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Electrospray ionization tandem mass spectrometry differentiation of *N*-phosphoryl-α-, β- and γ-amino acids

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Abstract

The fragmentation patterns of *N*-diisopropyloxyphosphoryl-L-α-Ala (DIPP-L-α-Ala), *N*-diisopropyloxyphosphoryl-D-α-Ala (DIPP-D-α-Ala), *N*-diisopropyloxyphosphoryl-β-Ala (DIPP-β-Ala) and *N*-diisopropyloxyphosphoryl-γ-amino butyric acid (DIPP-γ-Aba) were investigated by electrospray ionization tandem mass spectrometry (ESI-MS/MS). DIPP-D-α-Ala showed the same fragmentation pathways as DIPP-L-α-Ala. In the fragmentation of protonated DIPP-β-Ala, the characteristic fragment ion $[M+H-2C_3H_6-H_2O-CH_2CO]^+$ appeared and could be used to distinguish β-Ala from L-α-Ala and D-α-Ala through tandem mass spectra, even though they possess the same molecular weight. In the fragmentation of protonated DIPP-γ-Aba, the break of P–N bond occurred and an interesting protonated lactam ion with five-membered ring was generated. Furthermore, in the MS^3 spectrum of $[M+Na-2C_3H_6]^+$ ion of DIPP-γ-Aba, a strong intensity of unique fragment ion, namely lactam-sodium adduct with five-membered ring, was observed, which could be considered as a mark for γ-amino acids. The stepwise fragmentations of their $[M+Na]^+$ ions and $[M-H]^-$ ions showed that they all underwent a P–N to P–O bond migration through a five-membered or six-membered or even seven-membered ring transition state, respectively, which supported the great affinity of hydroxyl for phosphoryl group.

Keywords: *N*-phosphoryl amino acids; ESI-MS/MS; Characteristic fragment ions

1. Introduction

It is well known that all proteins, whether from the most ancient lines of bacteria or from the most complex forms of life, are constructed from the same ubiquitous set of 20 (L)-α-amino acids. In prebiotic chemistry there were many possible candidates for the building block of protein. A variety of compounds, such as α-amino acid and β-amino acid, were obtained in many experiments simulating the prebiotic earth [1]. Some studies about the reactivity differences among α-, β-, and γ-amino acids were reported previously. Rode and coworkers reported that in the salt-induced peptide formation reactions α-amino acids were more favorable than other kinds of amino acids [2]. Increasing work has been devoted to the study of the folding and organization of β-peptides (i.e., peptides composed of β-amino acids) which represents the smallest step away from α-amino acids in backbone space [3]. Jiang et al. have investigated the recognition of α-amino acids from β- and γ-amino acids by *N*-phosphorylation in peptide formation [4,5]. In addition, D-amino acids were recently found in various living higher organisms in the forms of free amino acids, peptides, and proteins [6].

It is worth noting that phosphorus plays an important role in the chemistry of life and has been selected by evolution for biochemical transformations [7,8]. The phosphorylation and dephosphorylation of proteins conduct the regulation of complex biochemical processes [9–11]. Phosphoryl amino acids are the smallest units of phosphoproteins, which have many interesting biomimic reactivities, such as ester exchange on phosphorus, esterification, N to O migration, and peptide formation [12–15]. When *N*-phosphoryl amino acids were incubated with nucleosides, peptides and nucleotides were simultaneously

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obtained [16,17]. Therefore, the hypothesis was proposed that *N*-phosphoryl amino acids might be regarded as the common seed for nucleic acids and prebiotic proteins [18].

Electrospray ionization mass spectrometry (ESI-MS) of the 20 diisopropylphosphoryl natural amino acids has been studied systematically [19,20]. In this paper, different kinds of *N*-phosphoryl amino acids, such as *N*-(*O*,*O*′-diisopropyl) phosphoryl-L-α-alanine (DIPP-L-α-Ala), *N*-(*O*,*O*′-diisopropyl) phosphoryl-D-α-alanine (DIPP-D-α-Ala), *N*-(*O*,*O*′-diisopropyl) phosphoryl-β-alanine (DIPP-β-Ala) and *N*-(*O*,*O*′-diisopropyl) phosphoryl-γ-amino butyric acid (DIPP-γ-Aba), were synthesized and investigated by electrospray ionization ion trap mass spectrometry (ESI-MS/MS) in positive and negative modes. Their fragmentation pathways were investigated in detail.

2. Experimental

All amino acids were purchased from Baitai Biochemical Co. (Shanghai, China). Each *N*-phosphoryl amino acid was prepared according to the published methods [21]. Each amino acid (0.1 mol) was dissolved in a mixture of 100 mL deionized water, 100 mL triethylamine, and 50 mL ethanol, and cooled in an ice-salt bath. Diisopropylphosphite (DIPPH, 0.1 mol) and 50 mL carbon tetrachloride were added to the mixture which was then stirred for 30 min. Further stirring for 20 min at room temperature was required. The mixture was extracted with diethyl ether and the aqueous phase was adjusted to pH 3–4 with 1N hydrochloric acid in an ice-salt bath, and then fully extracted with ethyl acetate (50 mL × 5). White solids or oily liquids (70–90%) were obtained after the evaporation of solvents. The solids were recrystallized from ethyl acetate and petroleum ether. However, the synthesis of DIPP-β-Ala and DIPP-γ-Aba was slightly different from that of DIPP-L-α-Ala and DIPP-D-α-Ala, that is, the reactant ratio (amino acid: DIPPH) was 1:1.5 and the mixture was adjusted to pH 1–2 after extraction. The yield of DIPP-β-Ala or DIPP-γ-Aba was about 80–90%.

Experiments were conducted on an esquire 3000 ESI-MS with an ion trap mass spectrometer in both positive and negative ionization modes (Bruker Daltonik Gmbh, Germany). The MS^n spectra were obtained by collision-induced dissociation (CID) with helium after isolation of the appropriate precursor ions. Ionization of analysis was carried out using the following setting of the ESI: nebulizer gas flow 7 psi, dry gas 4.5 L/min, dry temperature 300 °C, spray voltage 4000 V. Calibration of *m*/*z* was performed using a standard ESI-tuning-mixture. Scan range was 15–500 *m*/*z* and scan resolution was normal (13000 *m*/*z*/s). High resolution mass spectra were obtained with an ESI-Q-TOF-MS spectrometer (Micromass, England).

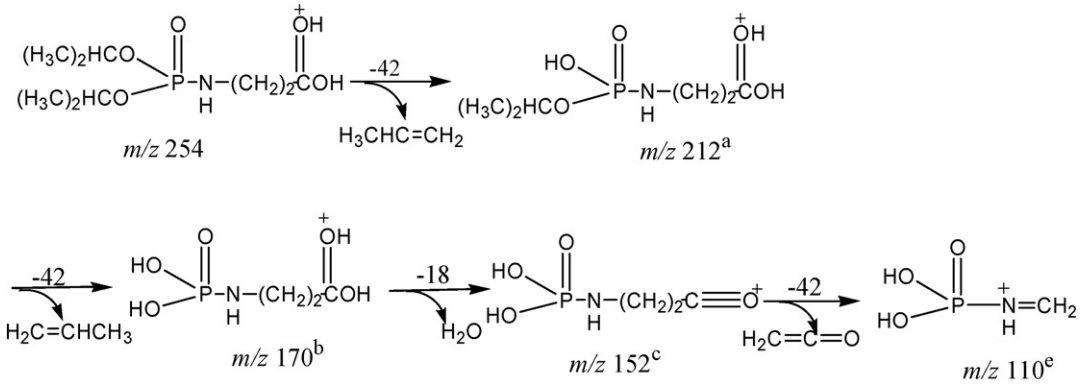
3. Results and discussion

3.1. Positive ion ESI-MS/MS spectra of DIPP-aa

The fragmentation patterns of $[M+H]^+$ ion of DIPP-D-α-Ala are same as those of DIPP-L-α-Ala and consistent with the literature results [19]. The main fragment ions derived from $[M+H]^+$ ions were also $[M+H-C_3H_6]^+$ and $[M+H-$

Table 1
Positive-ion ESI-MS/MS of protonated DIPP-aa [*m*/*z* (relative abundance, %), *m* = MW + 1]

Compounds	Precursor ions	Fragment ions							
		a (m-42)	b (m-84)	c (m-84-18)	d (m-84-18-28)	e (m-84-18-42)	f (m-18)	g (m-42-18)	h (m-84-18-80)
DIPP-L-α-Ala (MW = 253)	254	212 (69)	170(100)	152 (6)	124 (37)				
	212		170(100)		124 (3)				
	170			152 (20)	124 (100)				
	152				124 (100)				
DIPP-D-α-Ala (MW = 253)	254	212 (72)	170(100)	152 (7)	124 (38)				
	212		170(100)		124 (2)				
	170			152 (17)	124 (100)				
	152				124 (100)				
DIPP-β-Ala (MW = 253)	254	212 (88)	170(100)	152 (62)		110 (18)			
	212		170(100)	152 (40)		110 (5)			
	170			152 (100)		110 (13)			
	152					110 (100)			
DIPP-γ-Aba (MW = 267)	268	226 (92)	184(71)	**166 (100)**			**250**(5)	**208** (19)	**86** (31)
	226		184(73)	166 (100)				208 (17)	86 (28)
	184			166 (100)					86 (4)
	166								86 (100)
	250			166 (43)				208 (100)	86 (14)
	208			166 (100)					86 (10)



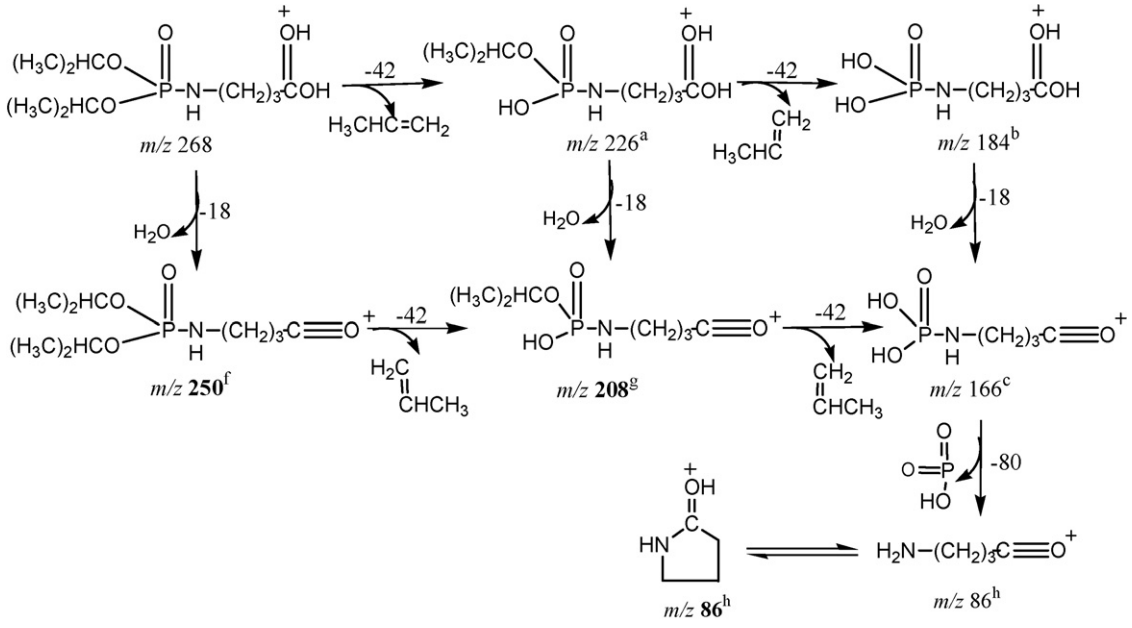
Scheme 1. Unique fragmentation pathways of protonated DIPP-β-Ala.

$2C_3H_6]^+$ ions (Table 1). The $[M+H-2C_3H_6-H_2O]^+$ and $[M+H-2C_3H_6-H_2O-CO]^+$ ions could also be formed by further dissociation. However, the $[M+H-2C_3H_6-H_2O-CH_2CO]^+$ ion at *m/z* 110 was observed only for DIPP-β-Ala, and its possible formation mechanism was proposed in Scheme 1. This ion could be used as a mark to distinguish DIPP-β-Ala from DIPP-L-α-Ala and DIPP-D-α-Ala even though they possess the same molecular weight. In terms of the fragmentation of DIPP-γ-Aba, the new fragment ions of f (*m/z* 250) and g (*m/z* 208) appeared in MS/MS spectrum (Table 1). While, $[M+H-2C_3H_6-H_2O]^+$ ion, namely c ion in Table 1, appeared in the MS/MS spectra for all kinds of DIPP-aa, but only for DIPP-γ-Aba it is the base peak. It is interesting to note that one water molecule could be eliminated from DIPP-γ-Aba before or after the elimination of one propylene molecule (Scheme 2). It could be due to the longer carbon chain leading to the preference of eliminating one water molecule from C-terminal. Further dissociation of ion c could yield a protonated lactam with five-membered ring, namely h ion in Table 1, as testified by high resolution mass spectrometer (Table 2). This fragmentation may be rationalized by the highly favorable structure of lactam with five-membered ring. Therefore, h ion at *m/z* 86 could be considered as the characteristic fragment ion of protonated DIPP-γ-Aba, which did not show up for the other DIPP-aa (Table 1).

Table 2
Experimental data by high resolution mass spectrometer

Fragment ions (*m/z*)	86	112	94	126	108
Experimental data	86.0941	112.0608	94.0545	126.0735	108.0753
Exact data by calculation	86.0606	112.0374	94.0269	126.0531	108.0425

It seems that the sodium adduct of DIPP-aa showed different fragmentation from the corresponding protonated DIPP-aa. The most significant point is that the MS/MS spectra of $[M+Na]^+$ ions all produced the characteristic fragment containing phosphoryl groups, i.e., the ions at *m/z* 163 and 121



Scheme 2. Unique fragmentation pathways of protonated DIPP-γ-Aba.

Table 3
Positive-ion ESI-MS/MS of $[M + Na]^+$ ions of DIPP-aa [*m/z* (relative abundance, %), *m* = MW + 23]

Compounds	Precursor ions	Fragment ions								
		a (m-42)	b (205)	c (m-84)	d (163)	e (121)	f (m-84-46)	g (m-84-80)	h (m-84-98)	i (m-84-18)
DIPP-L-α-Ala (MW = 253)	276	234 (26)	205(4)	192 (3)	**163** (**100**)	**121** (9)				
	234			192 (3)	163 (100)	121 (5)				
	205				163 (100)					
	192					121 (100)	146 (22)			
	163					121 (100)				
DIPP-D-α-Ala (MW = 253)	276	234 (33)	205(3)	192 (2)	**163** (**100**)	**121** (14)				
	234			192 (2)	163 (100)	121 (7)				
	205				163 (100)					
	192					121 (100)	146 (22)			
	163					121 (100)				
DIPP-β-Ala (MW = 253)	276	234 (66)		192 (11)	**163** (**100**)	**121** (22)		***112*** (4)	***94*** (4)	174 (5)
	234			192 (10)	163 (100)	121 (24)		112 (8)	94 (5)	174 (4)
	192					**121** (**100**)		***112*** (16)	***94*** (23)	**174** (**64**)
	163					121 (100)				
DIPP-γ-Aba (MW = 267)	290	248 (**100**)		206 (21)	**163** (**81**)	**121** (21)		***126*** (5)	***108*** (20)	188 (7)
	248			206 (10)	163 (100)	121 (19)		126 (4)	108 (27)	188 (7)
	206					121 (13)		***126*** (**59**)	***108*** (**100**)	
	163					121 (100)				

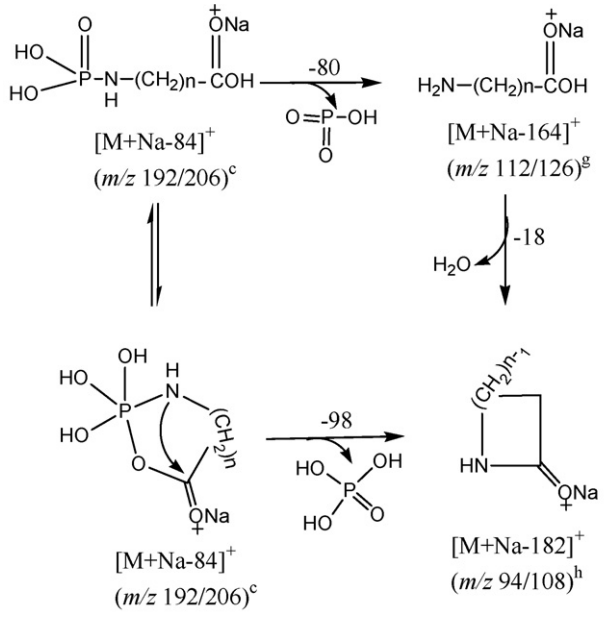
m/z 205: $[(i\text{-PrO})_2P(O)OH + Na]^+$, *m/z* 163: $[(i\text{-PrO})(HO)P(O)OH + Na]^+$, *m/z* 121: $[(HO)_2P(O)OH + Na]^+$.

corresponding to ion d $[(i\text{-PrO})(HO)P(O)OH + Na]^+$ and ion e $[(HO)_2P(O)OH + Na]^+$ (Table 3), respectively. It is implied that they all underwent a P–N to P–O bond migration through a five-membered ring, six-membered ring or seven-membered ring transition state, respectively, formed through the nucleophilic attack by the C-terminal carboxyl group on the phosphorus [22]. This phenomenon could be due to the strong affinity of the phosphoryl group for the hydroxyl group.

It is worth noting that the peak at *m/z* 163 was the base peak for DIPP-L/D-α-Ala or DIPP-β-Ala, but not for DIPP-γ-Aba. This phenomenon indirectly verified that the formation of the ion at *m/z* 163 might go through a cyclic transition state. Since seven-membered ring transition state was energetically less stable and its formation was a little more difficult, it was less favorable for the P–N to P–O bond migration. Hence, for DIPP-γ-Aba, the peak at *m/z* 163 was only 81% as the base peak.

The ions c at *m/z* 192 (Table 3) from DIPP-L-α-Ala and DIPP-D-α-Ala yielded the same daughter fragments, e and f ions. The base peak, namely e ion, was generated by the elimination of an amino acid residue, through a five-membered ring transition state formed by P–N to P–O bond migration [22]. In addition, for DIPP-β-Ala and DIPP-γ-Aba, c ion produced g ion and h ion, corresponding to relevant amino acid-sodium adducts and lactam-sodium adducts with four-membered or five-membered ring, respectively, by elimination of one $P(O)_2OH$ or one $(HO)_3P(O)$ molecule, as shown in Scheme 3. This fragmentation was illustrated in Fig. 1. The amino acid-sodium adducts and cyclic lactam-sodium adducts were also testified by high resolution mass spectrometer (Table 2).

In order to investigate the reason for the above different fragmentation pathways, the structures and energy of those sodium adducts were optimized and calculated with density functional theory at the B3LYP/6-31G** level with Gaussian 03 software at the Dell 6600 workstation. The results showed that the combinative location of Na^+ was carbonyl



n = 2: M = 253 for DIPP-β-Ala; n = 3: M = 267 for DIPP-γ-Aba.

Scheme 3. c, g, h ions from DIPP-β-Ala and DIPP-γ-Aba.

Table 4
Negative-ion ESI-MS/MS of DIPP-aa [*m*/*z* (relative abundance, %), *m* = MW − 1]

Compounds	Precursor ions	Fragment ions												
		a (m-60)	b (m-60-28)	c (m-60-42)	d (m-60-42-28)	e (m-42)	f (180)	g (138)	h (96)	i (79)	j (m-60-42-42)	k (m-18)	l (181)	m (139)
DIPP-L-α-Ala (MW = 253)	252	192 (100)	**164** (15)	150 (26)	122 (2)									
	192		164 (67)	150 (100)	122 (5)									
	164				122 (100)									
	150				122 (100)									
DIPP-D-α-Ala (MW = 253)	252	192 (100)	**164** (18)	150 (24)	122 (3)									
	192		164 (52)	150 (100)	122 (2)									
	164				122 (100)									
	150				122 (100)									
DIPP-β-Ala (MW = 253)	252	192 (100)		150 (58)		210 (26)	**180** (63)	**138** (81)	96(4)	79 (3)				
	192			150 (100)						79 (8)				
	150									79 (72)	**108**(**100**)			
	210			150 (100)				138 (14)						
	180							138 (100)						
	138								96(100)	79 (9)				
	96									79 (100)				
DIPP-γ-Aba (MW = 267)	266	206 (96)		164 (29)		224 (34)	**180** (63)	**138** (**100**)	96(7)	79 (34)		**248**(17)		
	206			164 (100)						79 (61)				
	164									79 (100)				
	224			164 (63)						79 (100)				
	180							138 (100)						
	138								96 (100)	79 (6)				
	96									79 (100)				
	248	206 (82)								79 (34)			**181**(100)	**139**(17)

m/*z* 180: $[(i\text{-PrO})_2P(NH)O]^-$, *m*/*z* 138: $[(i\text{-PrO})(HO)P(NH)O]^-$, *m*/*z* 96: $[(HO)_2P(NH)O]^-$, *m*/*z* 79: $[(O)_2PO]^-$, *m*/*z* 181: $[(i\text{-PrO})_2P(O)O]^-$, *m*/*z* 139: $[(i\text{-PrO})(HO)P(O)O]^-$.

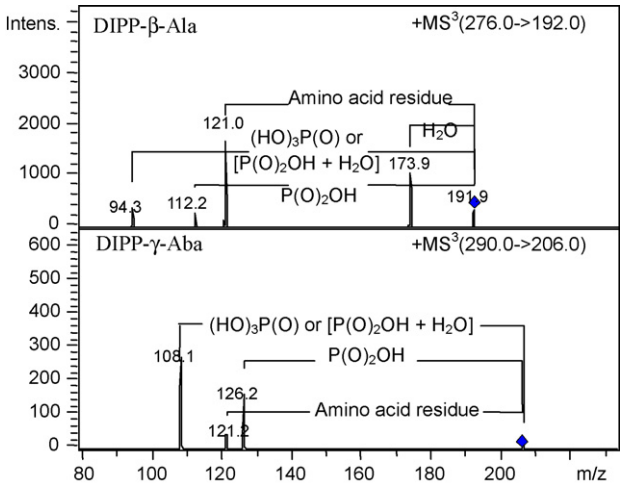


Fig. 1. MS^3 spectra of c ion from DIPP-β-Ala and DIPP-γ-Aba.

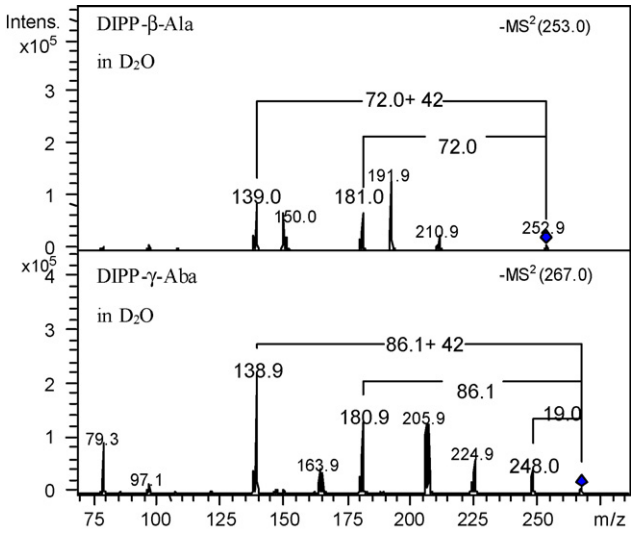


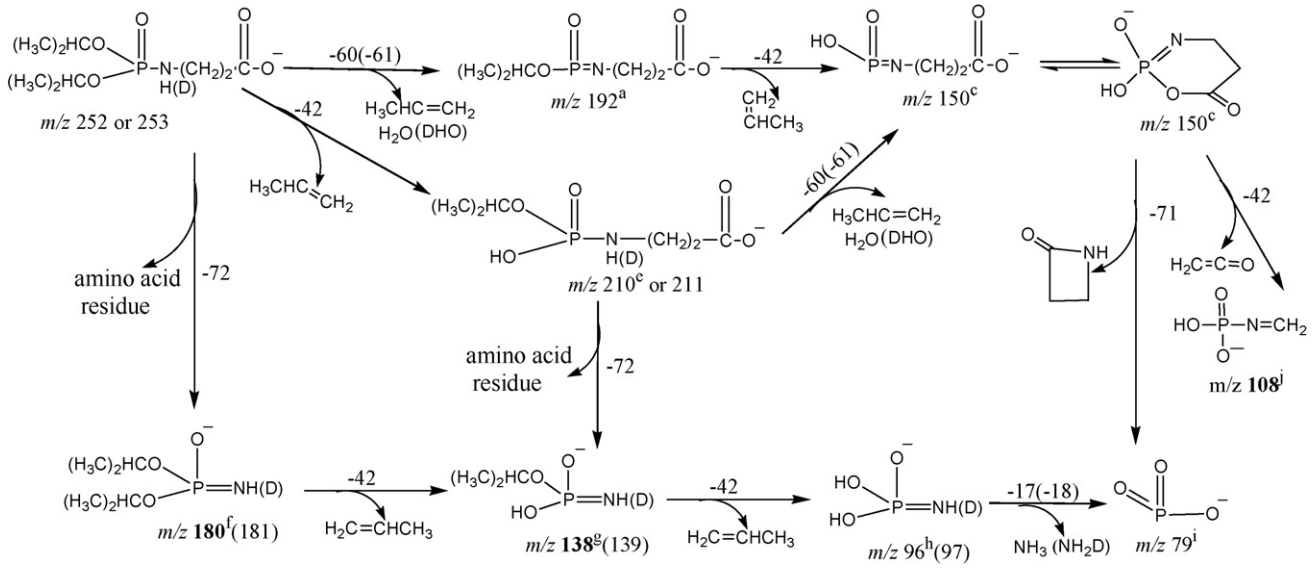
Fig. 2. MS^2 spectra of deuterated DIPP-β-Ala and DIPP-γ-Aba.

oxygen (Scheme 3). The binding energy of lactam with five-membered ring $(HO)_3P(O)$ and lactam with four-membered ring and sodium ion was 47.7 kJ/mol, 44.7 kJ/mol and 42.5kJ/mol, respectively. It can be concluded that lactam-sodium adduct with five-membered ring was most stable and $[(HO)_3P(O) + Na]^+$ ion was inferior. Moreover, according to the literature [4], seven-membered ring transition state was energetically less stable than six-membered ring transition state. Therefore, the stability of these sodium adducts and cyclic transition state are responsible for the different fragmentation pathways of different DIPP-aas.

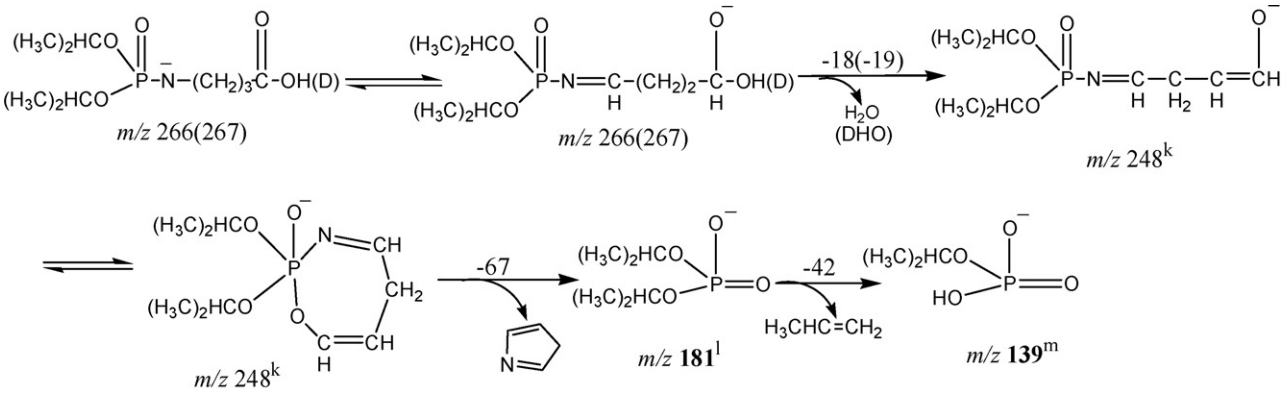
3.2. Negative ion ESI-MS/MS spectra of DIPP-aa

The fragmentations of $[M-H]^-$ ions of DIPP-L-α-Ala and DIPP-D-α-Ala were identical and consistent with the literature [19] which presented a five-membered ring transition state formed by the nucleophilic attack by the C-terminal carboxyl group on the phosphorus. The main fragment ions were possible due to an isopropyl alcohol or an isopropyl alcohol together with one propylene extrusion (Table 4), while, $[M-H-C_3H_6]^-$ and $[M-H-2C_3H_6]^-$ ions were not observed. However, the $[M-H-C_3H_6]^-$ ion, namely e ion in Table 4, appeared in the MS^2 spectra of deprotonated DIPP-β-Ala and DIPP-γ-Aba. The $[M-H\text{-}60\text{-}28]^-$ ion, namely b ion in Table 4, was observed only in the MS/MS spectra of DIPP-L-α-Ala and DIPP-D-α-Ala.

There were two characteristic fragments, f ion and g ion from the deprotonated DIPP-β-Ala and DIPP-γ-Aba, at *m/z* 180 and 138 (Table 4) observed, and the ion at *m/z* 138 is the base peak for deprotonated DIPP-γ-Aba. To prove the structure of these two ions, the active hydrogen atoms in DIPP-β-Ala and DIPP-γ-Aba were exchanged by deuterium atoms in D_2O. Then the fragments at *m/z* 180 and 138 were shifted to *m/z* 181 and 139 (Fig. 2), respectively, as expected (one active amino hydrogen



Scheme 4. Main fragmentation pathways of the $[M-H]^-$ ion of deuterated and non-deuterated DIPP-β-Ala.



Scheme 5. Formation and main fragmentation pathways of k ion (*m/z* 248) from DIPP-γ-Aba.

replaced by deuterium atom). Therefore, the ions at *m/z* 180 and 138 were reasonably identified as $(i\text{-PrO})_2P(NH)O^-$ and $(i\text{-PrO})(HO)P(NH)O^-$ containing phosphoryl group, respectively, and were formed by the elimination of corresponding amino acid residues (Scheme 4).

Since fragment ion $P(O)_2O^-$ (*m/z* 79) appeared in the MS^3 spectra of c ion (Table 4) from DIPP-β-Ala and DIPP-γ-Aba, it was assumed that the fragmentation underwent a six-membered (for DIPP-β-Ala, Scheme 4) or seven-membered (for DIPP-γ-Aba) ring transition state, then extrusion of one cyclic lactam. Furthermore, the base peak at *m/z* 108 (j ion in Table 4) came from the ketene CH_2CO loss from c ion (at *m/z* 150) of DIPP-β-Ala (Table 4) through six-membered ring transition state (Scheme 4).

In MS/MS spectrum of DIPP-γ-Aba, there was a novel k ion at *m/z* 248, generated by losing one water molecule from C-terminal, as supported by deuterium-labeling experiments (Fig. 2). In turn, the ion produced the characteristic ions at *m/z* 181 and 139, corresponding to $(i\text{-PrO})_2P(O)O^-$ and $(i\text{-PrO})(HO)P(O)O^-$, respectively, through a seven-membered ring transition state (Scheme 5) [19]. Therefore, the proposed seven-membered ring transition state was also testified by the further fragmentation of k ion at *m/z* 248.

4. Conclusions

In conclusion, the positive or negative ESI-MS/MS showed that DIPP-D-α-Ala gave the same fragmentation pathways as DIPP-L-α-Ala. The protonated DIPP-β-Ala had similar fragmentation patterns to DIPP-L-α-Ala. In addition, it gave characteristic fragment ion $[M+H-2C_3H_6-H_2O-CH_2CO]^+$ at *m/z* 110, which might be considered as a mark to distinguish β-Ala from L-α-Ala and D-α-Ala through MS^n spectra. In the fragmentation of protonated DIPP-γ-Aba, the break of P–N bond occurred and a protonated lactam ion with five-membered ring was generated. The dominant fragmentation pathway of $[M+Na-2C_3H_6]^+$ ion of DIPP-β-Ala was the elimination of one water molecular or amino acid residue, while that of DIPP-γ-Aba was the elimination of one $P(O)_2OH$ or one $(HO)_3P(O)$ molecule, generating an amino acid-sodium adduct or a lactam-sodium adduct with five-membered ring. The lactam-sodium adduct implicates the structural information of γ-amino acids, so it could be considered as a mark for the presence of γ-amino acids. The stepwise fragmentation of $[M+Na]^+$ ions and $[M-H]^-$ ions of DIPP-aa all underwent a five-membered, six-membered or seven-membered ring transition state leading to a P–N to P–O bond migration, which supported the great affinity of hydroxyl for phosphoryl group. The proposed fragmentation mechanisms of DIPP-aa prove that the multistage ESI-MS together with the D_2O experiment is a powerful tool to differentiate the structure of amino acids.

Acknowledgments

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