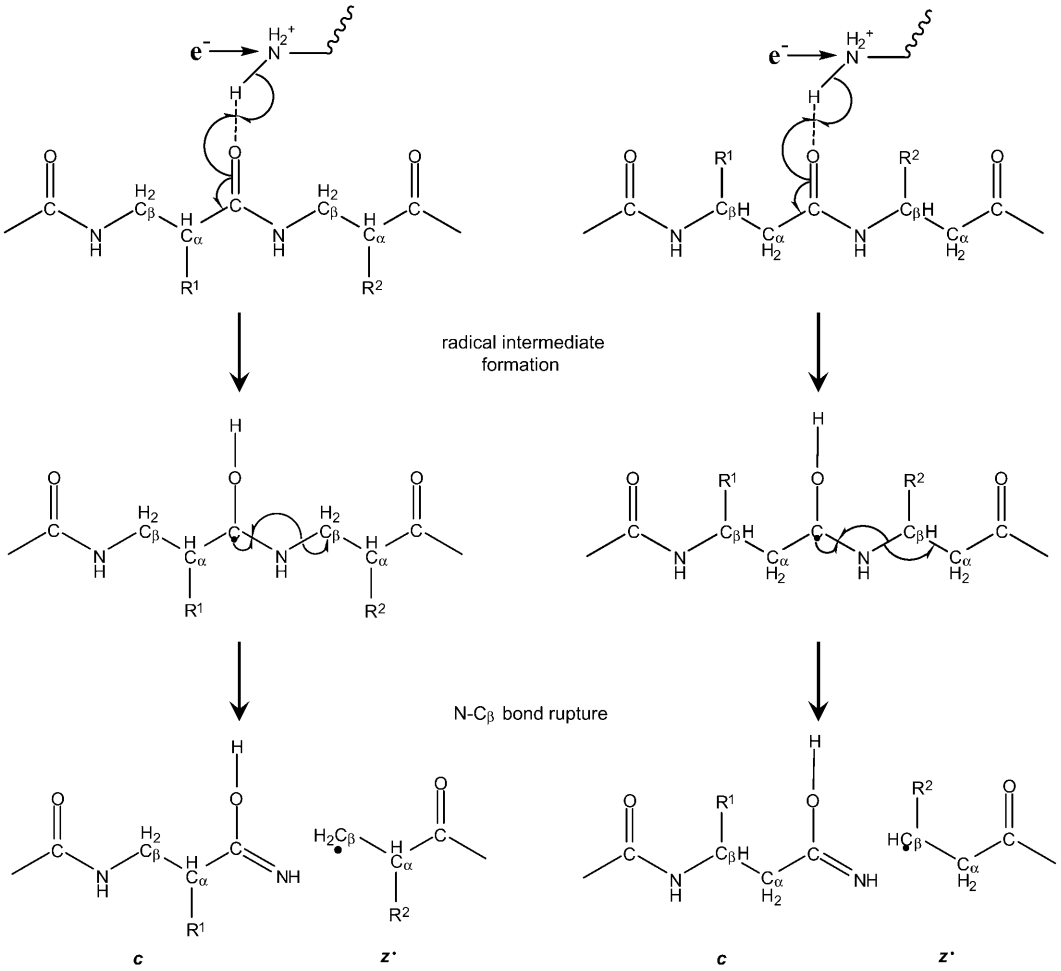
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200902480.



Scheme 1. Structures of β-amino acids of different types compared to an α-amino acid.

rated into the peptide backbone, resulting in increased flexibility and reduced side-chain size, as shown in Scheme 1. Distinguishing aspartyl from isoaspartyl residues is analytically challenging. Recently, O'Connor and co-workers introduced a new tandem mass-spectrometry-based method for the identification of isoaspartyl residues by a signature side-chain loss.[11,12] This method successfully employed electron-mediated tandem mass spectrometry (MS/MS) techniques, electron-capture dissociation (ECD),[13] and electron-transfer dissociation (ETD),[14] but not the commonly used slow heating techniques (e.g., collision-induced dissociation (CID) and infrared multiphoton dissociation (IRMPD)).[15] As previously reported, application of CID to various β-peptides primarily results in the preferential cleavage of the peptide bonds in the backbone, and formation of *b* and *y* ions, as observed for CID of α-peptides.[16] The principal difference between ECD/ETD and CID/IRMPD is in the types of sequence-specific product ions formed. In contrast to the peptide bond rupture in CID/IRMPD, the N–C_α bond ruptures preferentially in ECD/ETD of α-peptides.[17] The most commonly accepted possible mechanism of the N–C_α bond rupture in ECD/ETD of α-peptides, projected to β-amino acid templates, is shown in Scheme 2.

The second most abundant fragmentation pathway in ECD/ETD of α-peptides is the rupture of the C_α–C backbone bonds, with direct formation of radical *a* ions and sub-



Scheme 2. A possible β-peptide fragmentation following a McLafferty (Cornell)-type fragmentation pathway, leading to *c*- and *z*-ion formation, shown for the β^2-template (left), and the β^3-template (right) of a peptide backbone.

sequent formation of even-electron *y* ions, following C=O group release from *x* ions. Because it is the minor fragmentation channel, *a*/*y*-ion formation in ECD/ETD of α-peptides has received less attention. The most commonly invoked mechanism follows that originally suggested by McLafferty and co-workers: a process mediated by charge solvation at the backbone amide nitrogen.[17,18] As projected to β-amino acid templates, this pathway is presented in Scheme 3.

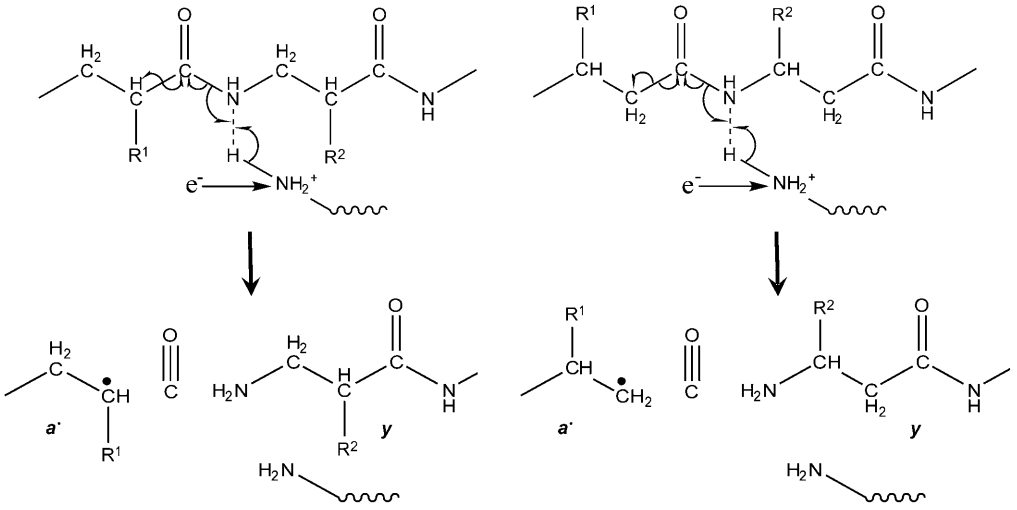
ECD- and ETD-based tandem mass spectrometry techniques are a relatively recent addition to the arsenal of methods for peptide and protein structure analysis based on mass spectrometry.[19] Nevertheless, they have captured the attention of research communities that use MS/MS for life-science-oriented research, as well as those that study fundamental effects in gas-phase radical-ion chemistry. A number of ECD/ETD-specific features make these fragmentation techniques complementary to CID.[20,21] In particular, biological application-oriented research benefits from using ECD/ETD for in-depth analysis of labile post-translational modifications located on peptides and proteins on a chromatographic timescale.[22] Distinguishing leucine from isoleucine[23] and, as discussed above, aspartyl from isoaspartyl by ECD/ETD are important steps toward complete de novo peptide sequencing.

Fundamental research-oriented attempts to describe the underlying, rich radical-ion chemistry aim to further improve the efficiency of these MS/MS techniques. The field has a number of open questions, including those related to quantifying the cleavage propensities of a peptide backbone, understanding the role of amino acid side-chain properties in the ECD/ETD process, understanding the pathways leading to the formation of radical ions, and the dynamics of hydrogen rearrangement between reaction products. Probing unnatural forms of amino acids and peptides by ECD/ETD is also one of the many objectives that need to be addressed to complete our understanding of these complex processes.[24] Interest in the structure of β-amino acids for fundamental ECD/ETD research is not based solely on the properties of the side chain, but also on the structural changes in the backbone. The influence of an additional CH_2 group in the peptide backbone on the cleavage propensities and formation of radical ions is not easily predictable for either ECD or ETD. Although ECD and ETD produce practically identical fragmentation patterns for α-peptides, differences in the extent of radical ion formation have been reported.[25] These differences were attributed to nonequivalent experimental conditions involving high vacuum ECD in Fourier transform ion cyclotron resonance mass spectrometry, (FT-ICR MS) compared to the low-to-medium vacuum ETD in ion trap mass spectrometry (ITMS).

Thus, the characterization of ECD and ETD techniques applied to a broad range of β-amino acids and β-peptides is of interest both for biological application-driven research and for the field of radical-ion chemistry. Here we aimed first to reveal the response of ECD and ETD to diverse β-amino acid substitutions in peptides. We selected β-variants of a neuropeptide substance P as the starting model systems, based on previous studies conducted on the structural and biological effects of β^2 and β^3-amino acid insertion into that neuropeptide.[3] We then attempted to determine the influence of various β-amino acid side chains on the inherent radical stabilization occurring in ECD/ETD to complement the general understanding of the electron-induced fragmentation of α-peptides.

Results

ECD/ETD of single β-amino acid substituted peptides: Figures 1 and 2 demonstrate the ECD fragmentation pattern response to β-amino acid substitution for various single-point β-variants of substance P. ETD fragmentation patterns obtained for these samples are similar to the ECD ones



Scheme 3. A possible β-peptide fragmentation process, presumably mediated by charge solvation at the backbone amide nitrogen in β-amino acids, leading to *y*- and *a*-ion formation, shown for the β^2-template (left), and the β^3-template (right) of a peptide backbone.

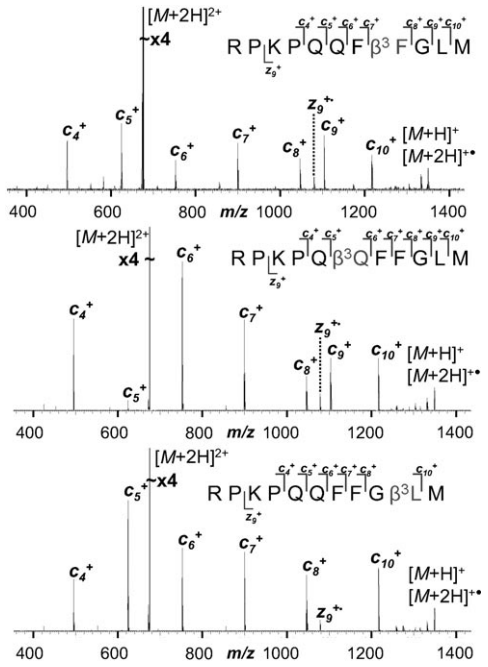


Figure 1. ECD FT-ICR MS of doubly-charged β-variants of substance P, including β^3-Phe_8 (top), β^3-Gln_6 (middle), and β^3-Leu_{10} (bottom), demonstrates the effect of single β-amino acid substitution on the N-terminal product ions. ETD performed in ion trap MS produces similar effects (see Supporting Information, Figure S1).

(Figures S1 and S2, Supporting Information). ECD/ETD mass spectra of substance P variants with the single β-amino acid substitution reported here can be compared to the typical ECD/ETD mass spectra of the wild-type substance P published elsewhere.[26] Interestingly, suppression of the fragmentation inside of the β-amino acid is observed for β^3 or β^2-homo leucine and alanine substitutions at the 9- and 10-positions, respectively (Figure 1, bottom, and Figure 2). However, for the substance P β^3-Gln_6 variant (Figure 1, middle panel) the c_5 product ion corresponding to this cleavage is still observed, but with drastically reduced intensity, whereas in the case of substance P β^3-Phe_8 (Figure 1, top panel) the cleavage efficiency is retained, and the corresponding c_7 product ion intensity is comparable to the ECD of the wild-type peptide. Figures 1 and 2 demonstrate only the case of the N-terminal product ions, for example, *c* ions, modulation by β-amino acid insertion.

Our previous results demonstrate abundant C-terminal product ions, or *z* ions, in ECD/ETD of a LLLLALLLK-NH_2 peptide.[25] Thus, we submitted the β-variants of this peptide to ETD ITMS to probe the influence of β-amino acid substitution on the formation of *z* ions (Figure 3). In particular, β^3-Leu_2 substitution (Figure 3, bottom) shows a clear reduction of z_8 relative intensity as compared to the previously reported ECD/ETD fragmentation pattern of the wild-type peptide.[25] Similarly, in the β^3-Leu_6 substituted peptide ETD mass spectrum (Figure 3, middle panel), the β-amino acid produced z_4 fragment ion intensity is reduced

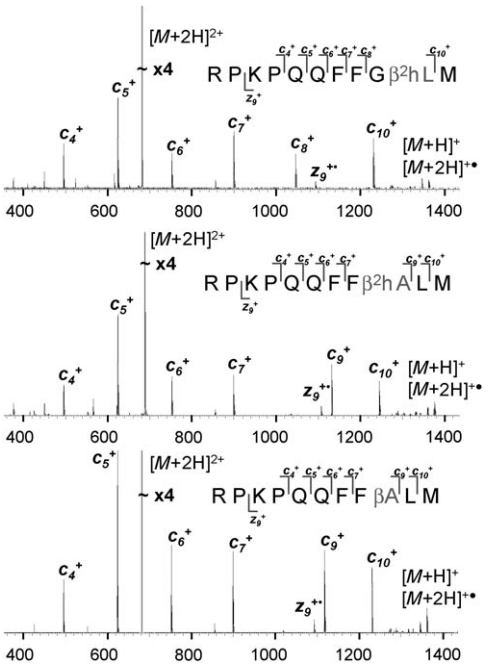


Figure 2. ECD FT-ICR MS of doubly-charged β-variants of substance P, including β^2-$homoLeu_{10}$ (top), β^2-$homoAla_9$ (middle), and β-Ala_9 (bottom), demonstrates the effect of single β-amino acid substitution on the N-terminal product ions. Similarly to Figure 1, suppression of cleavage inside the β-amino acid is observed. ETD performed in ion trap MS produces similar effects (see Supporting Information, Figure S2).

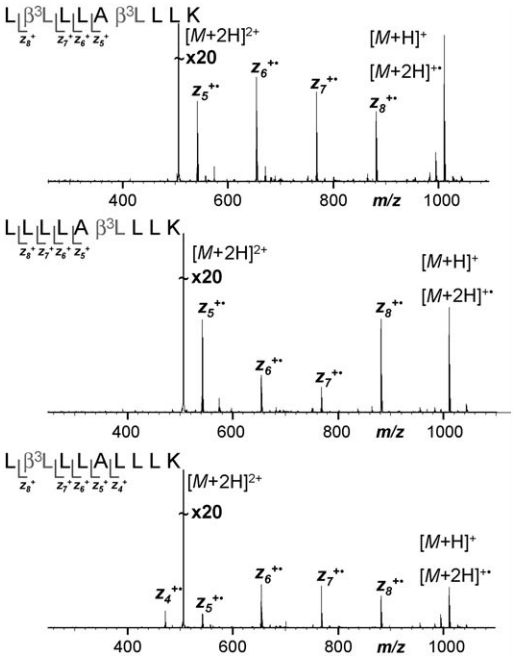


Figure 3. ETD ITMS of doubly-charged β-variants of LLLLALLLK–NH_2, including incorporation of β^3-Leu_2 and β^3-Leu_6 (top), β^3-Leu_2 (middle), and β^3-Leu_6 (bottom), demonstrates the effect of single and double β-amino acid substitution on C-terminal product ions. ECD performed in FT-ICR MS produces similar effects (see Supporting Information, Figure S3).

to such a degree that this product ion is no longer observed. Comparable results were obtained with ECD FT-ICR MS for the same samples (Figure S3 Supporting Information).

Surprisingly, ECD/ETD of the β^3-Leu$_7$ substituted peptide (Figure S4, Supporting Information) also shows a significant drop in z_4 ion abundance (the product ion is not observed under the employed experimental parameters). In this case, the reduction in N–C$_\beta$ bond cleavage efficiency is observed one amino acid away from the substitution site. ETD ITMS of the doubly β-amino acid substituted peptide, LLLLALLLK-NH$_2$, demonstrates both effects observed previously for single amino acid substitutions in this peptide (Figure 3, top panel, and Figure S4 in the Supporting Information, top panel). In other words, the intensity of the z_8 ion is reduced and the z_4 ion is not present.

ECD/ETD of β-amino acid multiply-substituted peptides: ECD and ETD mass spectra of a set of peptides comprising 11 amino acids, of which nine are β^3-amino acids, are shown in Figure 4, and Figures S5 and S6 of the Supporting Information. The peptide set is comprised of peptides with the following general sequence: H-R-βA-βA-βA-βA-X-βA-βA-βA-βA-K-OH, in which X is either β-Ala, β^3-Leu, β^3-Phe, β^3-Glu or β^3-Gln. Therefore, only N-terminal Arg and C-terminal Lys are α-amino acids, whereas the rest of the amino acids are in the β-form. The resulting ECD and ETD fragmentation patterns strongly differ from those reported for the α-analogues of these model peptides.[27] The extensive *c*-ion series, as well as the high mass-to-charge ratio *z* ions, are replaced by an almost complete radical *a*-ion series for all variants. High mass-to-charge ratio *y* ions are observed as well, and replace the corresponding *z* ions observed for α-peptides. Nevertheless, specific *c* ions are retained in ECD/ETD of these β-peptides. The c_{10} ion is observed in ECD/ETD of all the peptides, and is related to N-terminal cleavage to lysine, which is an α-amino acid. However, the c_5 ion is observed only for specific middle amino acids: when X is β-Ala (Figure 4, bottom) or β^3-Leu (Figure S6, Supporting Information), the c_5 ion is not produced, whereas when X is β^3-Glu (Figure 4, middle) or β^3-Gln (Figure S6, Supporting Information) a low intensity c_5 ion is produced. Finally, a peptide variant with X = β^3-Phe (Figure 4, top) produces a significant c_5 ion (second most intense product ion peak), highlighting the side-chain dependence of the fragmentation mechanism in β-peptides. The modulation of c_5 ion formation for these peptides as a function of amino acid side chain is in accordance with the ECD/ETD data reported above for substance P substitutions (Figures 1 and 2).

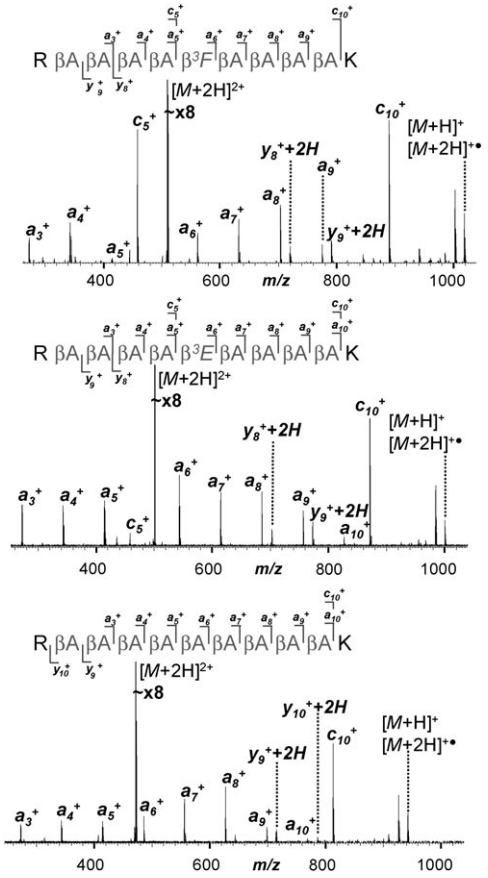


Figure 4. ECD FT-ICR MS of doubly-protonated β-peptides H-R-βA-βA-βA-βA-X-βA-βA-βA-βA-K-OH, in which X is β^3-Phe (top), β^3-Glu (middle), and β-Ala (bottom). The classical ECD/ETD fragmentation pathway, producing *c*- and *z*-type ions for the α-analogues of these peptides, does not occur; instead a complete series of *a* ions is present. Nevertheless, several *c* ions are observed for specific cases, which presumably relate to the side-chain properties, in accordance with Figures 1–3.

Figure 5 confirms the formation of *a/y* pairs in ETD of β-amino acids, as suggested by Scheme 1. The peptide sequence, H-K-βA-βA-βA-βA-β^3F-βA-βA-βA-βA-R-OH, employed in Figure 5 differs from the peptides used in Figure 4 by a swap between the N-terminal Arg and C-terminal Lys residues. As expected, the change in location of basic residues enhances charge neutralization at the N-terminal side of the peptide, thus favoring formation of C-terminal product ions. Therefore, an abundant z_6 ion is observed as a result of the N–C$_\beta$ bond cleavage of β^3-Phe, whereas in Figure 4 (top) its complementary c_5 ion is detected. The formation of *a/x* pairs, as seen in electron detachment dissociation (EDD) of multiply-deprotonated peptides, is not observed. Increased ETD efficiency is clearly observed for the triply-protonated precursors compared to the doubly-charged species. Nevertheless, the backbone cleavage sites remain, to follow the general trends described above for the doubly-protonated precursors.

ETD of a short β-peptide: ETD ITMS of the true β-peptide Ac-β^3hS-β^2hW-β^3hR-β^3hY-NH$_2$ is shown in Figure 6 (top). ETD ITMS of its α-analogue, Ac-SWRY-NH$_2$, is shown in Figure 6 (bottom) for comparison. A significant difference in fragmentation patterns is observed. Classical ECD/ETD product ions c_3 and z_3 obtained from an α-peptide are absent or significantly reduced in the β-peptide fragmentation spectrum. Moreover, a greater extent of secondary fragmentation and side-chain losses is seen for the β-peptide. The c_3 ions observed are due to N-terminal cleavage to Tyr, which is an aromatic amino acid like Phe. Thus, the effect is

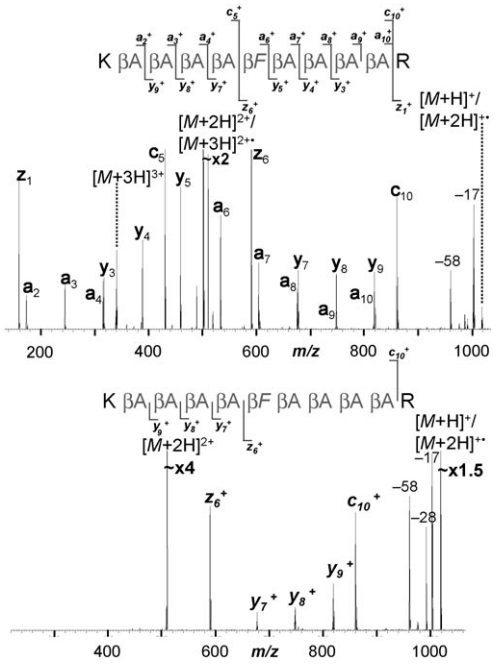


Figure 5. ETD LTQ Orbitrap FTMS of doubly (bottom) and triply (top) protonated β-peptides H-K-βA-βA-βA-βA-β^{3}F-βA-βA-βA-βA-R-OH. The basic residue inversion compared to the peptide reported in Figure 4 (middle) enhances the formation of C-terminal product ions. A corresponding series of *y* ions is observed in ETD of both doubly and triply charged precursors. Side-chain specific *c*- and *z*-ion formation is also confirmed.

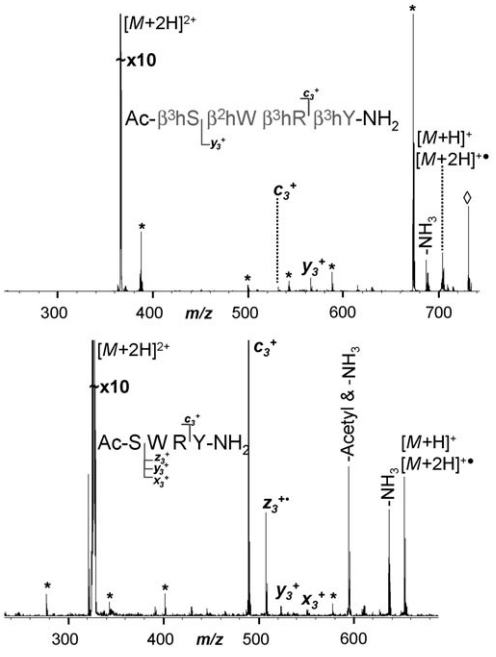


Figure 6. ETD ITMS of a short peptide Ac-Ser-Trp-Arg-Tyr-NH_2 in its α (bottom) and β-homo (top) form. The fragmentation patterns demonstrate similarities, although the intensities of *c* and *z* ions are clearly reduced in the β-peptide, and a higher proportion of unidentified and presumably secondary fragments, annotated with an asterisk (*), can be observed.

similar to that reported above for β^3-Phe substitution (Figures 1 and 4). Formation of y_3 ions is due to backbone N-terminal cleavage to another aromatic amino acid, Trp. In contrast to the more easily accessible and thus more widely employed β^3-amino acids, Trp in this peptide is in the β^2-homo form (Scheme 1).

Discussion

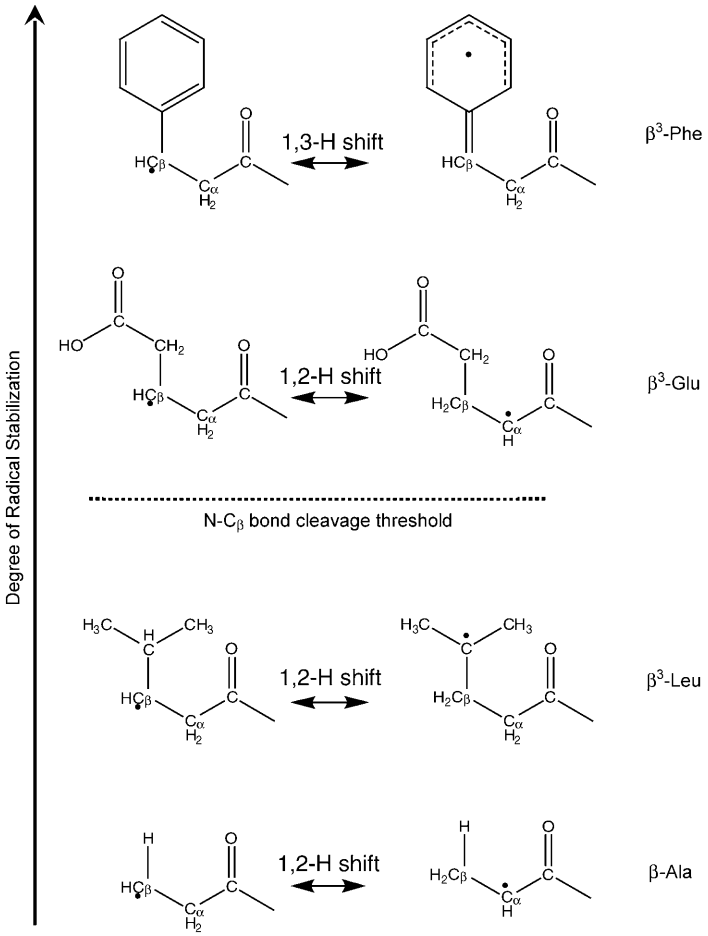
ECD/ETD characteristics of β-amino acids and β-peptide fragmentation: Peptide modification by replacement of an α-amino acid with a β-amino acid, by direct substitution with a β^2, β^3, β^2-homo or β^3-homo variant of the same or a different amino acid, results in similar fragmentation patterns for both ECD and ETD. Both fragmentation methods show that cleavages of the N–C_β backbone bonds belonging to the β-amino acid moiety are typically reduced to an undetectable level for all types of β-amino acid variants of Ala and Leu inserted into the α-peptides, whereas the β-variants of Phe, Tyr, Trp, Glu, and Gln allow the corresponding N–C_β bond cleavage to occur. However, an exception was observed for ECD/ETD of LLLLALLLK–NH_2 with β^3-Leu_2 substitution: the corresponding z_8 ion signal was reduced, but not completely removed. Presumably, not only the nature of the amino acid, but also its position in the sequence has an impact on the ECD/ETD fragmentation pattern.[12] Other ECD/ETD product ions (from the peptides substituted with a single β-amino acid, reported above) are retained, and the overall distribution of product ion abundance is preserved. An exception was observed for ECD/ETD of LLLLALLLK–NH_2 with β^3-Leu_7 substitution: the signal of the z_4 product ion disappeared, although it requires backbone cleavage one amino acid away from the substitution site. The corresponding z_3 ion is not observed in the peptide for either β- or α-forms of leucine in the 7-position. We shall note that *a* ion formation was not observed for the peptides substituted with a single β-amino acid employed here. Finally, comparison of Figure 4 and Figure S5 (Supporting Information) shows that ETD product ions may not be as abundant as ECD product ions due to less energetic fragmentation conditions in ion trap MS compared to FT-ICR MS, especially for the doubly-charged precursor ions.

ECD and ETD of multiple β-amino acid substitutions show two main trends. Firstly, the formation of abundant *a* ions is observed along the whole peptide backbone for every middle amino acid, X. Secondly, the formation of *c* ions near the substituted middle amino acids as a function of amino acid side chain follows the ECD/ETD fragmentation behavior reported above.

We explain the fragmentation characteristics summarized above following the currently accepted models for ECD/ETD and other radical-driven fragmentation processes for α-peptides.[28–31] These models explain the backbone cleavage propensities either through specific conformations, which determine the locations of the initial backbone radicals followed by local backbone fragmentation, or through a

distal radical migration to the cleavage site. Distinguishing these two pathways is not yet feasible.

Side-chain-mediated radical stabilization in single amino acid substituted peptides: Considering the degree of radical stabilization required to explain the formation of *c* and *z* ions, β-amino acids can be classified by side chains in the following manner (Scheme 4).[32] Firstly, β-amino acid side chains with little radical stabilization, such as β-Ala (Scheme 4, bottom), demonstrate significant or complete cleavage suppression. Secondly, moieties enabling strong radical delocalization, and hence stability, such as β^3-Phe (Scheme 4 top), show similar behavior to those of α-amino acids in ECD/ETD. Finally, despite a decrease in product ion intensities, N–C_β bond cleavages are nevertheless observed for β-amino acids containing a C=O group, such as β^3-Gln and β^3-Glu (Scheme 4, middle), indicating local stabilization of the intermediate carbonyl radical, provided by electronic delocalization into this moiety and possible interaction with the charged sites. Radical stabilization provided by the Glu residue has been reported previously, for example, by Simons and co-workers.[32] Amino acid side-chain structure also suggests higher radical stabilization provided by the Asp residue than by Glu, as reflected in the specific fragmentation patterns obtained by O'Connor and co-workers to distinguish between aspartic and isoaspartic acids.[11,12] Mentioned above as an exception, β^3-Leu may also provide radical stabilization, as recently confirmed for radical-driven tandem mass spectrometry of peptides containing Leu by Julian and Tsybin.[27,33] Although Leu is similar to Ile, its side chain provides a stronger radical stabilization, due to the possible formation of a tertiary radical versus a secondary radical for isoleucine.[23] Nevertheless, the intrinsic radical stabilization of leucine (Scheme 4) does not seem to be sufficient, as no N–C_β bond cleavage has been observed for β^3 or β^2-homo leucine substitutions in substance P. However, the case of the z_8 product ion retention in the LLLLALLLK–NH_2 peptide with β^3-Leu_2 could indicate a more complex combination of radical stabilization and structural effects on ECD/ETD fragmentation inside β-amino acids.



Scheme 4. The N–C_β bond cleavage efficiency is a function of the degree of β-amino acid side-chain radical stabilization. Consistently, N–C_β bond cleavage and formation of the *c*/*z* product ions are not observed or are very inefficient for amino acids that do not provide radical stabilization.

Our hypotheses for the absence of the z_4 ions in ECD/ETD of the LLLLALLLK–NH_2 dications (presumably protonated at the N-terminal amide and C-terminal Lys side chain) with either β^3-Leu_7 or β^3-Leu_6 substitution are based on either conformation selectivity or radical-driven chemistry. A conformation-based ECD/ETD model assumes that the N–C_β bond cleavage of the Leu_6 leading to the formation of the z_4 ions is possible if the N-terminal proton is first charge-solvated at the backbone carbonyl of Ala_5 (i.e., the C=O group between Ala_5 and Leu_6). However, substitution of β-amino acids for Leu_6 and Leu_7 increases the flexibility of the peptide backbone close to the C-terminus. Thus, the C-terminal protonated Lys side chain can reach farther than it does in the α-peptide, and can solvate more backbone carbonyls, thus probably preventing the formation of z_4 ions. As the N-terminal protonated amine should be preferentially neutralized compared to the protonated Lys, the radical that may lead to the formation of z_4 ions never appears at the backbone carbonyl. Similarly, the absence of z_3 ions in the α-peptide LLLLALLLK–NH_2 can be explained by the same protective effect, with a decreased flexibility of the C-terminal part of the peptide backbone. The feasibility of the alternative pathway of z_4 ion formation from charge solvation at the backbone carbonyl between Leu_6 and Leu_7 has yet to be proved.[34]

From the perspective of the radical-driven fragmentation process, the difference between retaining the z_8 product ions in the β^3-Leu_2-substituted peptide versus depleting the z_4 product ions in the β^3-Leu_6- and β^3-Leu_7-substituted peptides may be energy-dependent. More energy for radical migration and backbone activation is provided by charge neutralization of the N-terminal proton to the closer amino acid Leu_2 than to the distant Leu_6 or Leu_7 residues. The data presented here is insufficient to distinguish between the conformation-specific and radical-driven hypotheses, but it seems reasonable to consider both the conformation and the electronic structure as contributing to the ECD/ETD fragmenta-

tion. In the case of a double β-amino acid substitution (Figure 3, top panel), the additivity of multiple β-amino acid substitution effects on ECD/ETD emphasizes the hypothesis that electronic properties drive fragmentation, in which the two β-leucine amino acids could be seen as defects in the peptide lattice, inducing a local damping of the cleavage propensity.

Side-chain-mediated radical stabilization in multiple amino acid substituted peptides: ECD/ETD of H-R-βA-βA-βA-βA-X-βA-βA-βA-βA-K-OH and H-K-βA-βA-βA-βA-β^3F-βA-βA-βA-βA-R-OH peptides demonstrates not only amino acid dependent c_5-ion formation, but also a preference toward a minor fragmentation channel present in α-peptides (*a*/*y*-ion instead of *c*/*z*-ion formation, Scheme 3). Important facts in revealing the mechanism of these β-peptide fragmentations are the radical nature of the observed *a* ions, and the presence of even-electron *y* ions rather than *x* ions.

The most common consideration for explaining the formation of radical *a* ions and even-electron *y* ions as the minor channels in ECD/ETD, as manifested in β-peptides, is presented in Scheme 3.[17,35,36] Following this mechanism, electron-capture events should induce a hydrogen atom transfer, not to the backbone carbonyl oxygen, but to the backbone amide nitrogen, with subsequent formation of a nitrogen-centered radical. Consequently, $a^{\cdot}$- and *y*-products can be formed by radical rearrangement and subsequent rupture of the C–C backbone bond adjacent to the C=O group toward the N-terminus from the initially charge-solvated amide nitrogen. The reaction is accompanied by loss of CO. ECD literature suggests that the formation of *c*/*z* products following amide nitrogen radical formation is not favored in α-amino acids. Scheme 3 implies that the incorporation of a CH_2 group into the peptide backbone, either to the left of the backbone amide group (β^3-amino acid based peptide backbone) or to the right of it (β^2-amino acid based peptide backbone), may shift the preference for the charge solvation site. In general, the main criterion for deciphering oxygen protonation from nitrogen protonation in peptide backbones is the strength of the resulting hydrogen bond or the basicity of the hydrogen-bond acceptor group, CO versus NH, because the donor group, $R-NH_3^+$, is the same in both cases. The basicity of the carbonyl oxygen is greater than that of the amide nitrogen, favoring charge solvation on the carbonyl groups in regular α-amino acids and α-peptides.[37] However, due to the reorganization of the peptidic backbone and the incorporation of better electron-donating groups (CH_2 vs. CO), the basicity of the amide nitrogen may be enhanced in β-peptides, thus leading to charge solvation on the amide nitrogen and not on the amide oxygen.

However, we believe that charge solvation at the amide nitrogen remains thermodynamically unfavorable compared to solvation at the amide carbonyl oxygen, because the higher electronegativity of oxygen makes it a better hydrogen-bond acceptor, especially from a strongly electrophilic group such as $R-NH_3^+$. In the case where conformational constraints would account for the unavailability of a particular backbone carbonyl oxygen of number *n* to be solvated, the protonated amine from either an Arg or a Lys side chain would probably attempt to solvate the $(n-1)$ or $(n+1)$ carbonyl oxygen rather than the neighboring amide nitrogen, to form the most stable hydrogen bonds. The presence of an additional CH_2 group in the peptide backbone, as in β-amino acids and β-peptides, should not drastically increase the proton affinity of the amide nitrogen, as it is not a good electron-donating group, and therefore does not extensively modify the proton-accepting character of that amide nitrogen. Thus, the backbone modification alone presumably could not explain the increased probability of the charge being solvated preferentially at this position. We believe that charge solvation in β-peptides does not significantly differ from that observed in α-peptides; therefore amide nitrogen solvation should not prevail over carbonyl, despite additional structural flexibility. However, this hypothesis should be further investigated both experimentally and theoretically.

Therefore, the mechanism depicted in Scheme 3 may be used to explain the results shown in Figure 4 (preferential *a*-ion formation in multiply-substituted peptides), and it can then be extrapolated to describe other findings (single amino acid substitution-induced effects without *a*-ion formation). Interestingly, Figure 4 shows a possible competition in the formation of a_5 versus c_5 ions. Incorporation of β^3-Phe produces about 22 times more c_5 ions than a_5 ions (Figure 4, top). Substitution with β^3-Glu and β^3-Gln results in a substantial shift toward a_5 ion formation and drops the c_5/a_5 ratio by two orders of magnitude for both amino acids (Figure 4, middle, and Supporting Information Figure S6, bottom, respectively). Furthermore, insertion of β^3-Leu reduces the c_5 ion abundance (Supporting Information, Figure S6, top) to yield a c_5/a_5 ratio of only about 0.1. Finally, β-Ala incorporation leads to the complete disappearance of c_5 ions, but retains the a_5 ion relative abundance compared to other amino acids (Figure 4, bottom). Following the mechanism for *a*-ion formation depicted in Scheme 3, the competition for $c/z^{\cdot}$ and $a^{\cdot}/y$-ion formation would be attributed to radical formation either on the backbone amide nitrogen or on the backbone carbonyl carbon. The balance between these two pathways may thus account for the c_5/a_5 ratio described above as a function of amino acid side-chain properties.

Application of the reaction pathway suggested in Scheme 3 to explain the data in Figures 1–3 is less straightforward. In all the reported cases of single β-amino acid substitution, *a*-type ions are absent. Formation of *c*-type ions, as hypothesized above, is a function of the radical stabilization provided by the neighboring amino acids. Therefore, charge solvation on the backbone amide nitrogen of β-amino acids in singly-substituted peptides may not be as pronounced as in the multiply-substituted peptides. Presumably, the overall backbone influence may be required to induce the conformational change required for *a*-type ion formation. The backbone effects were considered to rationalize the en-

hanced *a*- and *y*-type ion formation observed for free acid peptides compared to amide peptides, as already described above.[29] Finally, formation of *a*-type ions implies rupture of the C_α–C backbone, which was demonstrated to be the dominant fragmentation channel in electron detachment dissociation (EDD) of peptide anions.[38]

Extension to true β-peptides reveals a higher extent of secondary fragmentation and pronounced side-chain loss in ECD/ETD as compared to that observed for α-peptides, as seen in Figure 6.[39,40] We believe that the complex and extensive fragmentation observed is primarily due to the large amount of energy available in the fragmentation of short peptides.

Radical *c*-type ion formation in ECD/ETD of β-amino acids: Comparative analysis of the isotopic distributions of product ions formed in ECD/ETD of peptides containing β-amino acids and their α-counterparts shows no significant differences between radical and prime ion contributions (data not shown). However, one exception was found, for c_7 radical ion formation in ETD and ETD with collision-induced dissociation of the charge-reduced species (ETD CRCID) of substance P–OH β^3-Phe_8. Firstly, the observation of c_7 ions is due to the strong radical stabilization properties of β^3-Phe, as discussed above. Secondly, ETD and ETD CRCID of the β^3-Phe_8-substituted free-acid substance P show no radical component in the isotopic cluster of c_7 ions, whereas ETD, and especially ETD CRCID of its α-analogue demonstrate an abundant radical component,[25] as seen in Figure S7 of the Supporting Information. According to the current understanding of radical ion formation in ECD/ETD, this effect is due to the shorter lifetime of the $[c+z^{\bullet}]$ complex and thus the reduced extent of hydrogen atom rearrangement between the products in the β-amino acid substituted peptide. Therefore, either the incorporation of a CH_2 group into the peptide backbone modifies the conformational space of the peptide sufficiently to eliminate conformations that contributed to $[c+z^{\bullet}]$ complexes with long lifetimes, or the fragmentation of this specific β-amino acid substituted peptide is more energetic.

Conclusion

ECD FT-ICR MS and ETD ITMS of β-peptides and regular α-peptides containing a diverse array of β-amino acids show a more distinct dependence on amino acid identity for β-peptides than for α-peptides. Analysis of the experimental data suggests that side-chain radical stabilization in β-amino acids plays a defining role in the efficiency and balance of N–C_β and C_α–C backbone bond cleavage. Although the radical stability of amino acids is known to direct ECD/ETD of α-amino acids and α-peptides, it is difficult to distinguish its influence from other effects (e.g., hydrogen bonding).[27] Interestingly, multiple additions of CH_2 groups into the peptide backbone shift the preferred product ion formation to the minor fragmentation channel observed in α-peptides. Considering the commonly accepted mechanism of *a*-ion formation through charge solvation on an amide nitrogen in relation to this observation implies that multiple incorporations of CH_2 groups into the peptide backbone shift the charge solvation preference in the peptide backbone. Nevertheless, the data shows that the radical stability of the amino acid side chains still directs the probability toward rupture of the C_α–C or N–C_β bond. From a practical point of view, distinct differences in ECD/ETD of α- versus β-peptides, e.g., *a/y* ions as opposed to *c/z* ions in ECD/ETD of poly-Ala peptides, should aid in distinguishing between these amino acids in peptide sequencing. The application of slow heating fragmentation methods, e.g., CID and IRMPD, would not allow this differentiation.

Although β-amino acids are known to influence substantially the conformations of even short peptides in solution, their influence on the electronic subsystem (molecular orbitals) of the peptide is yet to be understood. Further studies should address the characterization of ECD/ETD processes in larger β-peptides (comprising at least 10 amino acids) containing both β^2- and β^3-amino acids. Although large β-peptides could be produced,[41] their synthesis is challenging due to the strong tendency of these molecules to form stable secondary structure elements. The synthesis of β^2-amino acids is also more challenging than that of β^3-amino acids,[7] as reflected by the use of primarily β^3-amino acid based peptides in the current work. We believe that further comparative analysis of the ECD/ETD of β- and α-peptides may provide a better understanding of electron capture/transfer dissociation of peptide polycations, and clarify the currently debated mechanisms. In particular, as briefly discussed in this work, such a comparative approach could allow a revisiting of the *a*-ion formation pathway.

Experimental Section

Peptide synthesis and sample preparation: A β-turn mimicking tetrapeptide and a library of single-point β variants of neuropeptide substance P, using both β- and β-homo-amino acid incorporation, were designed, synthesized, and purified by HPLC at the Laboratory of Biomolecules, Université Pierre et Marie Curie (Paris, France). Commercially available β^3-alanine, β^3-leucine, β^3-glutamine, β^3-glutamic acid, and β^3-phenylalanine were purchased from Sigma Aldrich (Sigma Aldrich GmbH, Buchs, Switzerland), and incorporated by solid phase Fmoc biochemistry into the amide and free-acid substance P, LLLLALLLK–NH_2 model peptide, as well as into a series of poly-alanine peptides of the general sequence RAAAAXAAAAK–OH and KAAAAXAAAAR–OH, in which X is any α-amino acid or its β-analogue. Synthesis was performed using a microscale peptide synthesizer (Intavis Bioanalytical Instruments AG, Köln, Germany) at the Protein and Peptide Chemistry Facility (University of Lausanne, Switzerland), and the crude peptides were used for mass spectrometric analysis without further purification. Samples were dissolved in water to ≈1 mM concentration and further diluted in a standard spraying solution (H_2O/CH_3OH 50:50 volume ratio with 1% HCOOH) to a final peptide concentration of ≈1–10 μM.

Tandem mass spectrometry: ECD experiments were performed on a 7 T linear ion trap Fourier transform ion cyclotron resonance (LTQ FT-ICR) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a standard direct infusion electrospray ion source at a typical flow rate of 15 μL min^{-1}. Doubly-charged precursors were isolated in

LTQ (isolation window of 4 Th) and transferred to the ICR ion trap for subsequent tandem mass spectrometry, following standard procedures.[42] In summary, peptide dications were irradiated by a low-energy electron beam for about 50 ms before product ion excitation and detection. Magnetron motion phase optimization was performed following standard automated procedure.[43] Data analysis was carried out using XCalibur 2.0.7 and Xtract 3.1 software (ThermoFischer Scientific, Bremen, Germany).

Low-resolution ETD experiments were performed on a Paul ion trap mass spectrometer (HCTultra PTM discovery system, Bruker Daltonics GmbH, Bremen, Germany).[44] A microfluidic chip-based ionization source (Cube source, Agilent Technologies, Cheshire, UK) delivering a flow rate of $\approx 300\,nL\,min^{-1}$ was employed for electrospray ionization. Peptide dications were isolated (isolation window of 4 Th) prior to ion–ion reactions performed following standard MS/MS procedures. To summarize, the ETD reaction time was 120 ms; a low mass cut-off was set at 210 *m/z*; and experiments were performed with and without supplemental activation of the charge-reduced species. Data analysis was carried out using Bruker's Data Analysis (version 3.4) software (Bruker Daltonics GmbH, Bremen, Germany). High resolution ETD experiments on β-peptide H-K-βA-βA-βA-βA-β^3F-βA-βA-βA-βA-R-OH were performed on the LTQ Orbitrap Velos FTMS (Thermo Fisher Scientific) using standard ETD experimental parameters.

β-Amino acid structures and β-peptide fragmentation nomenclature: Four major types of β-amino acids are shown in Scheme 1. Two criteria must be taken into consideration for proper labeling. The first criterion refers to the position of the β-carbon relative to the peptide termini: if the carbon bound to the side chain is on the C-terminal side of the amino acid, then it is labeled β^2; it is labeled β^3 if the side chain is on the N-terminal side. The second criterion is whether the additional methylene group incorporated into the backbone is the β-carbon from the side chain or an additional CH_2 group that increases the mass of the amino acid by 14.015 Da. In the latter case, the resulting nomenclature would be a β-homo (either type 2 or 3 depending on the side-chain position) amino acid.

The basis for the nomenclature suggested here for the fragmentation of β-peptides or peptides containing β-amino acids was defined by Roepstorff et al. for α-peptides.[45] The difference in the backbone motif of the β-peptides in comparison to the α-peptides introduces additional possible fragmentation channels and hence requires appropriate modification of the product ion terminology to fit the specific case of peptides containing β-amino acids (see Scheme S1, Supporting Information). For the β^2 template, the additional C_β–C_α backbone cleavage will produce $c_n^*(c_n+CH_2)$ and $z_{(m-n)}^*(z_{(m-n)}\text{-}CH_2)$ product ions, for which m is the total number of residues in the peptide. The β^3 template cleaved at the same backbone bonds gives rise to different fragments: $a_n^*(a_n-CH_2)$ and $x_{(m-n)}^*(x_{(m-n)}+CH_2)$. The corresponding products for the β-homo-amino acids are (c_n+CH_2) and $z_{(m-n)}$ for β^2-homo-amino acids, and a_n and $(x_{(m-n)}+CH_2)$ for β^3-homo-amino acids. The nomenclature for β-peptide fragmentation introduced here is a custom one, but may be considered as a suggestion for future use as a conventional rule. However, additional notations will need to be adopted to fully describe fragmentations of disubstituted as well as cyclic beta amino acids.

Acknowledgements

We are grateful to Prof. Pierre Vogel and Prof. Janne Jänis for the discussions and comments on the manuscript. We thank Dr. Marc Moniatte, Diego Chiappe, and Jérôme Vialaret (Proteomics Core Facility, EPFL, Lausanne) for their assistance in ETD experiments. We also thank Catherine Servis (Protein and Peptide Chemistry Facility, UNIL, Lausanne) for peptide synthesis, and Thermo Fisher Scientific (Bremen, Germany) for granting access to 7 T LTQ FT-ICR MS and LTQ Orbitrap Velos FTMS. Financial support was provided by EPFL and the Swiss National Science Foundation (project 200021-125147/1).

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Received: September 8, 2009
Published online: March 16, 2010

