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Fragmentation of deprotonated cyclic dipeptides by electrospray ionization mass spectrometry

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The fragmentation pathways of deprotonated cyclic dipeptides have been studied by electrospray ionization multi-stage mass spectrometry (ESI-MS^n) in negative mode. The results showed that the fragmentation pathways of deprotonated cyclic dipeptides depended significantly on the different substituents, the side chains of amino acid residues at the diketopiperazine ring. In the spectra of deprotonated cyclic dipeptides, the ion [M−H−substituent radical]$^-$ was firstly observed in the ESI mode. The characteristic fragment ions [M−H−substituent radical]$^-$ and [M−H−(substituent−H)]$^-$ could be used as the symbols of particular cyclic dipeptides. The hydrogen/deuterium (H/D) exchange experiment, the high-resolution mass spectrometry (Q-TOF) and theoretical calculations were used to rationalize the proposed fragmentation pathways and to verify the differences between the fragmentation pathways. The relative Gibbs free energies (ΔG) of the product ions and possible fragmentation pathways were estimated using the B3LYP/6−31++G(d, p) model. The results have some potential applications in the structural elucidation and interpretation of the mass spectra of homologous compounds and will enrich the gas-phase ESI-MS ion chemistry of cyclic dipeptides.

Keywords: cyclic dipeptides; electrospray ionization; fragmentation pathway; theoretical calculation; radical anion

Introduction

Cyclic dipeptides [(piperazine 2,5-diones or 2,5-diketopiperazines (DKPs)] are relatively simple compounds and, therefore, are among the most common peptide derivatives found in nature. In comparison to linear peptides, cyclic peptides are more bioavailable and more stable to degradative peptidases, and their research has been fundamental to many aspects of peptide chemistry. It was found that naturally occurring and synthesized cyclic dipeptides exhibit a multitude of interesting biological activities as antiviral, antibiotic, antimicrobial and antitumor agents.[1−4] There are many reports in the literature that cyclic dipeptides were identified in several beverages and foods, especially fermented and thermally treated ones, which include aged sake, beer, cocoa, dried squid,[5] hydrolyzed vegetable protein,[6] roasted coffee,[7,8] roasted malt and others. However, identification of the structures of cyclic dipeptides in foods and beverages still has low throughput because of the limited availability of standards.[5] With the advent of mild ionization techniques, electrospray ionization mass spectrometry (ESI-MS) using low-energy collision-induced dissociation (CID) has become an increasingly important tool for the analysis of various kinds of compounds with excellent sensitivity. But, for full structure elucidation based on their mass spectrometry data, it is necessary to understand all the gas-phase chemistry involved in each fragmentation step and to clarify the relationships between all the different possible pathways. Researchers have synthesized their own cyclic dipeptides of interest to achieve some identification by mass spectrometry for each instance.[7−9] Up to now, some reports have been published concerning the systemic studies of the fragmentation of protonated cyclic dipeptides by ESI-MS in positive mode. Y. H. Chen *et al*. have identified DKPs in chicken essence and verified the molecular structures of the DKPs using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Moreover, they found that the protonated molecule often produces $[M + H - CO - NH_3]^+$ ion accompanied by losing CO and NH_3.[5] A. E. M. Crotti's group analyzed a series of five-membered lactones (both saturated and unsaturated) and lactam by ESI-MS/MS and electrospray ionization multi-stage mass spectrometry (ESI-MS^n).[10] It was observed that the main fragment ions of their protonated molecules are derived from neutral losses of CO and/or H_2O (NH_3 for the lactam). Recently, they used ESI-MS/MS to investigate the fragmentation of a series of DKPs previously isolated from *Aspergillus fumigatus* and found that losing CO directly from the protonated molecule was a fragmentation process common to all the compounds analyzed. Furthermore, their results revealed a series of ions that are diagnostic of the substituents at the DKPs' ring.[11] With regard to deprotonated ions, there are just some elucidations about the collision-induced spectra of a variety of peptides.[12] Many studies[13−15] of small deprotonated peptides suggested that the fragmentation modes of the $[M - H]^-$ ions depended significantly on the different substituents at the side chain of amino acids.

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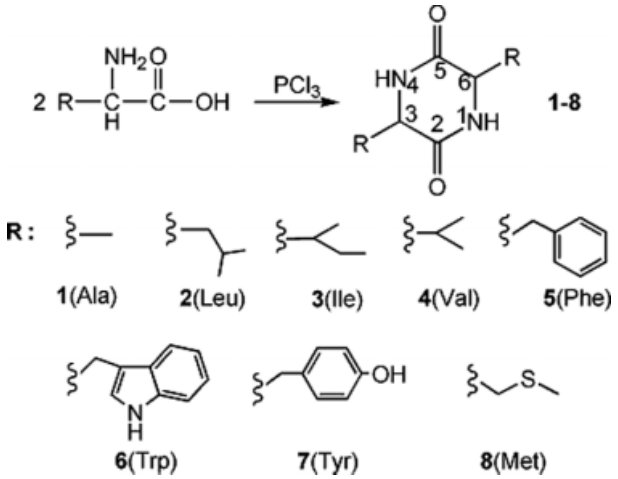
For example, the study on the fragmentation reactions of a variety of deprotonated dipeptides and tripeptides containing phenylalanine revealed that the benzyl-group has a substantial effect on the fragmentation reactions observed.[13] Although there are many reports about the fragmentation pathways of deprotonated peptides and protonated cyclic dipeptides, to the best of our knowledge, much less is known so far concerning the investigation of structure–fragmentation relationships of deprotonated cyclic dipeptides. J. H. Bowie's group has studied the mass spectra of some deprotonated cyclic dipeptides in the negative chemical ionization (CI) mode.[16] It was found that there were two major fragmentations of $[M - H]^-$ ions for symmetrical and unsymmetrical cyclic dipeptides. One fragmentation pathway was the characteristic side-chain loss that could be used to identify the particular cyclic dipeptide, and the other fragmentation pathway was an unusual loss of RCHO (R is the substituent) that involved initial 1,2 migration of R to the carbon of the adjacent carbonyl group. Nevertheless, there is not report describing studies of the fragmentation of deprotonated cyclic dipeptides in ESI negative mode. It is known that the fragmentation pathways of protonated and deprotonated species generated by ESI are quite different from those generated by other ionization techniques, and now, ESI-MS is used extensively, so an understanding of the fragmentation mechanisms of deprotonated cyclic dipeptides in ESI mode has critical significance. It can be applied to the fragmentation elucidation of larger more complex natural products and compounds of biological significance that may contain cyclic dipeptides as part of their structures. At the same time, this study can also enrich the gas-phase ion chemistry of ESI-MS under low-energy CID condition. In this study, eight symmetrical cyclic dipeptides were synthesized and their fragmentation pathways in ESI negative mode were studied to evaluate the role of the substituents at the DKPs ring on the fragmentation of the deprotonated cyclic dipeptides. The fragmentation mechanisms were investigated by multi-stage mass spectrometry incorporating hydrogen/deuterium (H/D) exchange experiments. Furthermore, the results were validated by the ESI-MS/MS data of high-resolution accurate-mass quadrupole-time of flight (Q-TOF) and theoretical calculations. The relative Gibbs free energies (ΔG) of the product ions and possible fragment ions were estimated using the B3LYP/6–31++G (d, p) model.

Experimental

Chemicals

Compounds **1–8** were synthesized from L-amino acids and phosphorus trichloride. Each L-amino acid (20 mM) was dissolved in tetrahydrofuran (THF) (30 ml), and then PCl_3 (10 mM) was added in batches. The mixture was stirred and refluxed for 2 h. After evaporation of the solvent, H_2O was added and the solution was adjusted to pH 7–8 with saturated aqueous $NaHCO_3$. Then, the reaction mixture was filtered and the solid was washed with water (3 × 10 ml). The crude cyclic dipeptide was obtained. Analytical sample was obtained by column chromatography (silica gel, methanol/ethyl acetate = 1:19 to 1:4) or recrystallization from acetone and petroleum ether as white solid. The yield of cyclic dipeptide was 20–60%. The structures were characterized by ^1H NMR, ^{13}C NMR, ESI-MS, high resolution mass spectrometer (HRMS) and IR methods. The symmetrical cyclic dipeptides investigated in this study



Scheme 1. Synthesis of cyclic dipeptides.

are cyclo(Ala-Ala)(**1**), cyclo(Leu-Leu)(**2**), cyclo(Ile-Ile)(**3**), cyclo(Val-Val)(**4**), cyclo(Phe-Phe)(**5**), cyclo(Trp-Trp)(**6**), cyclo(Tyr-Tyr)(**7**) and cyclo(Met-Met)(**8**). Methanol (HPLC grade) was obtained from Tedia Company and deionized water (Milli-Q) was used throughout the study. The reaction and the structures of cyclic dipeptides are shown as Scheme 1.

Mass spectrometry

Mass spectrometric analysis was performed by a Bruker Esquire 3000 (Bruker Dalton, Germany) ion trap mass spectrometer interfaced with an ESI source. Ionization of analytes was carried out using the following setting of ESI: nebulizer gas flow 7 psi, dry gas 4 l/min, dry temperature 300 °C and capillary voltage 4000 V. Calibration of *m/z* was performed using a standard ESI-tuning-mix. Scan range was 15–500 *m/z* and scan resolution was normal (13 000 *m/z*/s). ESI-MS^n spectra were obtained by CID experiments with helium after isolation of the appropriate precursor ions. High-resolution mass spectra were performed on an ESI-Q-TOF-MS spectrometer (Micromass, England). Solutions of the compounds (0.1 mg/ml) were prepared by dissolving the compounds in methanol.

Computational methods

Using density functional theory, the geometries of the reactant, intermediates and products in all reaction channels were optimized at the B3LYP/6–31++G (d, p) level. All theoretical calculations were performed using the GAUSSIAN03[17] suite of software.

Results and Discussion

Negative ESI-MS/MS spectra of cyclic-dipeptides

ESI-MS^n spectra of compounds **1–8** in negative mode were studied in detail and the data are shown in Tables 1 and 2. Comparison among the structures of compounds **1–8**, which differ only in the side-chain substituent at C(3) and C(6) of DKP ring, revealed that the fragmentation patterns are correlative with the types of these substituents at C(3) and C(6). Therefore, in accordance with the different substituent at DKP ring, the cyclic dipeptides are divided into three groups (1) compounds **1–4** (R is an alkyl substituent), (2) compounds **5–7** (R contains an aromatic substituent) and

(3) compound **8** (R is an alkyl substituent containing a heteroatom). A significant difference was observed in the cleavage of deprotonated cyclic dipeptides in low-energy CID process due to the effect of the side-chain substituents on fragmentation. The ESI-MS^n spectral data showed that there are three main fragmentation pathways (path a, b and c), as shown in Scheme 2. The fragmentations from ions **A** occur at the side chain with consecutive elimination of a CO and a saturated substituent molecule (R + H), elimination of an unsaturated substituent molecule (R − H) or elimination of a substituent radical to form ions **D, B1** or **C1**, respectively. The first pathway (a) occurs only for compounds **1–4.** The second pathway (b) occurs for compounds **6** and **7** and the third pathway (c) occurs for compounds **1–5**. It seems strange that for compounds **1–5**, the formation of the product ion **C1** involves an elimination of an odd-electron loss and a substituent radical might be lost in the fragmentation process.

The electron impact ionization (EI) mass spectra of small organic compounds, recorded at 70 eV, are often performed to identify unknown compounds because of its excellent reproductibility and structure-specific nature of the dissociation products. In EI spectra, the loss of free radical is very common in the fragmentation process. However, for the need of volatility of the sample, the application of EI is limited in peptides analysis. Now, soft ionization methods such as ESI and matrix assisted laser desorption ionization (MALDI) have been widely applied to produce protonated and deprotonated proteins or peptides, and these ions yielded under CID conditions have been used more and more to determine amino acid sequences.[18–20] Comparison with EI, CID process provides lower energy, and the unusual losses, such as the loss of a radical, are rarely observed. However, there are still some reports about the observation of the radical loss under CID conditions.[11,21–25] K. Whitehead's study about seven mycosporine-like amino acids (MAAs) showed that the small loss of 15 Da was the result of the loss of a group radical from the protonated MAA molecule.[24] Subsequently, K.H.M. Cardozo *et al*. confirmed the small loss was a methyl radical by (H/D)exchange, ESI-MS/MS and high-resolution mass spectrometry (Q-TOF).[25] Recently, by theoretical calculations, they further demonstrated that losing a radical in the CID process for the protonated MAA molecule was reasonable and the elimination of mass 15 Da involved a methyl radical.[26] However, most of the observations of radical loss under CID condition come from cations in positive mode. To the best of our knowledge, only a few reports have been published so far describing studies of the fragmentation of odd-electron peptide radical anions through low-energy CID in ESI negative mode.[27] Considering the ability of CID to produce odd-electron radical cation and anion, it is supposed that the odd-electron loss from ions **A** to form ions **C1** in the fragmentation reaction of cyclic dipeptides might be a radical loss.

To validate the mechanisms, the deuterium-labeled experiments were first used to investigate the fragmentation pathways of the deprotonated cyclic dipeptide. For example, the ESI-MS^2 spectra of deuterium-labeled and non-deuterium-labeled compounds **5** and **6** are shown in Fig. 1. Figure 1(**5a, 6a**) shows the spectra of deuterated sample and Fig. 1(**5b, 6b**) shows those of non-deuterated sample. As shown in Fig. 1, compound **5**, as compounds **1–4**, always in the same way eliminates the substituent radical from ion **A** to form ion **C1**. In Fig. 1(**5a**), the deuterium-labeled deprotonated molecule at *m/z* 294 of compound **5** gives a product ion **C1** $[M(D) - H - 91]^-$ at *m/z* 203, which is formed by the elimination of a benzyl radical. After successive elimination of another benzyl radical from ion **C1**, the product ion**C2** at *m/z* 112 is formed that still includes the active hydrogen. The fragment ion at *m/z* 146 is produced by a loss of amino acid residue from deprotonated molecule ion **A**, accompanied by the active hydrogen lost. Corresponding to the deuterated sample, the non-deuterated deprotonated molecule **A** is observed at *m/z* 293 [shown in Fig. 1(**5b**)], and it can successively eliminate two benzyl radicals to produce the ions **C1** and **C2** at *m/z* 202 and 111, respectively. Therefore, the deuterium-labeled experiment of compound **5** can prove that the processes of the formation of ions **C1** and **C2** do not relate to the loss of active hydrogen. Moreover, the base peak at *m/z* 146 is formed by losing an amino acid residue that includes the active hydrogen. However, different from compound **5**, compounds **6** and **7** often eliminate an unsaturated substituent molecule to form ions **B1** and **B2** as shown in Scheme 2. Furthermore, the process of the formation of ions**B1** and **B2** does not relate to the loss of active hydrogen either. In Fig. 1(**6a**), the deuterium-labeled deprotonated molecule at *m/z* 372 of compound **6** gives a base peak ion $[M(D) - H - 129]^-$ at *m/z* 243, corresponding to ion **B1**. It is formed by a neutral loss of the unsaturated substituent molecule, 3-methylene-indole, which has a bigger conjugated structure. The fragment ion at *m/z* 114 corresponding to ion **B2** is produced by continuously losing another unsaturated substituent molecule. The non-deuterated deprotonated molecule at *m/z* 371 likewise produces ions **B1** and **B2** at *m/z* 242 and 113, respectively as shown in Fig. 1(**6b**). Therefore, the deuterium-labeled experiment of compound **6** proves that the ions **B1** and **B2** still contain the active hydrogen after elimination of the unsaturated substituent molecules. Apparently, there is not any fragment ion produced by a radical lose for compound **6**. So the deuterium-labeled results of compounds **5** and **6** indirectly testify to the fragmentation pathway b and c in Scheme 2. Moreover, the results also indicate that the deprotonation has occurred directly at the nitrogen of the DKP ring, not at the side chain of compounds **6** and **7**.

As compound **6**, compound **7** can also eliminate its stable neutral substituent molecule, 4-methylenecyclohexa-2,5-dienone $O{=}C_6H_4{=}CH_2$, to form ions **B1** and **B2** at *m/z* 219 and 113, respectively. However, according to the compound **5**, cyclo(Phe-Phe) with benzyl substituent, its base peak in the deprotonated ESI-MS/MS spectrum is ion **F** formed by the elimination of a phenylalanine residue molecule, since it cannot eliminate a stable substituent molecule with a bigger conjugative structure as those of compounds **6** and **7** (data in Table 1). Since the produced fragment ion **F** of compound **5** contains a bigger conjugative structure, it is observed as the base peak. The substituent of compound **8** contains a sulfur atom, so the fragmentation reactions are always driven by the sulfur atom. Just like their protonated molecule ion, the heterolytic cleavage takes place near the sulfur atom. Then, the deprotonated cyclo(Met-Met) is prone to eliminate one or two CH_3SH molecule to form ions**H1** ($[M - H - CH_3SH]^-$) or H2 ($[M - H - 2CH_3SH]^-$) at *m/z* 213 or 165, respectively. The ion **H1** is observed as the base peak in the ESI-MS^2 spectra of the deprotonated compound **8**. To test the mechanism proposed above, ESI-MS/MS of the deprotonated cyclic dipeptides was carried out by high-resolution mass spectra. High-resolution mass data allowed us to determine the elemental composition for each ionic species detected in ESI-MS/MS spectra. The data are also listed in Table 1, and the results are in agreement with the proposed fragmentation pathways.

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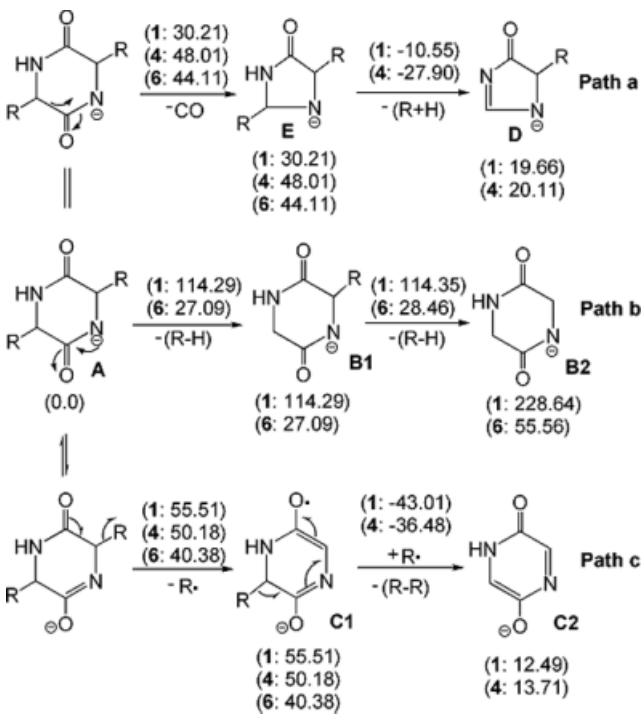
Table 1. ESI-MS^2 data of deprotonated cyclic-dipeptides

Compounds		Type of ions (relative abundance %) A	B1	B2	C1	C2	D	F	H1	H2
Cyclo(Ala-Ala)(**1**)		141.0(3)			125.8(19)		96.7(100)	70.1(2)		
	a	141.0664			126.0429		97.0528	70.0293		
	b	141.0664			126.0398		97.0402	70.0277		
Cyclo(Leu-Leu)(**2**)		225.0(5)			167.7(52)	110.7(11)	138.7(100)			
	a	225.1603			168.0899	111.0195	139.0634			
	b	225.1603			168.0925	111.0184	139.0881			
Cyclo(Ile-Ile)(**3**)		225.0(60)			167.7(100)	110.9(9)	138.7(56)			
	a	225.1603			168.0899	111.0195	139.0634			
	b	225.1603			168.0941	111.0234	139.0954			
Cyclo(Val-Val)(**4**)		197(37)			153.7(100)	110.6(26)	124.9(17)			
	a	197.1296			154.0748	111.0200	125.0483			
	b	197.1290			154.0757	111.0207	125.0737			
Cyclo(Phe-Phe)(**5**)		293.0(17)			201.7(47)	110.9(13)		145.8(100)		
	a	293.1296			202.0748	111.0200		146.0612		
	b	293.1290			202.0610	111.0145		146.0523		
Cyclo(Trp-Trp)(**6**)		371.0(20)	241.6(100)	112.7(22)						
	a	371.1513	242.0935	113.0357						
	b	371.1508	242.0984	113.0422						
Cyclo(Tyr-Tyr)(**7**)		325.0(4)	218.6(100)	112.7(33)						
	a	325.1194	219.0775	113.0356						
	b	325.1189	219.0808	113.0412						
Cyclo(Met-Met)(**8**)		261.0(18)	184.6(2)						212.7(100)	164.8(6)
	a	261.0731	185.0384						213.0697	165.0663
	b	261.0731	185.0510						213.0748	165.0734

a, the exact mass of ions by theoretical calculation; b, The actual mass of ions determined by high-resolution ESI-Q-TOF-MS; R, substituent. **A**, $[M-H]^-$; **B1**, $[A-(R-H)]^-$; **B2**, $[B1-(R-H)]^-$; **C1**, $[A-R]^-$; **C2**, $[C1-R]^-$; **D**, $[A-CO-(R+H)]^-$; **F**, [**A** − residue]$^-$; **H1**, [**A** − part of sbustituent]$^-$; **H2**, [**H1** − part of sbustituent]$^-$.

Table 2. ESI-MS^n data of deprotonated cyclic-dipeptides

Compounds	Precursor ions $[M-H]^-$	Fragment ions (relative abundance %)
Cyclo(Ala-Ala)(**1**)	141.0(3)	96.7(100),125.8(19),70.1(2)
Cyclo(Leu-Leu)(**2**)	225.0(5)	167.7(52), 138.7(100), 110.7(11)
Cyclo(Ile-Ile)(**3**)	225.0(60)	167.7(100), 138.7(56), 110.9(9)
	168.0(9)	110.8(100)
Cyclo(Val-Val)(**4**)	197(37)	153.7(100), 124.9(17), 110.6(26)
Cyclo(Phe-Phe)(**5**)	293.0(17)	274.7(26), 201.7(47), 183.6(4), 145.8(100), 110.9(13)
	275.0(16)	183.7(100)
	202.0(4)	110.9(100)
	146.0(100)	42.8(69)
Cyclo(Trp-Trp)(**6**)	371.0(20)	241.6(100), 112.7(22)
	242.0(37)	112.6(100)
Cyclo(Tyr-Tyr)(**7**)	325.0(4)	218.6(100), 112.7(33)
	219.0(13)	112.7(100)
Cyclo(Met-Met)(**8**)	261.0(18)	212.7(100), 184.6(2),164.8(6)
	213.0(12)	184.7(13), 164.7(100), 147.7(19), 136.8(26), 120.7(6), 108.6(3)



Scheme 2. Proposed fragmentation pathways of deprotonated cyclic dipeptides. The values above the arrows (in kcal/M) represent the relative Gibbs free energies (ΔG) for each individual fragmentation reaction.

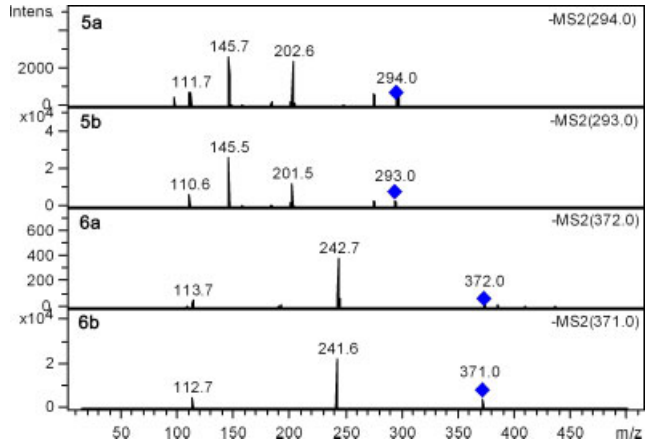
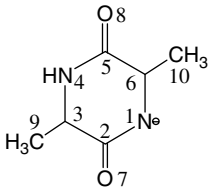


Figure 1. Deuterium-labeled (**a**) and non-deuterium-labeled (**b**) ESI-MS^2 spectra of compounds **5** and **6** in negative mode.

Table 3. Bond lengths (Å) for neutral [M] and deprotonated [M − H] compound **1**



	M	$[M-H]^-$	*D*-value[a]
C(2)–N(1)	1.361	1.320	−0.041
C(2)–O(7)	1.224	1.258	+0.034
N(1)–C(6)	1.462	1.452	−0.010
C(5)–C(6)	1.535	1.535	0.000
C(5)–N(4)	1.361	1.357	−0.004
C(5)–O(8)	1.224	1.237	+0.013
N(4)–C(3)	1.462	1.462	0.000
C(3)–C(2)	1.535	1.577	+0.042
C(3)–C(9)	1.528	1.526	−0.002
C(6)–C(10)	1.528	1.533	+0.005

[a] Difference between bond lengths of neutral and deprotonated compound **1**.

Theoretical calculations of deprotonated cyclic dipeptides

The fragmentation pathways of deprotonated cyclic dipeptides revealed that the different fragmentation pathways are closely related to their substituents at the DKP ring. In some cases, the deprotonation is found to occur directly at the nitrogen of the lactam group. Furthermore, using density functional theory, the geometries of the deprotonated molecule were optimized at the B3LYP/6–31++G (d, p) level. The results indicated that nitrogen atom of cyclic dipeptide is the most favorite deprotonation site than carbon atom. Since the cyclic dipeptides studied are all symmetrical molecules, N(1) site is completely equal to N(4) site as shown in Table 3. Thus, the deprotonation is assumed directly at N(1) atom of cyclic dipeptides instead of C(3) or C(6). Because compound **1** is the simplest compound studied, compound **1** is used as an example for theoretical calculations. The geometries of the neutral and the deprotonated compound **1** were fully optimized and the bond lengths were calculated using B3LYP/6–31++G (d, p) model. The bond lengths data for neutral and deprotonated compound **1** are shown in Table 3. Some differences can be obviously observed that the bond lengths of C(2)–C(3), C(2)–O(7), C(5)–O(8) and C(6)–C(10) in deprotonated species become longer comparing with those in neutral molecule of compound**1**. The C(2)–C(3) bond length in the deprotonated molecule is approximately 0.042 Å longer than the corresponding one in the neutral molecule, accordingly that of C(2)–O(7) bond is 0.034 Å longer. The C(5)–O(8) bond length in the deprotonated molecule is approximately 0.013 Å longer than the corresponding one in the neutral molecule, accordingly that of C(6)–C(10) bond is 0.005 Å longer. In the same manner, if the deprotonation takes place at N(4), the bond lengths of C(5)–C(6), C(5)–O(8), C(3)–C(9) and C(2)–O(7) in deprotonated molecule will similarly be longer than those in neutral molecule. The bond lengths of C(2)–O(7) and C(5)–O(8) in the anion are much longer than those in the neutral because deprotonation at atom N(1) results in a decrease of the C(2)–N(1) distance by the negative charge migrating to the oxygen atom. Therefore, the change of the C(2)–C(3) and C(6)–C(10) bond after deprotonation may be an initial evidence for the direction of further fragmentation reactions.

As shown in Scheme 2, the MS^n data suggested that the fragmentation of the deprotonated cyclic dipeptides follows three major competitive pathways starting from the deprotonated molecule ions **A**: (path a) consecutive elimination of a molecule CO and a saturated substituent molecule (R + H) to form ions **D**, (path b) elimination of unsaturated substituent molecule (R − H) to form ions **B1** and **B2** and (path c) elimination of substituent radical to form ions **C1** and **C2**. Path a is observed only for compounds **1**–**4**; path b is observed for compounds **6** and **7** and path c is observed for compounds **1**–**5**. Furthermore, the ions **C1** are observed as base peaks for compounds **3** and **4** and as secondary intense peaks for compounds **1**, **2** and **5**. To rationalize the relative abundances of some product ions in the spectra of cyclic dipeptides, the B3LYP/6–31++G (d, p) model was used to calculate the relative Gibbs free energies for the reactant and product ions. It is considered that in CID-ESI-MS process, the energy that is transferred to the precursor ion due to the collision activation can be even higher than the activation barrier for some specific fragmentation reactions, thus the difference between the initial and final energy states can be used to understand the mechanism, and it is not necessary to consider the energies of any transition state species.[26] Therefore, the relative Gibbs free energies of compounds **1**, **4** and **6**, which represent three different pathways a, c and b, respectively, were calculated and one of the **A** isomers was assumed as the reference (0.0 kcal/M). The data of calculation are shown in Scheme 2.

Most of the species of product ions in the ESI-MS spectra of compounds **1**–**5** (listed in Tables 1 and 2) are similar, except their different base peaks. This means that they follow similar fragmentation pathways, and their different substituents play a crucial role in the intensity of some peaks. For compounds **1** and **4**, the formation of ions **E** from the deprotonated molecule **A** (via path a) is an endergonic process, as evidenced by the relative energies of **A** (0.0 kcal/M) and **E** (30.21 kcal/M for **1**, 48.01 kcal/M for **4**) as shown in Scheme 2. The process can be interpreted in terms of the energy that is required for the heterolytic cleavage of the C(2)–C(3) bond, which length in the deprotonated molecule of compound **1** is approximately 0.042 Å longer than that in the neutral molecule, and for successive losing a CO molecule. This energy content is considered to be transferred to the deprotonated

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molecule owing to the CID process. The analysis of the relative ΔG revealed that the formation of the ions **E** from ions **A** is a thermodynamically disfavored process. However, the product ions **E** can spontaneously transform to ions **D** (**1**: −10.55 kcal/M, **4**: −27.90 kcal/M) by losing a molecule (R + H). Therefore, the ion **D** is observed as the base peak for compound **1**, while the relative abundances of ion **D** is relatively lower (17%) for compound **4**. Another reason will be interpreted later.

It is reported that the transfer of energy content on the CID process is high enough to induce the homolytic cleavage of the chemical bond.[11,26] Apparently, the fragmentation reaction to produce a radical by homolytic cleavage is a thermodynamically disfavored process, since the product ion and the lost fragment are all radicals. However, for compounds **3** and **4**, their base peaks are all ions **C1** produced by losing a substitute radical (via path c), while ions **C1** are secondary intense peak in the ESI-MS/MS spectrum for compounds **1**, **2** and **5** (as shown in Tables 1 and 2). In addition, the activation barrier from ions **A** to **C1** is a little higher than the other fragmentation pathways as shown in Scheme 2, except comparing to the path b for compound **1**. In the previous study of our group, the amides and esters were found to undergo hydrolysis reaction catalyzed by amino-group during ion trap mass analysis in ESI conditions.[28] Other studies have also reported in-trap hydrolysis, for example, the thioester analogs of the alkenyldiarylmethanes,[29] the esters of some alkenyldiarylmethanes and benzophenones[30] and the trimethylsilyl esters of long-chain fatty acids.[31] Prompted by the mechanism of the hydrolysis reactions in ion trap and the mechanism of CI, which produces ions by gas-phase ion-molecule reactions, we speculated the lost substitute radical R· might collide with ions **C1**, involving elimination of molecule R–R. First, the deprotonated molecule (ion **A**) gains enough energy from the CID process and eliminates a substitute radical R. Then, the substitute radical R reacts with ions **C1** to eliminate dual-substitute molecule (R-R) to form ions **C2**. This continuous process is a radical-driven process. By theoretical calculations, the first step is a thermodynamically disfavored process ($\Delta G > 0$, **1**: 55.51 kcal/M; **4**: 50.18 kcal/M). However, the second step is a thermodynamically more favored process ($\Delta G < 0$, **1**: −43.01 kcal/M; **4**: −36.48 kcal/M). It means that the lost substitute radical R can drive product ion **C1** losing dual-substitute molecule (R-R) to form product ion **C2**, which has a more stable conjugative structure.

As shown in Scheme 2, the analysis of the relative ΔG reveals that, in the case of compounds **1** and **4**, the total energy needed for ion **A** to produce ion **C1** (path c) is lower than that of ion **D** (path a). However, the base peak of compound **1** is not ion **C1** but ion **D**, which is different to compound **4**. One of the reasons is that the lost radical ($R^{\bullet}$) of compound**4** is a secondary radical and more stable than the corresponding primary radical of compound **1**, so the relative abundance of the product ion **C1** in the spectrum of compound **4** (100%) is apparently higher than that in the spectrum of compound **1** (19%). Another reason is that for compound **1**, either producing ion **D** (via path a) or producing ion **C1** (path c) all needs to go over an activation barrier and then spontaneously transform to product ion. The energy barrier involved in path a ($\Delta G = 30.21$ kcal/M) is apparently lower than that in path c ($\Delta G = 55.51$ kcal/M). Therefore, it is not difficult to imagine that ion **A** of compound **1** is apt to produce ion **D** along path a, resulting in ion **D** as the base peak instead of ion **C1**, and ion **A** of compound **4** is apt to produce ion **C1** as the base peak along path c.

For the spectra of compounds **6** and **7**, the product ions **D** and **C1** are not observed in the ESI-MS/MS spectrum. Conversely, the product ions **B1** and **B2** are not formed in the spectra of compounds **1**–**5**. For compound **6**, the process from the deprotonated molecule **A** to form product ion **B1** (via path b, $\Delta G = 27.09$ kcal/M) is a thermodynamically more favored process in comparison with the formation of ion **D** (path a, $\Delta G = 44.11$ kcal/M) and **C1** (path c, $\Delta G = 40.38$ kcal/M). Moreover, the lost unsaturated substituent molecule (R–H), 3-methylene-indole, is very stable considering its special conjugative structure. Therefore, for compound **6**, cyclo(Trp-Trp), it is obvious that the formation of the product ion **B1** is relatively easier than that of the product ions **D** and **C1**. So in the ESI-MS/MS spectrum of compound **6**, the base peak is ion **B1** and the secondary intense peak is the ion **B2** formed by the elimination of another unsaturated substituent molecule. For compound **1**, however, the deprotonated form of ion **A** is expected to produce predominantly the product ion **D** (30.21 kcal/M) or **C1** (55.51 kcal/M) instead of **B1** (not observed, 114.29 kcal/M), as the former is a thermodynamically more favored process.

Conclusions

In summary, ESI-MS^n spectra of eight cyclic dipeptides in negative mode were investigated and the fragmentation pathways were elucidated. Under low-energy CID conditions, the deprotonated cyclic dipeptides tend to eliminate an unsaturated substituent molecule (R − H), a substituent radical, successively eliminate a CO and a saturated substituent molecule (R + H). Using (H/D) exchange experiments and combining with the high-resolution MS, it is first found that in negative-ion ESI mode, the cyclic dipeptides with alkyl substituents tend to eliminate their substituent radical to form radical ions **C1** and **C2**. At the same time, the cyclic dipeptides with aromatic substituents (Trp, Tyr) tend to eliminate the substituent molecule (R − H) to form ions **B1** and **B2**. Therefore, the characteristic side-chain losses can be used for the identification of the composing amino acids in the cyclic dipeptides. The results of theoretical calculations successfully demonstrated that their formation can be rationalized on the basis of the relative Gibbs free energies. These observations may have some potential applications in the structural elucidation and interpretation of mass spectra of homologous compounds.

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