



## RESEARCH ARTICLE

# Novel C<sub>β</sub>–C<sub>γ</sub> Bond Cleavages of Tryptophan-Containing Peptide Radical Cations

Tao Song,<sup>1</sup> Qiang Hao,<sup>1,2</sup> Chun-Hin Law,<sup>1</sup> Chi-Kit Siu,<sup>2</sup> Ivan K. Chu<sup>1</sup><sup>1</sup>Department of Chemistry, The University of Hong Kong, Hong Kong, China<sup>2</sup>Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, China**Abstract**

In this study, we observed unprecedented cleavages of the C<sub>β</sub>–C<sub>γ</sub> bonds of tryptophan residue side chains in a series of hydrogen-deficient tryptophan-containing peptide radical cations (M<sup>•+</sup>) during low-energy collision-induced dissociation (CID). We used CID experiments and theoretical density functional theory (DFT) calculations to study the mechanism of this bond cleavage, which forms [M – 116]<sup>+</sup> ions. The formation of an α-carbon radical intermediate at the tryptophan residue for the subsequent C<sub>β</sub>–C<sub>γ</sub> bond cleavage is analogous to that occurring at leucine residues, producing the same product ions; this hypothesis was supported by the identical product ion spectra of [LGGGH – 43]<sup>+</sup> and [WGGGH – 116]<sup>+</sup>, obtained from the CID of [LGGGH]<sup>•+</sup> and [WGGGH]<sup>•+</sup>, respectively. Elimination of the neutral 116-Da radical requires inevitable dehydrogenation of the indole nitrogen atom, leaving the radical centered formally on the indole nitrogen atom ([Ind]<sup>•–2</sup>), in agreement with the CID data for [WGGGH]<sup>•+</sup> and [W<sub>1-CH<sub>3</sub></sub>GGGH]<sup>•+</sup>; replacing the tryptophan residue with a 1-methyltryptophan residue results in a change of the base peak from that arising from a neutral radical loss (116 Da) to that arising from a molecule loss (131 Da), both originating from C<sub>β</sub>–C<sub>γ</sub> bond cleavage. Hydrogen atom transfer or proton transfer to the γ-carbon atom of the tryptophan residue weakens the C<sub>β</sub>–C<sub>γ</sub> bond and, therefore, decreases the dissociation energy barrier dramatically.

**Key words:** C<sub>β</sub>–C<sub>γ</sub> cleavages of tryptophan, Peptide radical cations, Low-energy collision-induced dissociation, Density functional theory calculations

## Introduction

Gas phase dissociation of enzymatically cleaved peptide ions is a key technology for peptide sequencing and, ultimately, protein identification [1, 2]. Dissociations of protonated even-electron peptides under the conditions of low-energy collision-induced dissociation (CID) commonly

reveal charge-directed cleavages at different amide bonds along the peptide backbone, as described by the “mobile proton” model [3–7], forming sequence-informative b or y ions [8, 9]. During low-energy CID processes, the side chains of the amino acid residue often remain intact (except for the loss of H<sub>2</sub>O or NH<sub>3</sub>), thereby introducing the analytical challenge of distinguishing isobaric residues (e.g., leucine and isoleucine) in peptides; the challenge can be overcome by using high-energy CID to induce side chain cleavages of the bonds between the β- and γ-carbon atoms of the amino acid residues [10, 11].

Radicals can also induce bond cleavages in the side chains of the amino acid residues. Such side chain cleavages, together with the formation of c and z ions resulting from the cleavages of N–C<sub>α</sub> bonds along the

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Correspondence to: Chi-Kit Siu; e-mail: chiksiu@cityu.edu.hk, Ivan K. Chu; e-mail: ivankchu@hku.hk

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peptide backbone, are common fragmentation channels for the hydrogen-rich odd-electron peptide radical cations [M+nH]<sup>•(n-1)+</sup> that are generated in electron-capture dissociation (ECD) [12–15] or electron-transfer dissociation (ETD) experiments [16–18]. These characteristic side chain losses assert the presence of specific amino acid residues in peptides. For example, losses of 30, 33, 61, 129, and 87 Da are potential indicators of the presence of serine, cysteine, methionine, tryptophan, and arginine residues, respectively [14, 15, 18]. Facile distinction of isomeric leucine and isoleucine is also realized by the respective diagnostic losses of 43 and 29 Da, respectively, that result from their C<sub>β</sub>–C<sub>γ</sub> bond cleavages [18, 19]. At present, 11 amino acid residues can be identified by their characteristic side chain losses, which also provide additional information relating to backbone fragmentations and, thus, greatly enhance the confidence of peptide identification [20].

Many investigations have been performed recently regarding the side chain losses occurring during the dissociations of classical molecular peptide radical cations (M<sup>•+</sup>), which are odd-electron peptide cations that are hydrogen-deficient relative to their even-electron protonated counterparts ([M+nH]<sup>n+</sup>). M<sup>•+</sup> species can be effectively generated through oxidative dissociations of transition metal/peptide complexes [21–25], laser photolysis of peptides containing photolabile tags [26], or dissociation of peptides containing labile groups [27–34]. Examinations of several model M<sup>•+</sup> species, containing aliphatic, aromatic, and/or basic amino acid residues, have revealed that their fragmentation behaviors are greatly governed by competition between the radical mobility within these radical cations and their fragmentations [20, 35–48]; in arginine-containing M<sup>•+</sup> species, where the charge is tightly sequestered by the very basic side chain of the arginine residue, radical migrations are facilitated and, as a result, radical-induced charge-remote fragmentations (such as side chain, C<sub>α</sub>–C bond, and N–C<sub>α</sub> bond cleavages) are favored [20, 37, 44–46, 49]. Abundant side chain losses from the amino acid residues most likely involve α- or γ-centered radical intermediates.

Facile 3-methyleneindolenine (129 Da) loss through side chain C<sub>α</sub>–C<sub>β</sub> bond cleavage of the tryptophan residue is commonly observed in the dissociations of both hydrogen-rich [15, 18] and hydrogen-deficient radical peptide systems [20, 30, 36, 46]. Loss of a neutral species having a mass of 116 Da through cleavage at the C<sub>β</sub>–C<sub>γ</sub> bond of a tryptophan residue has also been observed in several ETD/ECD experiments [15, 18, 50], therefore, such a fragmentation potentially provides diagnostic information to directly implicate the presence of a tryptophan residue in a parent peptide. In this study, we report the first observation of this unique 116-Da neutral loss from molecular peptide radical cations. Using both experimental and theoretical methods, we have examined its chemical identity and the mechanistic details of its formation via the C<sub>β</sub>–C<sub>γ</sub> bond cleavages of tryptophan residues.

## Experimental

### Materials

Fmoc-protected unmodified amino acids and the Wang resin were obtained from Advanced ChemTech (Louisville, KY, USA). Fmoc-protected 1-methyl tryptophan and α-methyl tryptophan were purchased from Matrix Scientific (Columbia, SC, USA). All other chemicals were obtained commercially (Aldrich or Sigma, St Louis, MO, USA; Bachem, King of Prussia, PA, USA). Oligopeptides and the Cu(II)(terpy)(NO<sub>3</sub>)<sub>2</sub> (terpy=2,2':6',2''-terpyridine) and [Co(III)(salen)Cl] [salen=N, N'-ethylenebis(salicylideneiminato)] complexes were synthesized according to procedures described in the literature [51–53].

### Methylation of Peptides

A solution of HCl (ca. 2 M) in MeOH was prepared through the dropwise addition of acetyl chloride (800 μL) into anhydrous MeOH (5 mL) and then stirring for 5 min at room temperature. This solution (1 mL) was added to the peptide (10 mg) and then the mixture was stirred for 3 h at room temperature. The resulting solution was dried using a SC250DDA Speedvac Plus (Thermo Electron Corporation, Waltham, MA, USA). The methylated peptide was mixed with the metal complexes in each experiment without any further purification.

### Mass Spectrometry

All mass spectrometry experiments were conducted using a quadrupole ion trap mass spectrometer (Finnigan LCQ, ThermoFinnigan, San Jose, CA, USA). The molecular peptide radical cations M<sup>•+</sup> were generated through one-electron oxidative dissociations of transition metal/peptide complexes [21–25]. Their abundances were optimized using [Cu<sup>II</sup>(terpy)M]<sup>2+</sup> complexes (for M = GGGGW, GWGGG, WGGGG, GGGGW-OMe, and GGGFW) or [Co<sup>III</sup>(salen)M]<sup>2+</sup> complexes (for M = WGGGR, GWGGR, WGGGK, GWGGK, WGGGH, GWGGH, LGGGH, W<sub>α-CH<sub>3</sub></sub>GGGH, W<sub>1-CH<sub>3</sub></sub>GGGH, WGGFLR, WGGFLK, WGGFLH, WVYIHPR, WVYIFPK, and WVYIHPF). The fragmentation chemistries of those radical peptide cations were independent of the choice of the metal complexes. Samples typically comprised 600 μM Cu(II)(terpy) or Co(III)(salen) complex and 50 μM oligopeptide in a H<sub>2</sub>O/MeOH (50:50) solution. A syringe pump (Cole Parmer, Vernon Hills, IL, USA) was used for direct infusion of the electrospray samples (flow rate: 30 μL/h). CID spectra were acquired using helium as the collision gas. The injection time and excitation time for CID in the ion trap were 200 and 50 ms, respectively; the amplitude of the excitation was optimized for each experiment.

### Computational Methods

Electronic energies were calculated in the framework of density functional theory (DFT) using the unrestricted (U)

hybrid functional formulated with a mixture of the Hartree-Fock exchange energy and Becke's three-parameter 1988 gradient-corrected exchange energy, and the Lee-Yang-Parr (LYP) correlation energy [54]. Atomic orbitals were described by a Gaussian-type split valence shell 6-31++G (d,p) basis set including polarization and diffuse functions for all atoms [55, 56]. Harmonic vibrational frequencies of all optimized structures were calculated to confirm that the structures were at local minima (all real frequencies) or were transition states (one imaginary frequency). The local minima associated with each transition structure were verified using the intrinsic reaction coordinate (IRC) method. Relative enthalpies at 0 K ( $\Delta H_0^\circ$ ) were calculated from the electronic energies and zero-point vibrational energies (ZPVE) obtained within the harmonic approximation. All DFT calculations were conducted using the Gaussian 03 software package [57].

## Results and Discussion

### Peptides Containing Non-Basic Residue

The CID spectrum of [GGGGW]<sup>++</sup> (Figure 1a) features a predominant ion at  $m/z$  388, which corresponds to a loss

of CO<sub>2</sub> from the C-terminal carboxylic group, and its subsequent neutral loss of 116 Da at  $m/z$  272, presumably from the cleavage at the side chain C<sub>β</sub>-C<sub>γ</sub> bond of the tryptophan residue, and a typical fragment ion of [c<sub>4</sub> + 2H]<sup>+</sup> at  $m/z$  246 [36, 37]. Cleavage at the C<sub>α</sub>-C<sub>β</sub> bond of the tryptophan residue to lose 3-methyleneindolenine with a mass of 129 Da is known, but loss through C<sub>β</sub>-C<sub>γ</sub> cleavage is unprecedented. It is noteworthy that we did not observe the direct loss of 116 Da from the CID of [GWGGG]<sup>++</sup> (Figure 1b) and [WGGGG]<sup>++</sup> (Figure 1c), where the tryptophan residue is, respectively, located in the middle and at the N-terminus of the peptide sequence; they fragmented predominantly via typical N-C<sub>α</sub> bond cleavages at tryptophan residues [36, 37], forming [z<sub>4</sub> - H]<sup>++</sup> (Figure 1b) and [z<sub>5</sub> - H]<sup>++</sup> (i.e., NH<sub>3</sub> loss) (Figure 1c) species. Absence of the direct 116 Da loss from the CID of [GGGGW]<sup>++</sup>, [GWGGG]<sup>++</sup>, and [WGGGG]<sup>++</sup> strongly suggests that the formation of an  $\alpha$ -carbon-centered radical at the tryptophan residue in [GGGGW - CO<sub>2</sub>]<sup>++</sup> plays an essential role prior to subsequent C<sub>β</sub>-C<sub>γ</sub> bond cleavage [46]. We observed no analogous fragmentations from the protonated [GGGGW + H]<sup>+</sup> species, from which the loss of H<sub>2</sub>O and the formation of b/y ions were predominant (Figure S1). It seems clear

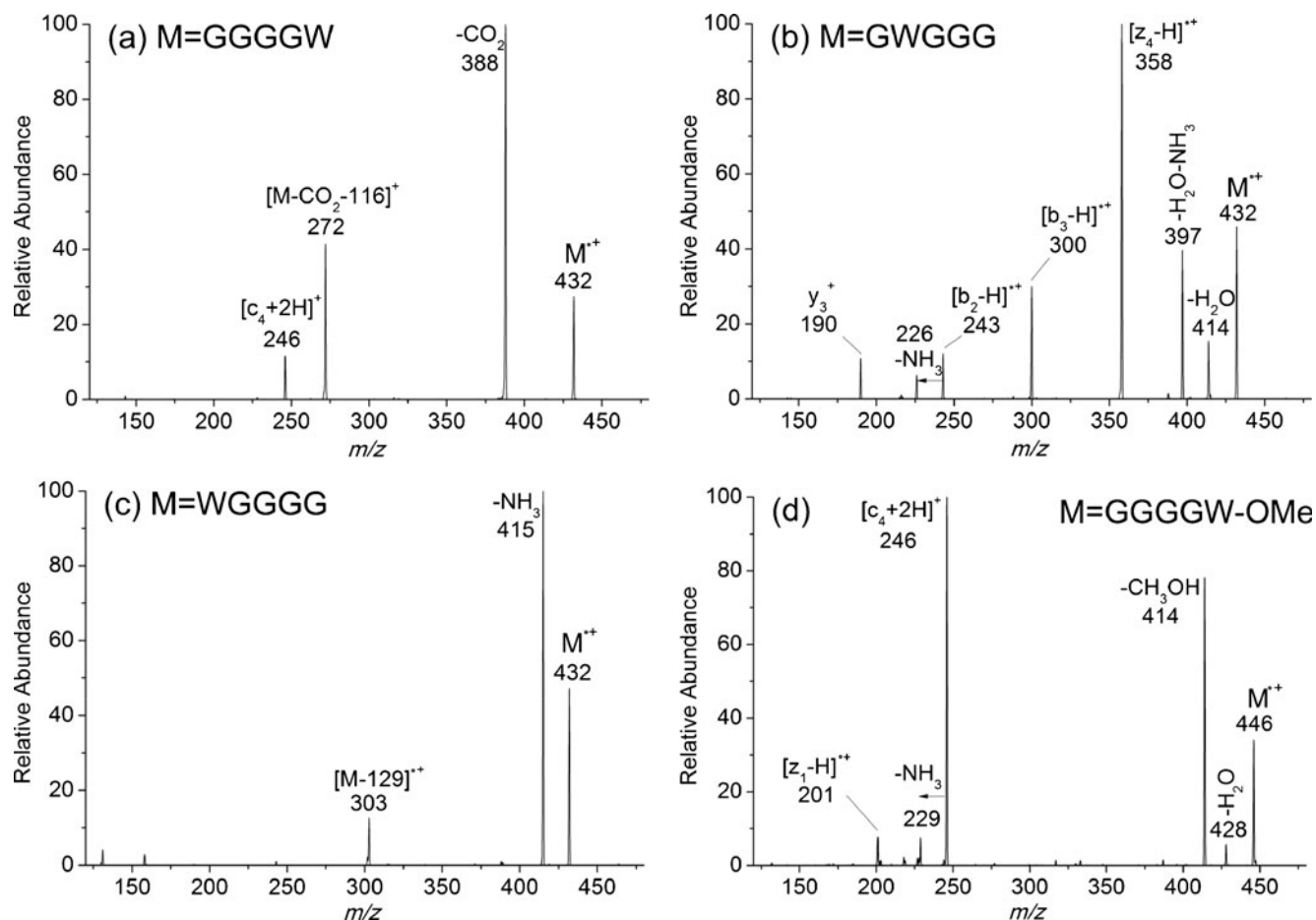


Figure 1. CID spectra of (a) [GGGGW]<sup>++</sup>; (b) [GWGGG]<sup>++</sup>; (c) [WGGGG]<sup>++</sup>; (d) [GGGGW-OMe]<sup>++</sup>

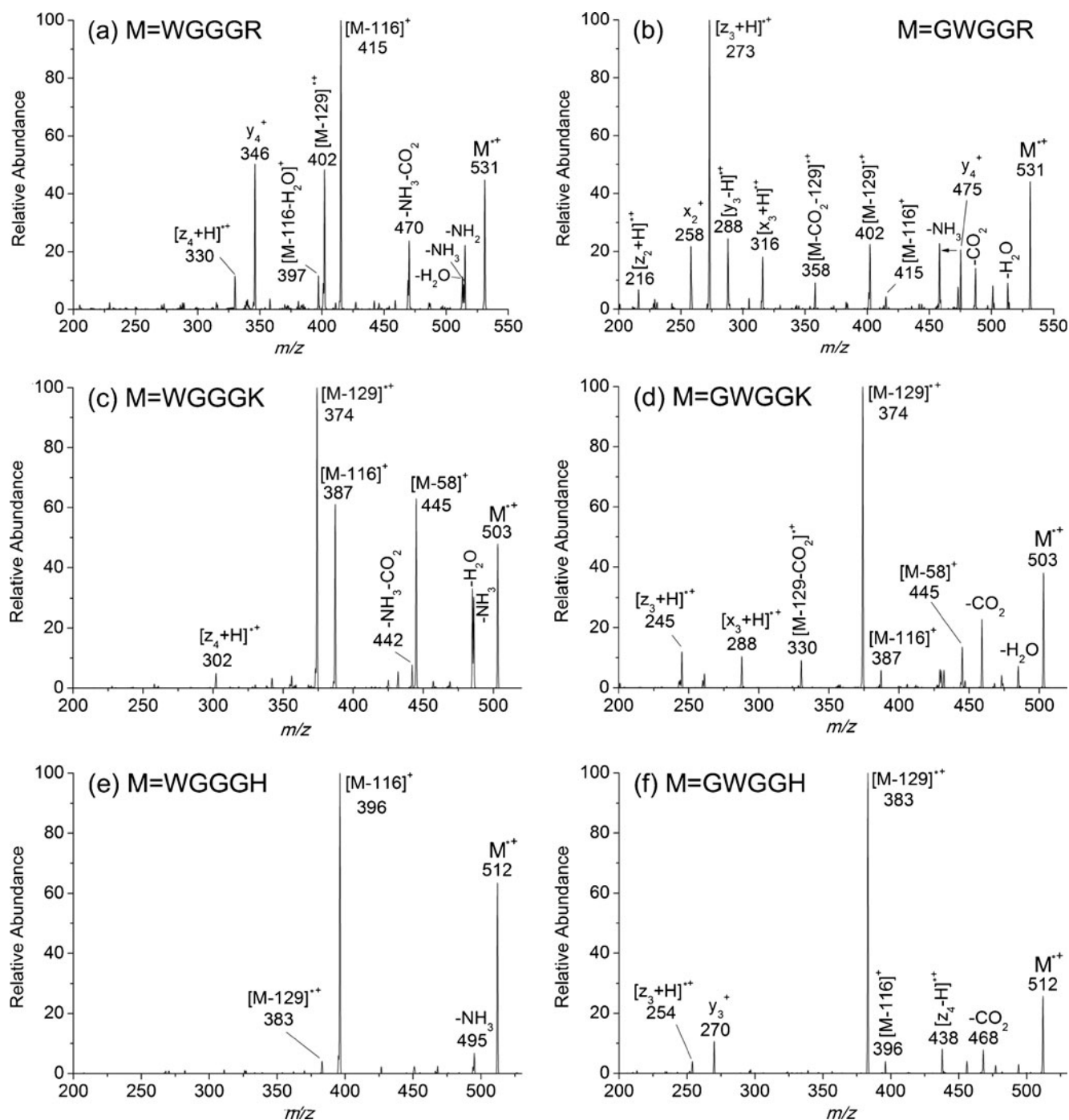


Figure 2. CID spectra of (a) [WGGGR]<sup>•+</sup>; (b) [GWGGR]<sup>•+</sup>; (c) [WGGGK]<sup>•+</sup>; (d) [GWGGK]<sup>•+</sup>; (e) [WGGGH]<sup>•+</sup>; (f) [GWGGH]<sup>•+</sup>

that propagation of the radical is inefficient among the  $\alpha$ -carbon centers along the backbones of peptide radical cations containing only glycine and tryptophan residues, thereby suppressing some of the competitive fragmentation reactions [42], allowing the [GGGGW - CO<sub>2</sub>]<sup>•+</sup> fragment ion, possessing its initial radical site at the C-terminal  $\alpha$ -carbon atom of the tryptophan residue, to readily undergo subsequent C<sub>β</sub>-C<sub>γ</sub> bond cleavage. Through C-terminal methylation, we experimentally eliminated the possibility of forming the  $\alpha$ -carbon-

centered radical through CO<sub>2</sub> loss; the CID spectrum of [GGGGW-OMe]<sup>•+</sup> revealed neither CO<sub>2</sub> loss nor its consecutive 116-Da loss (Figure 1d).

### Peptides Containing Basic Residue

We indirectly confirmed the prerequisite for the  $\alpha$ -carbon-centered radical to be located at the tryptophan residue to trigger C<sub>β</sub>-C<sub>γ</sub> bond cleavages through examination of the arginine-containing [WGGGR]<sup>•+</sup> and

[GWGGR]<sup>++</sup> species, in which the mobile proton is sequestered by the guanidine group of the arginine side chain, thereby facilitating the radical migrations among the π-systems, β-carbon atoms, and α-carbon atoms (including the α-carbon-centered radical at the tryptophan residue) through hydrogen atom transfers (HATs) along the peptide [43]. As expected, the CID of [WGGGR]<sup>++</sup> (Figure 2a) and [GWGGR]<sup>++</sup> (Figure 2b) produced in situ [M - 116]<sup>+</sup> species, albeit with much lower abundance for the latter case; the presence of the arginine residue in the peptide also catalyzed several other known fragmentations [35, 43, 48], including the formation of [z<sub>4</sub> + H]<sup>++</sup>, y<sub>4</sub><sup>+</sup>, and [M - 129]<sup>++</sup> species in Figure 2a. We confirmed the identities of the major fragment ions by replacing a glycine residue with an alanine residue; for example, the CID spectrum of [GWAGR]<sup>++</sup> (Figure S2, supplementary material) notably featured the [M - 116]<sup>+</sup> ion with a mass shifted to *m/z* 429, due to the mass difference between glycine and alanine residues. The dramatic decrease in abundance of the [M - 116]<sup>+</sup> ion obtained from the CID of [GWGGR]<sup>++</sup> relative to that of [WGGGR]<sup>++</sup> is in accordance with the fact that the N-terminal α-carbon-centered radical features stronger captodative stabilization than do the middle α-carbon-centered radicals [42, 43, 47].

Parenthetically, we also observed the C<sub>β</sub>-C<sub>γ</sub> bond cleavages of tryptophan residues in the CID spectra of [WGGGK]<sup>++</sup> (Figure 2c) and [GWGGK]<sup>++</sup> (Figure 2d) and of [WGGGH]<sup>++</sup> (Figure 2e) and [GWGGH]<sup>++</sup> (Figure 2f), producing [M - 116]<sup>+</sup> ions at *m/z* 387 and 396, respectively, because lysine (K) and histidine (H) residues can also facilitate HATs to some extent [43]. Similar to the arginine-containing peptides, the relative abundance of the [M - 116]<sup>+</sup> ion produced from the lysine- and histidine-containing peptides was also sensitive to the position of the tryptophan residue; in general, the C<sub>β</sub>-C<sub>γ</sub> bond cleavage was more competitive when the tryptophan residue was located at the N-terminus. We also observed other side chain cleavages; for example, the CID of [WGGGK]<sup>++</sup> and [GWGGK]<sup>++</sup> induced C<sub>β</sub>-C<sub>γ</sub> bond cleavages of the lysine residues, giving [M - 58]<sup>+</sup> ions at *m/z* 445. The [M - 129]<sup>++</sup> ion was generated in all cases; it also arose from the tryptophan residue, but instead via its C<sub>α</sub>-C<sub>β</sub> bond cleavage.

Table 1 displays the fragmentation behavior of various leucine enkephalin and angiotensin derivatives, with C<sub>β</sub>-C<sub>γ</sub> bond cleavages of tryptophan residues, forming [M - 116]<sup>+</sup> ions similar to those in Figures 1 and 2. This abundant bond cleavage has the potential application of allowing the identification of N-terminal tryptophan residues during peptide sequencing. Furthermore, from the information in Table 1 regarding the two most competitive fragmentations, it is noteworthy that most of the competing fragmentations are side chain losses from various residues (e.g., leucine, lysine, tryptophan)—of great importance for improving the searching engines used for peptide identifications.

**Table 1.** Sequences (M) and Relative Abundances of 116 Da Neutral Losses Generated by C<sub>β</sub>-C<sub>γ</sub> Bond Cleavage of Tryptophan. The Relative Abundances for Two Most Competitive Fragmentations are also Shown

Sequence (M)	[M - 116] <sup>+</sup> , %	Two most competitive fragmentations (Xxx <sup>a</sup> , relative abundance %)
GGGFW	95 <sup>b</sup>	[M - CO <sub>2</sub> ] <sup>++</sup> (100), [c <sub>4</sub> +2H] <sup>+</sup> (20)
WGGFLR	50	[M - 43] <sup>+</sup> ( <i>Leu</i> , 100), [M - 56] <sup>+</sup> ( <i>Leu</i> , 83)
WGGFLK	67	[M - 58] <sup>+</sup> ( <i>Lys</i> , 100), [M - 129] <sup>++</sup> ( <i>Trp</i> , 64)
WGGFLH	100	[M - NH <sub>3</sub> ] <sup>++</sup> (18), [M - 129] <sup>++</sup> ( <i>Trp</i> , 13)
WVYIHPR	14	[M - 129] <sup>++</sup> ( <i>Trp</i> , 100), [M - NH <sub>3</sub> ] <sup>++</sup> (70)
WVYIFPK	100	[M - 58] <sup>+</sup> ( <i>Lys</i> , 58), [M - 129] <sup>++</sup> ( <i>Trp</i> , 41)
WVYIHPF	100	y <sub>s</sub> <sup>+</sup> (30), [M - 129] <sup>++</sup> ( <i>Trp</i> , 22)

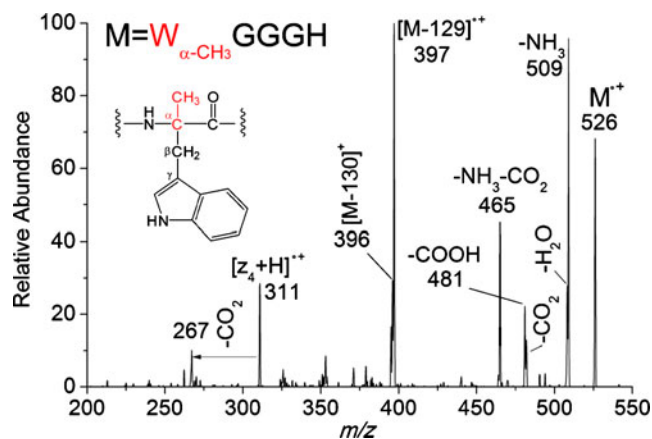
<sup>a</sup>Residue corresponding to the side chain fragmentation, if applicable.

<sup>b</sup>The relative abundances of [M - CO<sub>2</sub> - 116]<sup>+</sup>, %.

### Role of α-Carbon-Centered Radical of Tryptophan

We further confirmed the prerequisite for an α-carbon-centered radical at the tryptophan residue as a key intermediate to trigger subsequent C<sub>β</sub>-C<sub>γ</sub> bond cleavage, as described in the previous section, through an experimental study using [W<sub>α-CH<sub>3</sub></sub>GGGH]<sup>++</sup>, which has a methyl group substituted at the α-carbon atom of the tryptophan unit (i.e., no α-hydrogen atom) to prevent the radical migrating to the α-carbon atom of the tryptophan residue. In the CID spectrum of [W<sub>α-CH<sub>3</sub></sub>GGGH]<sup>++</sup> (Figure 3), the signal arising from the loss of 116 Da, which had been the most-abundant signal in the CID spectrum of [WGGGH]<sup>++</sup> (Figure 2e), was absent; instead, the [M - 129]<sup>++</sup> ion arising from the C<sub>α</sub>-C<sub>β</sub> bond cleavage of the tryptophan residue, that does not require the presence of the α-carbon-centered radical of tryptophan [30], became the most abundant fragment ion.

The involvement of an α-carbon-centered radical intermediate in the C<sub>β</sub>-C<sub>γ</sub> side chain cleavage of amino acid residues has been observed previously; comprehensive studies have revealed the mechanisms of C<sub>β</sub>-C<sub>γ</sub> bond cleavage of the isobaric leucine and isoleucine (X<sub>Ic</sub>) residues, as indicated in Scheme S1. Fragmentations of X<sub>Ic</sub>-containing peptide radical cations lead to the formation of characteristic product ions resulting from losses of <sup>•</sup>CH<sub>2</sub>CH<sub>3</sub> (29 Da) from isoleucine and <sup>•</sup>CH(CH<sub>3</sub>)<sub>2</sub>



**Figure 3.** CID spectrum of [W<sub>α-CH<sub>3</sub></sub>GGGH]<sup>++</sup>. “W<sub>α-CH<sub>3</sub></sub>” represents a modified tryptophan residue with the α-hydrogen substituted by a methyl group, which prevents the α-radical formation of tryptophan

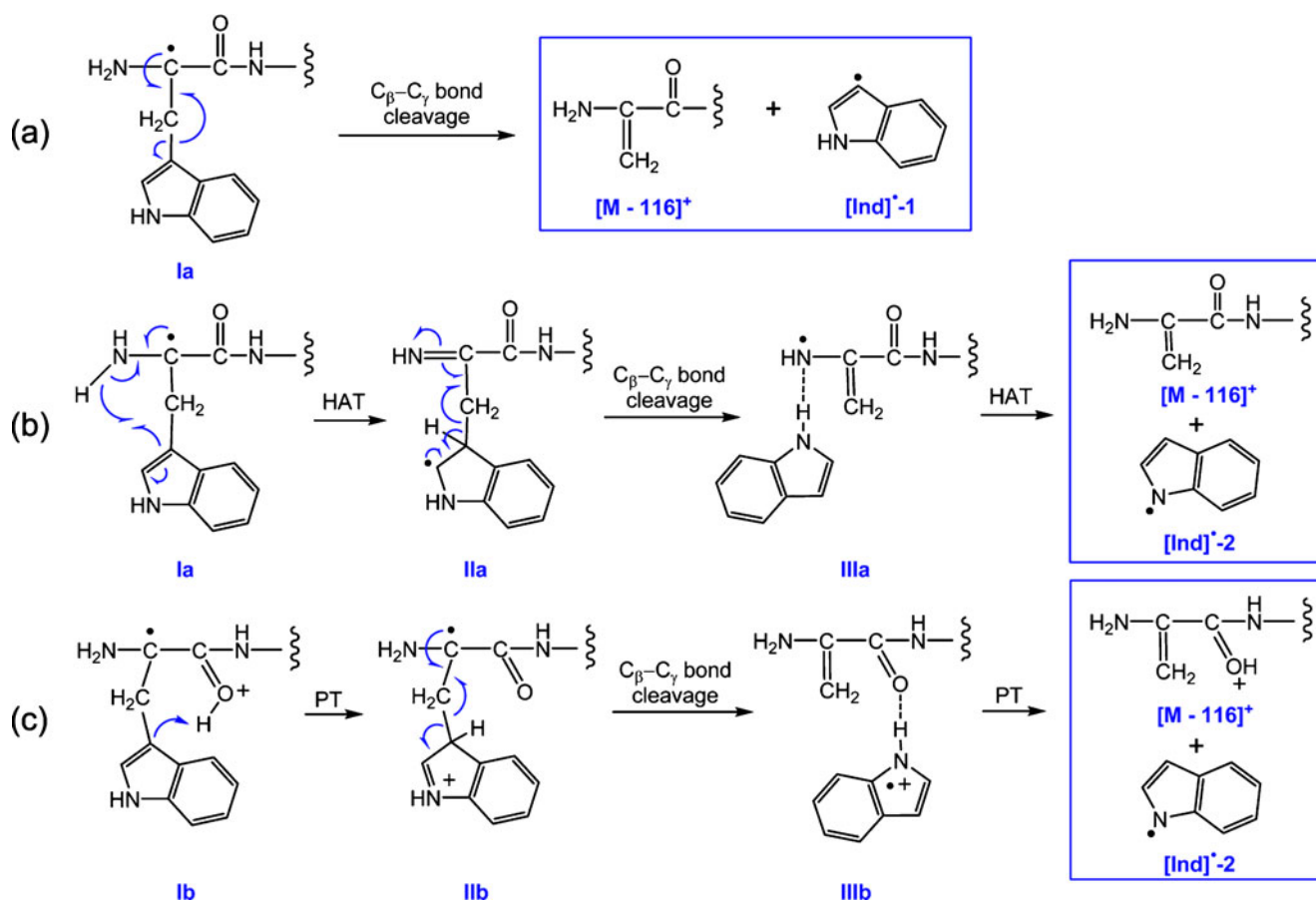
(43 Da) from leucine through C<sub>β</sub>-C<sub>γ</sub> side chain cleavages of the (iso)leucine residues, allowing the two peptides to be distinguished. We speculate that the C<sub>β</sub>-C<sub>γ</sub> bond cleavages of tryptophan residues described herein follow a similar mechanism as that for leucine residues, generating product ions with the same structure: that is, [M - 116]<sup>+</sup> and [M - 43]<sup>+</sup> ions from C<sub>β</sub>-C<sub>γ</sub> bond cleavages of the tryptophan and leucine residues, respectively, as displayed in Scheme 1a and Scheme S1a. This hypothesis is supported by the identical CID spectra of [WGGGR - 116]<sup>+</sup> and [LGGGR - 43]<sup>+</sup> (Figure 4), originally derived from [WGGGR]<sup>•+</sup> and [LGGGR]<sup>•+</sup>, respectively.

### Identity of the 116-Da Neutral Species

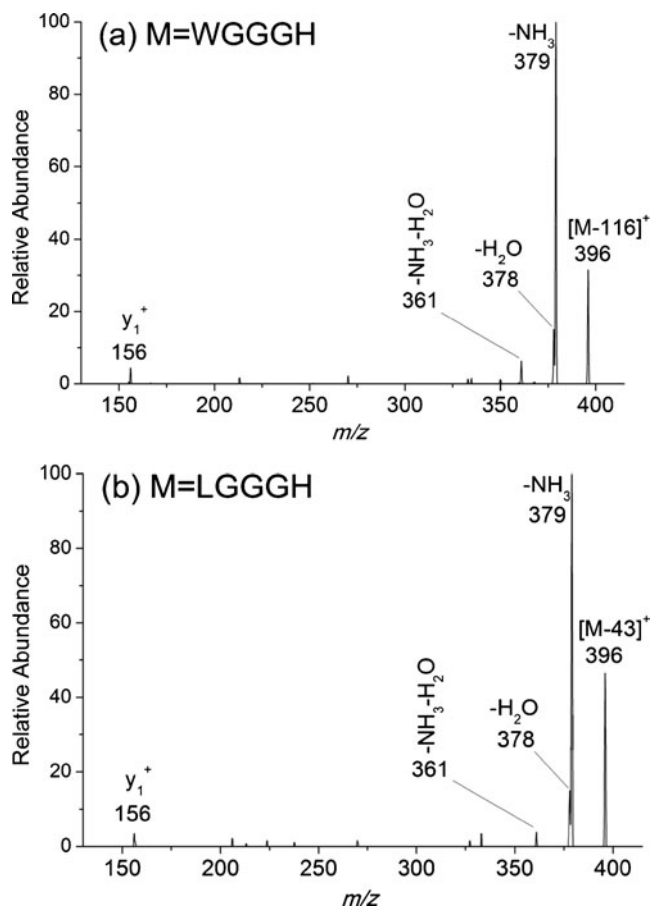
The neutral molecules that are lost from the homolytic C<sub>β</sub>-C<sub>γ</sub> bond cleavages of the α-carbon-centered radicals of leucine and isoleucine are the 2-propyl radical ([2-prop]<sup>•</sup>) and the ethyl radical ([eth]<sup>•</sup>), respectively (Scheme S1). A similar pathway (Scheme 1a) for 3-indolyl radical ([Ind]<sup>•</sup>-1) formation from tryptophan residue is not favorable, judging from its radical stabilization energy (RSE), which we evaluated employing a widely used method at the ROMP2/6-311+G(3df,2p)//UB3LYP/6-31G(d) level [58] (details relating to the calculation for each radical fragment are available in Table S1 of the supplementary material); the RSE of [Ind]<sup>•</sup>-1 of -16.3 kcal/mol

is significantly lower than those of [eth]<sup>•</sup> and [2-prop]<sup>•</sup> (3.3 and 5.6 kcal/mol, respectively; Table 2). The energy barrier against the C<sub>β</sub>-C<sub>γ</sub> bond cleavage of tryptophan following the mechanism presented in Scheme 1a, evaluated at the UB3LYP/6-31++G(d,p) level of theory for the α-tryptophylmethylamino radical as a model system (Figure S3b), is 50.8 kcal/mol, substantially higher than those against the C<sub>β</sub>-C<sub>γ</sub> bond cleavage of the α-leucyl analogue (31.9 kcal/mol, Figure S3a) and the backbone cleavages for peptide radical cations (<40.0 kcal/mol) [42, 43]. Taken together, although the product ions are the same, our theoretical calculations suggest that the C<sub>β</sub>-C<sub>γ</sub> bond cleavages of tryptophan residues are not as simple as the direct bond cleavages of leucine or isoleucine residues; as a result, [Ind]<sup>•</sup>-1 might not be the structure of the lost 116-Da neutral species.

One notable difference between leucine and tryptophan is that the former has an aliphatic side chain, while the latter has an aromatic indole ring with a labile hydrogen atom on the nitrogen atom at position 1 of the indole ring (<sup>1</sup>N). This labile hydrogen atom might be involved in the side chain loss, resulting in a much more stable 1-indolyl radical ([Ind]<sup>•</sup>-2) with an RSE of 10.1 kcal/mol, comparable with those of [2-prop]<sup>•</sup> (5.6 kcal/mol) and [eth]<sup>•</sup> (3.3 kcal/mol) (Table 2). We used the model [W<sub>1-CH<sub>3</sub></sub>G<sub>3</sub>GGH]<sup>•+</sup>, with the hydrogen atom on <sup>1</sup>N replaced by a methyl group, to examine the role played by this



Scheme 1. Possible mechanisms for the C<sub>β</sub>-C<sub>γ</sub> cleavage of tryptophan residue examined in this study



**Figure 4.** CID spectra of (a)  $[M - 116]^+$  from  $[WGGGH]^+$ ; (b)  $[M - 43]^+$  from  $[LGGGH]^+$

hydrogen atom. As revealed in Figure 5, CID of  $[W_{1-CH_3}GGGH]^+$  did not produce the  $[M - 130]^+$  ion, which would be the analogue of  $[M - 116]^+$  resulting from the C<sub>β</sub>-C<sub>γ</sub> bond cleavage of the tryptophan residue. This experiment demonstrates that dehydrogenation of the indole nitrogen atom is an inevitable step in the loss of the 116-Da neutral species.

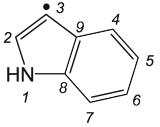
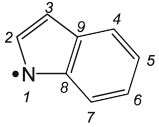


Interestingly, the most abundant ion from  $[W_{1-CH_3}GGGH]^+$  was the  $[M - 131]^+$  species, which also originated from the

C<sub>β</sub>-C<sub>γ</sub> bond cleavage of  $W_{1-CH_3}$  with the loss of 131 Da corresponding to neutral 1-methyl-1*H*-indole. This finding suggests that the hydrogen atom on the indole nitrogen atom, although it could change the form of the final product, was not an indispensable factor for the C<sub>β</sub>-C<sub>γ</sub> bond cleavage. The counterpart of the  $[M - 131]^+$  ion in the CID of  $[WGGGH]^+$  is the  $[M - 117]^+$  species, which is not observable in Figure 2e, indicating that the product of  $[M - 116]^+$  plus  $[Ind]^*-2$  is more favorable than that of  $[M - 117]^+$  plus 1-methyl-1*H*-indole. The formation of  $[Ind]^*-2$  requires the transfer of one hydrogen atom to the γ-carbon atom of the tryptophan residue (or position 3 of the indole ring). This process could occur either before or after C<sub>β</sub>-C<sub>γ</sub> bond cleavage. The aforementioned calculations revealed that the direct C<sub>β</sub>-C<sub>γ</sub> bond cleavage of a tryptophan residue is associated with a very high energy barrier relative to that of a leucine residue. In addition to the stabilities of the products, this high energy barrier might also result from the stronger sp<sup>3</sup>-sp<sup>2</sup> type of C<sub>β</sub>-C<sub>γ</sub> bond of tryptophan (greater s character) than the sp<sup>3</sup>-sp<sup>3</sup>-type bond of leucine (less s character), consistent with the shorter C<sub>β</sub>-C<sub>γ</sub> bond length of tryptophan (1.514 Å) than that of leucine (1.555 Å), as indicated in Figure S3. Therefore, it is highly possible that one hydrogen atom transfers to the γ-carbon atom of the tryptophan side chain prior to cleavage of the C<sub>β</sub>-C<sub>γ</sub> bond, which transforms the γ-carbon atom from sp<sup>2</sup> to sp<sup>3</sup> hybridization, thereby weakening the C<sub>β</sub>-C<sub>γ</sub> bond. The C<sub>β</sub>-C<sub>γ</sub> bond cleavage followed by HAT from the indole nitrogen atom to the peptide backbone in the ion-molecule complex results in the  $[M - 116]^+$  ion and  $[Ind]^*-2$ . Because there is no labile hydrogen atom on the indole ring of  $[W_{1-CH_3}GGGH]^+$ , during CID, the 1-methyl-1*H*-indole produced in the course of the C<sub>β</sub>-C<sub>γ</sub> bond cleavage cannot undergo HAT back to the peptide backbone; as a result, the  $[M - 131]^+$  ion is formed instead of the HAT product  $[M - 130]^+$ .

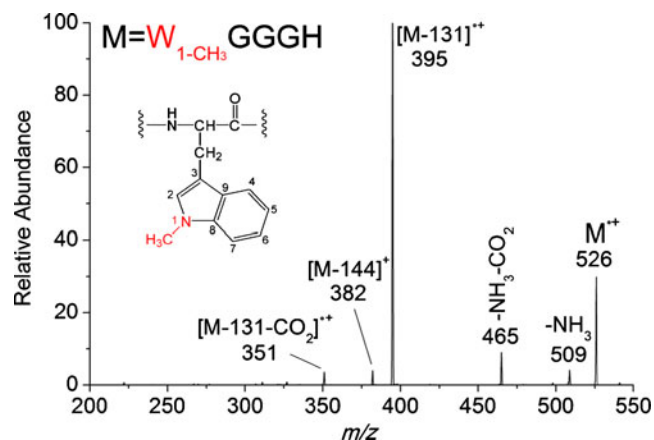
### Alternative Mechanisms for the C<sub>β</sub>-C<sub>γ</sub> Bond Cleavage

Our present experimental observations for the facile C<sub>β</sub>-C<sub>γ</sub> bond cleavages of N-terminal tryptophan residues can be

**Table 2.** Relative Energies and Radical Stabilization Energies (RSE)<sup>a</sup> in kcal/mol for Possible 116 Da Neutral Losses, Produced by C<sub>β</sub>-C<sub>γ</sub> Bond Cleavage of Tryptophan. The RSE Values for 29 and 43 Da Neutral Losses from Isoleucine and Leucine, Respectively, are also Shown

	Tryptophan		Isoleucine	Leucine
				
Relative Energy	0.0	-29.0	-	-
RSE	-16.3	10.1	3.3	5.6

<sup>a</sup>Please refer to the Table S1 of the supplementary material for the details regarding the calculation of RSE.



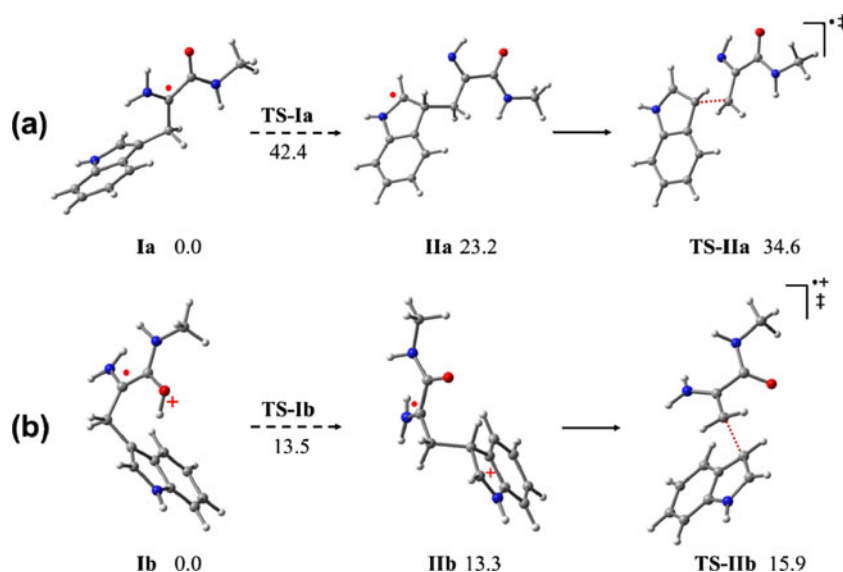
**Figure 5.** CID spectrum of  $[W_{1-CH_3}GGGH]^+$ . “ $W_{1-CH_3}$ ” represents a modified tryptophan residue with the 1-hydrogen of indole ring substituted by a methyl group, which prevents the dehydrogenation of the indole nitrogen of tryptophan

rationalized in terms of two proposed mechanisms (Scheme 1b and c). Scheme 1b depicts a charge-remote radical-driven cleavage, in which HAT from the amino group of the tryptophan residue to the  $\gamma$ -carbon atom of its side chain, followed by homolytic cleavage of the C<sub>β</sub>–C<sub>γ</sub> bond, gives a molecule–ion complex (IIIa) formed from the radical peptide backbone and the 1H-indole; subsequent HAT from the indole nitrogen atom to the amino nitrogen atom generates the neutral 116-Da molecule. For an M<sup>•+</sup> species containing a 1-methyl-tryptophan residue, the dissociation of IIa will give  $[M - 131]^+$  directly, instead of the ion molecule complex (IIIa). Scheme 1c depicts a charge-assisted radical-driven cleavage, which involves proton transfer (PT) from the peptide backbone to the  $\gamma$ -carbon atom of the tryptophan residue, thereby weakening the C<sub>β</sub>–C<sub>γ</sub> bond; subsequent heterolytic cleavage of this bond,

followed by PT from the indole nitrogen atom to the backbone, could also result in the loss of the neutral species having a mass of 116 Da.

To examine these two mechanisms, we performed DFT calculations using the  $\alpha$ -tryptophylmethylamino radical and radical cation, respectively, as model systems. Figure 6 presents the key structures for the considered mechanisms; all related structures are provided in Figure S4. For the mechanism depicted in Scheme 1b, the isomer IIa, resulting from HAT from the amino group to the  $\gamma$ -carbon atom of the tryptophan residue through TS-Ia, is 23.2 kcal/mol higher in enthalpy higher than the low-lying conformer Ia. The barrier against the homolytic cleavage of the C<sub>β</sub>–C<sub>γ</sub> bond of IIa is 11.4 kcal/mol (34.6 kcal/mol relative to the lowest-energy isomer Ia), substantially lower than that for direct C<sub>β</sub>–C<sub>γ</sub> bond cleavage of tryptophan (50.8 kcal/mol). A similar mechanism involving a HAT from the amide group has also examined; its barrier is higher (by 4.5 kcal/mol) than that against the mechanism depicted in Scheme 1b (Figure S5). The reaction is even more favorable through Scheme 1c; PT from the amide oxygen atom gives the isomer IIb, which is only 13.3 kcal/mol higher in enthalpy than that of the low-lying conformer Ib. The heterolytic cleavage of the C<sub>β</sub>–C<sub>γ</sub> bond of IIb has a barrier of only 2.6 kcal/mol (15.9 kcal/mol relative to Ib). The calculations for these model systems suggest that the mechanism depicted in Scheme 1c is favored as long as there is a free proton available in the system. When the peptide radical cations contain highly basic residues (e.g., arginine), the charge-remote process—the mechanism depicted in Scheme 1b—would become possible for the C<sub>β</sub>–C<sub>γ</sub> bond cleavages of tryptophan residues.

Parenthetically, the CID spectrum of  $[W_{1-CH_3}GGGH]^+$  provides mechanistic insight regarding the C<sub>α</sub>–C<sub>β</sub> bond cleavage of tryptophan. The  $[M - 143]^+$  ion, the counterpart of  $[M - 129]^+$  in the CID of  $[WGGGH]^+$ , was not observed in



**Figure 6.** Some critical conformers and transition structures of  $\alpha$ -tryptophylmethylamino (a) radical and (b) radical cation involved in the elimination of neutral 116 Da radical via mechanisms shown in Scheme 1b and c, respectively. Relative enthalpies at 0 K are in kcal/mol. All related structures are shown in Figure S4



the CID spectrum of [W<sub>1-CH<sub>3</sub>GGGH</sub>]<sup>++</sup>, indicating that removal of the hydrogen atom from the indole nitrogen atom is an essential step for the loss of the neutral species having a mass of 129 Da. This result is consistent with the experimental findings for tryptophan-containing peptide radical cations with the initial radical site located on the indole nitrogen atom [30]. Similar to the C<sub>β</sub>-C<sub>γ</sub> bond cleavage, the C<sub>α</sub>-C<sub>β</sub> bond cleavage can occur without the participation of the indole nitrogen atom, instead forming the product ion [M - 144]<sup>+</sup> during the CID of [W<sub>1-CH<sub>3</sub>GGGH</sub>]<sup>++</sup>.

## Conclusion

Loss of a neutral species having a mass of 116 Da, generated from the C<sub>β</sub>-C<sub>γ</sub> bond cleavage of tryptophan, has been observed in the dissociations of a series of tryptophan-containing peptide radical cations; this feature could be a diagnostic fragmentation revealing the presence of tryptophan residues in peptides. To the best of our knowledge, this fragmentation has not been observed previously in the CID spectra of peptide radical cations generated from the dissociative oxidation of metal complexes. Both experimental and theoretical analyses revealed that the α-carbon-centered radical of the tryptophan residue is a crucial intermediate. Furthermore, dehydrogenation of the indole nitrogen atom is a prerequisite for the loss of the 116-Da neutral species, but it is not an inevitable step for the C<sub>β</sub>-C<sub>γ</sub> bond cleavage. From RSE calculations and comparisons of the CID spectra of [WGGGH]<sup>++</sup> and [W<sub>1-CH<sub>3</sub>GGGH</sub>]<sup>++</sup>, we confirmed the identity of the neutral 116-Da species to be [Ind]<sup>-2</sup>, with the radical positioned on the indole nitrogen atom. DFT calculations of the proposed mechanisms revealed that HAT or PT to the γ-carbon atom of the tryptophan residue weakened the C<sub>β</sub>-C<sub>γ</sub> bond, thereby decreasing the dissociation energy barriers dramatically.

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## References

1. Aebersold, R., Goodlett, D.R.: Mass spectrometry in proteomics. *Chem. Rev.* **101**, 269–295 (2001)
2. Stults, J.T.; Arnott, D.: In: Burlingame, A.L. (ed.) Academic Press, *Methods Enzymol* **402**, 245–289 (2005)
3. Burlet, O., Orkiszewski, R.S., Ballard, K.D., Gaskell, S.J., Bertrand, M. J.: Charge Promotion of Low-Energy Fragmentations of Peptide Ions. *Rapid Commun. Mass Spectrom.* **6**, 658–662 (1992)
4. Jones, J.L., Dongré, A.R., Somogyi, Á., Wysocki, V.H.: Sequence Dependence of Peptide Fragmentation Efficiency Curves Determined by Electrospray Ionization/Surface-Induced Dissociation Mass Spectrometry. *J. Am. Chem. Soc.* **116**, 8368–8369 (1994)
5. Dongré, A.R., Jones, J.L., Somogyi, Á., Wysocki, V.H.: Influence of Peptide Composition, Gas-Phase Basicity, and Chemical Modification on Fragmentation Efficiency: Evidence for the Mobile Proton Model. *J. Am. Chem. Soc.* **118**, 8365–8374 (1996)
6. Tsapraillis, G., Nair, H., Somogyi, Á., Wysocki, V.H., Zhong, W., Futrell, J.H., Summerfield, S.G., Gaskell, S.J.: Influence of Secondary Structure on the Fragmentation of Protonated Peptides. *J. Am. Chem. Soc.* **121**, 5142–5154 (1999)
7. Wysocki, V.H., Tsapraillis, G., Smith, L.L., Breci, L.A.: Special Feature: Commentary – Mobile and Localized Protons: A Framework for Understanding Peptide Dissociation. *J. Mass Spectrom.* **35**, 1399–1406 (2000)
8. Paizs, B., Suhai, S.: Fragmentation pathways of protonated peptides. *Mass Spectrom. Rev.* **24**, 508–548 (2005)
9. Wysocki, V.H., Cheng, G., Zhang, Q., Herrmann, K.A., Beardsley, R. L., Hilderbrand, A.E.: In: Laskin, J., Lifshitz, C. (eds.) Principles of Mass Spectrometry Applied to Biomolecules, pp. 279–300. Wiley Interscience, New York (2006)
10. Johnson, R.S., Martin, S.A., Biemann, K., Stults, J.T., Watson, J.T.: Novel Fragmentation Process of Peptides by Collision-Induced Decomposition in a Tandem Mass Spectrometer: Differentiation of Leucine and Isoleucine. *Anal. Chem.* **59**, 2621–2625 (1987)
11. Papayannopoulos, I.A.: The Interpretation of Collision-Induced Dissociation Tandem Mass-Spectra of Peptides. *Mass Spectrom. Rev.* **14**, 49–73 (1995)
12. Zubarev, R.A., Horn, D.M., Fridriksson, E.K., Kelleher, N.L., Kruger, N.A., Lewis, M.A., Carpenter, B.K., McLafferty, F.W.: Electron Capture Dissociation for Structural Characterization of Multiply Charged Protein Cations. *Anal. Chem.* **72**, 563–573 (2000)
13. Zubarev, R.A.: Electron-Capture Dissociation Tandem Mass Spectrometry. *Curr. Opin. Biotechnol.* **15**, 12–16 (2004)
14. Cooper, H., Hudgins, R., Håkansson, K., Marshall, A.: Characterization of Amino Acid Side Chain Losses in Electron Capture Dissociation. *J. Am. Soc. Mass Spectrom.* **13**, 241–249 (2002)
15. Fung, Y.M.E., Chan, T.W.D.: Experimental and Theoretical Investigations of the Loss of Amino Acid Side Chains in Electron Capture Dissociation of Model Peptides. *J. Am. Soc. Mass Spectrom.* **16**, 1523–1535 (2005)
16. Syka, J.E.P., Coon, J.J., Schroeder, M.J., Shabanowitz, J., Hunt, D.F.: Peptide and Protein Sequence Analysis by Electron Transfer Dissociation Mass Spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 9528–9533 (2004)
17. Hogan, J.M., Pitteri, S.J., Chrisman, P.A., McLuckey, S.A.: Complementary Structural Information from a Tryptic N-Linked Glycopeptide via Electron Transfer Ion/Ion Reactions and Collision-Induced Dissociation. *J. Proteome Res.* **4**, 628–632 (2005)
18. Han, H.L., Xia, Y., McLuckey, S.A.: Ion Trap Collisional Activation of c and z' Ions Formed via Gas-Phase Ion/Ion Electron-Transfer Dissociation. *J. Proteome Res.* **6**, 3062–3069 (2007)
19. Chung, T.W., Tureček, F.: Backbone and Side-Chain Specific Dissociations of z Ions from Non-Tryptic Peptides. *J. Am. Soc. Mass Spectrom.* **21**, 1279–1295 (2010)
20. Sun, Q.Y., Nelson, H., Ly, T., Stoltz, B.M., Julian, R.R.: Side Chain Chemistry Mediates Backbone Fragmentation in Hydrogen Deficient Peptide Radicals. *J. Proteome Res.* **8**, 958–966 (2009)
21. Hopkinson, A.C., Siu, K.W.M.: Principles of Mass Spectrometry Applied to Biomolecules, pp. 301–335. Wiley Interscience, New York (2006)
22. Hopkinson, A.C.: Radical Cations of Amino Acids and Peptides: Structures and Stabilities. *Mass Spectrom. Rev.* **28**, 655–671 (2009)
23. Barlow, C.K., McFadyen, W.D., O'Hair, R.A.J.: Formation of Cationic Peptide Radicals by Gas-Phase Redox Reactions with Trivalent Chromium, Manganese, Iron, and Cobalt Complexes. *J. Am. Chem. Soc.* **127**, 6109–6115 (2005)
24. Laskin, J., Yang, Z.B., Lam, C., Chu, I.K.: Charge-Remote Fragmentation of Odd-Electron Peptide Ions. *Anal. Chem.* **79**, 6607–6614 (2007)
25. Chu, I.K., Rodriguez, C.F., Lau, T.C., Hopkinson, A.C., Siu, K.W.M.: Molecular Radical Cations of Oligopeptides. *J. Phys. Chem. B* **104**, 3393–3397 (2000)
26. Ly, T., Julian, R.R.: Residue-Specific Radical-Directed Dissociation of Whole Proteins in the Gas Phase. *J. Am. Chem. Soc.* **130**, 351–358 (2008)
27. Wee, S., Mortimer, A., Moran, D., Wright, A., Barlow, C.K., O'Hair, R. A.J., Radom, L., Easton, C.J.: Gas-Phase Regiocontrolled Generation of

- Charged Amino Acid and Peptide Radicals. *Chem. Commun.* (Cambridge, UK) 4233–4235 (2006)
28. Hao, G., Gross, S.S.: Electrospray Tandem Mass Spectrometry Analysis of S- and N-Nitrosopeptides: Facile Loss of NO and Radical-Induced Fragmentation. *J. Am. Soc. Mass Spectrom.* **17**, 1725–1730 (2006)
  29. Ryzhov, V., Lam, A.K.Y., O'Hair, R.A.J.: Gas-Phase Fragmentation of Long-Lived Cysteine Radical Cations Formed Via NO Loss from Protonated S-Nitrosocysteine. *J. Am. Soc. Mass Spectrom.* **20**, 985–995 (2009)
  30. Knudsen, E.R., Julian, R.R.: Fragmentation Chemistry Observed in Hydrogen Deficient Radical Peptides Generated from N-nitrosotryptophan Residues. *Int. J. Mass Spectrom.* **294**, 83–87 (2010)
  31. Lam, A.K.Y., Ryzhov, V., O'Hair, R.A.J.: Mobile Protons Versus Mobile Radicals: Gas-Phase Unimolecular Chemistry of Radical Cations of Cysteine-Containing Peptides. *J. Am. Soc. Mass Spectrom.* **21**, 1296–1312 (2010)
  32. Masterson, D.S., Yin, H., Chacon, A., Hachey, D.L., Norris, J.L., Porter, N.A.: Lysine Peroxycarbamates: Free Radical-Promoted Peptide Cleavage. *J. Am. Chem. Soc.* **126**, 720–721 (2003)
  33. Hodyss, R., Cox, H.A., Beauchamp, J.L.: Bioconjugates for Tunable Peptide Fragmentation: Free Radical Initiated Peptide Sequencing (FRIPS). *J. Am. Chem. Soc.* **127**, 12436–12437 (2005)
  34. Lee, M., Kang, M., Moon, B., Oh, H.B.: Gas-phase peptide sequencing by TEMPO-mediated radical generation. *Analyst* **134**, 1706–1712 (2009)
  35. Wee, S., O'Hair, R.A.J., McFadyen, W.D.: Comparing the Gas-Phase Fragmentation Reactions of Protonated and Radical Cations of the Tripeptides GXR. *Int. J. Mass Spectrom.* **234**, 101–122 (2004)
  36. Bagheri-Majidi, E., Ke, Y.Y., Orlova, G., Chu, I.K., Hopkinson, A.C., Siu, K.W.M.: Copper-Mediated Peptide Radical Ions in the Gas Phase. *J. Phys. Chem. B* **108**, 11170–11181 (2004)
  37. Siu, C.K., Ke, Y., Orlova, G., Hopkinson, A.C., Siu, K.W.M.: Dissociation of the N–C<sub>α</sub> Bond and Competitive Formation of the [z<sub>n</sub> – H]<sup>+</sup> and [c<sub>n</sub> + 2H]<sup>+</sup> Product Ions in Radical Peptide Ions Containing Tyrosine and Tryptophan: The Influence of Proton Affinities on Product Formation. *J. Am. Soc. Mass Spectrom.* **19**, 1799–1807 (2008)
  38. Wee, S., O'Hair, R.A.J., McFadyen, W.D.: Side-Chain Radical Losses from Radical Cations Allows Distinction of Leucine and Isoleucine Residues in the Isomeric Peptides Gly-XXX-Arg. *Rapid Commun. Mass Spectrom.* **16**, 884–890 (2002)
  39. Laskin, J., Futrell, J.H., Chu, I.K.: Is Dissociation of Peptide Radical Cations an Ergodic Process? *J. Am. Chem. Soc.* **129**, 9598–9599 (2007)
  40. Moran, D., Jacob, R., Wood, G.P.F., Coote, M.L., Davies, M.J., O'Hair, R.A.J., Easton, C.J., Radom, L.: Rearrangements in Model Peptide-Type Radicals via Intramolecular Hydrogen-Atom Transfer. *Helv. Chim. Acta* **89**, 2254–2272 (2006)
  41. Yang, Z.B., Lam, C., Chu, I.K., Laskin, J.: The Effect of the Secondary Structure on Dissociation of Peptide Radical Cations: Fragmentation of Angiotensin III and Its Analogues. *J. Phys. Chem. B* **112**, 12468–12478 (2008)
  42. Ng, D.C.M., Song, T., Siu, S.O., Siu, C.K., Laskin, J., Chu, I.K.: Formation, Isomerization, and Dissociation of  $\alpha$ -Carbon-centered and  $\pi$ -Centered Glycylglycyltryptophan Radical Cations. *J. Phys. Chem. B* **114**, 2270–2280 (2010)
  43. Song, T., Ng, D.C.M., Quan, Q., Siu, C.-K., Chu, I.K.: Arginine-Facilitated  $\alpha$ - and  $\pi$ -Radical Migrations in Glycylarginyltryptophan Radical Cations. *Chem. Asian J.* **6**, 888–898 (2011)
  44. Xu, M., Song, T., Quan, Q., Hao, Q., Fang, D.C., Siu, C.K., Chu, I.K.: Effect of the N-terminal Basic Residue on Facile C<sub>α</sub>–C Bond Cleavages of Aromatic-Containing Peptide Radical Cations. *Phys. Chem. Chem. Phys.* **13**, 5888–5896 (2011)
  45. Ly, T., Julian, R.R.: Tracking Radical Migration in Large Hydrogen Deficient Peptides with Covalent Labels: Facile Movement does not Equal Indiscriminate Fragmentation. *J. Am. Soc. Mass Spectrom.* **20**, 1148–1158 (2009)
  46. Laskin, J., Yang, Z., Ng, C.M.D., Chu, I.K.: Fragmentation of  $\alpha$ -Radical Cations of Arginine-Containing Peptides. *J. Am. Soc. Mass Spectrom.* **21**, 511–521 (2010)
  47. Chu, I.K., Zhao, J., Xu, M., Siu, S.O., Hopkinson, A.C., Siu, K.W.M.: Are the Radical Centers in Peptide Radical Cations Mobile? The Generation, Tautomerism, and Dissociation of Isomeric  $\alpha$ -Carbon-centered Triglycine Radical Cations in the Gas Phase. *J. Am. Chem. Soc.* **130**, 7862–7872 (2008)
  48. Wee, S., O'Hair, R.A.J., McFadyen, W.D.: The Role of the Position of the Basic Residue in the Generation and Fragmentation of Peptide Radical Cations. *Int. J. Mass Spectrom.* **249**, 171–183 (2006)
  49. Zhang, L.Y., Reilly, J.P.: Radical-Driven Dissociation of Odd-Electron Peptide Radical Ions Produced in 157 nm Photodissociation. *J. Am. Soc. Mass Spectrom.* **20**, 1378–1390 (2009)
  50. Savitski, M.M., Nielsen, M.L., Zubarev, R.A.: Side-Chain Losses in Electron Capture Dissociation to Improve Peptide Identification. *Anal. Chem.* **79**, 2296–2302 (2007)
  51. Chan, W.C., White, P.D.: Fmoc Solid Phase Peptide Synthesis: A Practical Approach, pp. 41–76. Oxford University Press, New York (2000)
  52. Henke, W., Kremer, S., Reinen, D.: Cu<sup>2+</sup> in Five-Coordination: A Case of a Second-Order Jahn-Teller Effect. 1. Structure and Spectroscopy of the Compounds Cu(terpy)X<sub>2</sub>·nH<sub>2</sub>O. *Inorg. Chem.* **22**, 2858–2863 (1983)
  53. Varkey, S.P., Ratnasamy, C., Ratnasamy, P.: Zeolite-Encapsulated Manganese(III)salen Complexes. *J. Mol. Catal. A Chem.* **135**, 295–306 (1998)
  54. Becke, A.D.: Density-Functional Thermochemistry. III. The Role of Exact Exchange. *Chem. Phys.* **98**, 5648–5652 (1993)
  55. Hehre, W.J., Ditchfield, R., Pople, J.A.: Self-Consistent Molecular Orbital Methods. XII. Further Extensions of Gaussian-Type Basis Sets for Use in Molecular Orbital Studies of Organic Molecules. *J. Chem. Phys.* **56**, 2257–2261 (1972)
  56. Clark, T., Chandrasekhar, J., Spitznagel, G.W., Schleyer, P.v.R.: Efficient Diffuse Function-Augmented Basis Sets for Anion Calculations. III. The 3-21+G Basis Set for First-Row Elements, Li–F. *J. Comput. Chem.* **4**, 294–301 (1983)
  57. Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Montgomery, Jr., J.A., Vreven, T., Kudin, K.N., Burant, J.C., Millam, J.M., Iyengar, S.S., Tomasi, J., Barone, V., Mennucci, B., Cossi, M., Scalmani, G., Rega, N., Petersson, G.A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Li, X., Knox, J.E., Hratchian, H.P., Cross, J.B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R.E., Yazyev, O., Austin, A.J., Cammi, R., Pomelli, C., Ochterski, J.W., Ayala, P.Y., Morokuma, K., Voth, G.A., Salvador, P., Dannenberg, J.J., Zakrzewski, V.G., Dapprich, S., Daniels, A.D., Strain, M.C., Farkas, O., Malick, D.K., Rabuck, A.D., Raghavachari, K., Foresman, J.B., Ortiz, J.V., Cui, Q., Baboul, A.G., Clifford, S., Cioslowski, J., Stefanov, B.B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Martin, R.L., Fox, D.J., Keith, T., Al-Laham, M.A., Peng, C.Y., Nanayakkara, A., Challacombe, M., Gill, P.M.W., Johnson, B., Chen, W., Wong, M.W., Gonzalez, C., Pople, J.A.: Gaussian 03, Revision C.02; Gaussian, Inc., Wallingford CT (2004)
  58. Zipse, H.: Radical stability – A theoretical perspective. *Top. Curr. Chem.* **263**, 163–189 (2006)