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Article

Elucidation of *O*-Phosphoryl and *N*-Phosphoryl Amino Acids by Electrospray Ionization Tandem Mass Spectrometry

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Mass spectroscopic characteristics of phosphoryl amino acids were studied in detail by positive and negative electrospray ionization mass spectrometry (ESI-MS) in conjunction with tandem mass spectrometry (MS/MS). Besides *N*-diisopropyloxyphosphoryl amino acids (*N*-DIPP-AA), *O*-phospho- and *O*-diisopropyloxyphosphoryl amino acids (*O*-DIPP-AA) were studied and compared to *N*-DIPP-AA. The fragmentation pathways of $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ ions of phosphoryl amino acids were summarized. In addition to several similar patterns, each of them showed its characteristic fragmention.

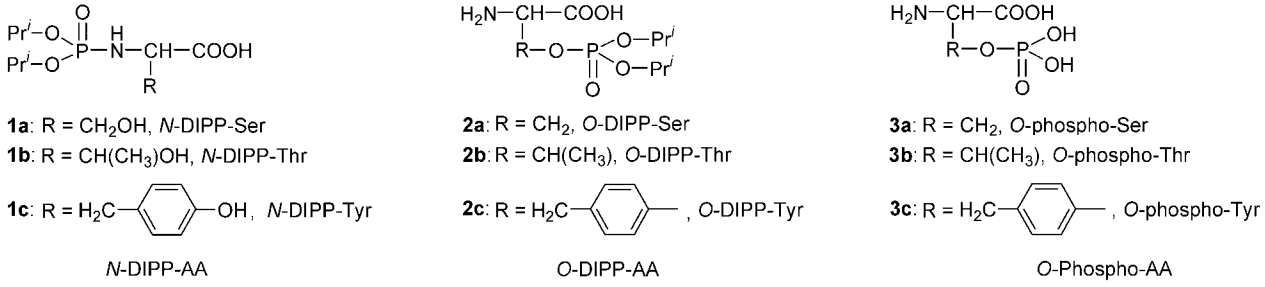
Keywords *N*-phosphoryl amino acid, *O*-phosphoryl amino acid, electrospray ionization tandem mass spectrometry

Introduction

N-Phosphoryl amino acids play a special and important role in biological systems.[1] Simultaneously, *O*-phosphorylation at the hydroxyl groups of serine, threonine and tyrosine residues often acts as a molecular switch controlling the protein activity in different pathways such as metabolism, signal transduction, cell division, *etc*.[2,3] It is well known that phosphorylation of proteins is a key biological reaction. Therefore, identification of phosphoryl group in protein is an important task in protein analysis. However, proteins are too large to be studied directly by normal methods of structural analysis. Phosphoryl amino acids are the smallest units of phosphoproteins. At the same time, synthesis of peptide of dialkyl phosphoryl as protecting group has been developed in recent years, since it is easier to de-protect and cheaper than Boc. *etc*.[4,5]

In our previous work, phosphorylated amino acids and peptides were analyzed using several kinds of mass spectrometers.[6-8] There are some regular cleavage patterns that have been reported for *N*-diisopropyloxyphosphoryl amino acids (*N*-DIPP-AA) in positive and negative modes.[9,10] However, some important *O*-phosphoryl amino acids have not been studied and compared to *N*-DIPP-AA by ESI-MS in detail. In the present article, *N*-DIPP-, *O*-DIPP- and *O*-phospho amino acids (serine, threonine and tyrosine) (Scheme 1) were analyzed by ESI-MS, which is often regarded as a soft ionization technique for the structure elucidation of involatile and thermally labile compounds, especially biological materials.[11,12] Fragmentation patterns of $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ ions of phosphoryl amino acids were compared to each other. It was found that *N*-DIPP-AA and *O*-DIPP-AA could be distinguished by their ESI-MS characteristics. Furthermore, the ion at *m/z* 181 or 97 in negative mode could be a mark for

Scheme 1 Structures of phosphoryl amino acids



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identification of phosphoryl amino acids.

Experimental

Instruments

Mass spectra were recorded on a Bruker Esquire 3000 ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ion source. Samples were typically dissolved in methanol or water at concentration of about 10^{-5} mol/L and were introduced into the electrospray needle by a mechanical infusion through a Cole-Parmer 74900 syringe pump (Cole-Parmer Instrument Company) at a flow rate of 4 μL/min. The ESI source potentials were capillary 4.0 kV, lens 15.0 V, lens 260.0 V and capillary exit offset is at 75.9 V. The mass spectrometer was scanned at the rate of 300 mass units per second. At least ten scans were averaged to obtain each spectrum. Nitrogen as nebulizer gas with a flow rate of 4 L/min (nebulizer pressure of 48.23 kPa) at 300 ℃ was used. MS^n spectra were obtained by low-energy CID with helium after isolation of the appropriate precursor ions. The collision conditions were maintained at 0.60—1.20 V (fragmentation voltage amplitude) and 40 ms of fragmentation time. Data acquisition and processing were carried out using Data analysis 5.0 software supplied with the instrument. Calibration was carried out using the tuning solution.

Reagents

O-Phosphoryl amino acids and *N*-phosphoryl amino acids were synthesized according to the method[13-15] respectively, and were characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS. Chemicals for synthesis were purchased from BaiTai Co., Ltd. HPLC grade methanol was used to dissolve the samples.

Results and discussion

ESI-MS/MS spectra of positive ions of *N*-DIPP-AA, *O*-DIPP-AA and *O*-phospho-AA

There were two types of ions for *N*-DIPP-AA in positive ESI-MS/MS mode: one was $[M+H]^+$ ion and the other was $[M+Na]^+$ ion. The main fragment ions derived from $[M+H]^+$ ions were $[M+H-C_3H_6]^+$ and $[M+H-2C_3H_6]^+$ ions (Table 1). The $[M+H-2C_3H_6-H_2O]^+$ and $[M+H-2C_3H_6-HCOOH]^+$ ions could be formed by the further dissociation. For most of *N*-DIPP-AA the base peak was formed by elimination of one propylene molecule.

In MS/MS spectra of $[M+Na]^+$ ions of N-DIPP-AA, there were similar fragment ions such as $[M+Na-C_3H_6]^+$, $[M+Na-2C_3H_6]^+$ as compared to the fragmentation of $[M+H]^+$ ions. However, the types of $[M+Na-2C_3H_6-H_2O]^+$ and $[M+Na-2C_3H_6-HCOOH]^+$ ions were not observed. Some abundant characteristic fragment ions containing phosphoryl groups appeared, for example, the ions at *m/z* 205, 163 and 121 corresponding to $[(i\text{-PrO})_2P(O)OH+Na]^+$, $[(i\text{-PrO})(HO)P(O)OH+Na]^+$ and $[(HO)_2P(O)OH+Na]^+$ ions respectively. Strangely, for most of N-DIPP-AA, the base peak of $[M+Na]^+$ MS/MS spectra was neither the $[M+Na-C_3H_6]^+$ ion nor the $[(i\text{-PrO})_2P(O)OH+Na]^+$ ion but it was the ion at *m/z* 163, corresponding to $[(i\text{-PrO})(HO)P(O)OH+Na]^+$. It has been reported that the phosphoryl group has a strong affinity for the hydroxyl group, which might be related to the active role of phosphorus in living systems. The base peak *m/z* 163 ion corresponding to cationized phosphoric acid isopropyl ester was the result of the rearrangement reaction leading to the P—N to P—O bond migration.[8]

Two types of ions of *O*-phosphoryl amino acids were studied, one was $[O\text{-DIPP-AA}+H]^+$ ion and the other was $[O\text{-phospho-AA}+H]^+$ ion. The main fragment ions derived from $[O\text{-DIPP-AA}+H]^+$ ions were $[O\text{-DIPP-AA}+H-C_3H_6]^+$ and $[O\text{-DIPP-AA}+H-2C_3H_6]^+$ ions (Table 1). The base peak of *O*-DIPP-AA was formed by elimination of one propylene molecule. Comparing positive MS/MS spectra of *O*-phosphoryl amino acids with those of *N*-phosphoryl amino acids, they have some similar dissociation shown in Scheme 2. The main fragment ions derived from $[O\text{-phospho-AA}+H]^+$ ions were $[O\text{-phospho-AA}+H-H_2O]^+$ and $[O\text{-phospho-AA}+H-HCOOH]^+$ ions similar to those of *N*-DIPP-AA.

Table 1 ESI-MS/MS positive-ion data for phosphoryl amino acids [*m/z* (relative abundance, %)]

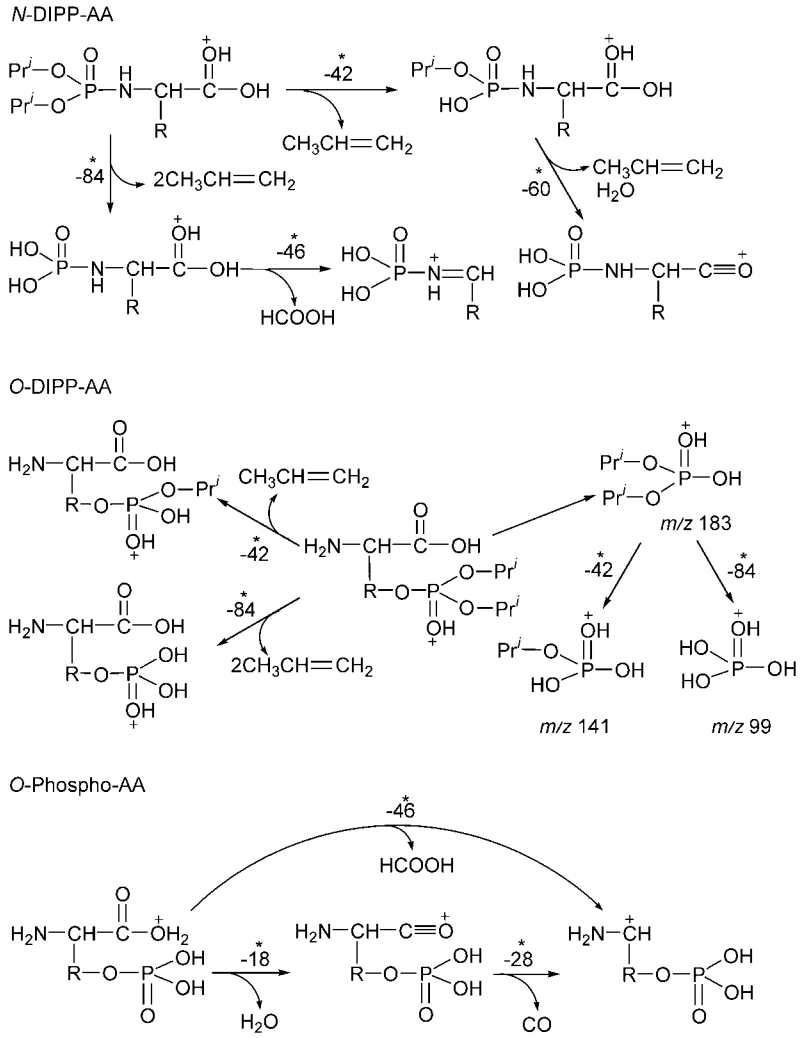
Compd	Precursor ion $[M+H]^+$	Fragment ion
1a	270 (70)	228 (100), 186 (69), **183 (4)**
1b	284 (22)	242 (100), 200 (66), **183 (11)**
1c	346(39)	304 (100), 262 (72), 216 (20)
2a	270 (52)	228 (100), 186 (38), **183 (47)**, 141(16), 130 (8), 99 (13)
2b	284 (60)	242 (100), 200 (77), **183 (40)**, 154 (5), 141 (8), 102 (14)
2c	346 (54)	304 (100), 262 (81), 216 (20)
3a	186 (92)	88 (100), 70 (5)
3b	200 (23)	182 (40), 154 (100), 136 (5), 102(5)
3c	262 (53)	244 (3), 216 (100)

For most of *N*-DIPP-AA, there are few of ions at *m/z* 183 because the rearrangement is difficult to form ion at *m/z* 183 in the positive mode. However, in the MS/MS spectra of $[O\text{-DIPP-Ser}+H]^+$ and $[O\text{-DIPP-Thr}+H]^+$, there are distinct ions at *m/z* 183. Figure 1 shows positive spectra of *N*-DIPP-Ser in contrast to *O*-DIPP-Ser. So, some of *N*-DIPP-AA and *O*-DIPP-AA may be distinguished by the relative intensity of the peak at *m/z* 183.

ESI-MS/MS spectra of $[M-H]^-$ ions of *N*-DIPP-AA, *O*-DIPP-AA and *O*-phospho-AA

Unexpectedly, in negative mode the $[M-H-C_3H_6]^-$, $[M-H-2C_3H_6]^-$ ions were not observed for *N*-DIPP-

Scheme 2 Fragmentation pathways of protonated phosphoryl amino acids (*: data from MS/MS)



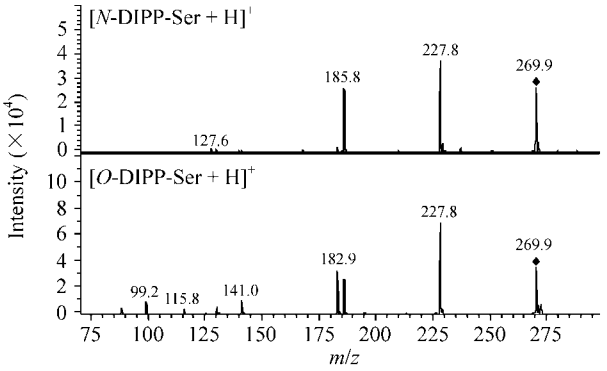


Figure 1 MS/MS spectra of protonated *N*-DIPP-Ser and *O*-DIPP-Ser.

AA. The main fragmentation pattern of $[M-H]^-$ ions was the $[M-H-C_3H_6-H_2O]^-$ and $[M-H-2C_3H_6-H_2O]^-$ ions. In addition, two characteristic fragment ions containing phosphoryl groups appeared, the ions at *m/z* 181 and 139 corresponding to $(i\text{-PrO})_2P(O)O^-$ and $(i\text{-PrO})(HO)P(O)O^-$, respectively (Table 2). For negative-ion ESI-MS/MS, it has been reported that a unique fragmentation from the *N*-DIPP- AA containing a free *β*-OH or -COOH group was observed to yield the characteristic fragment ion $(RO)_2P(O)O^-$ (*m/z* 181).[9]

For negative ESI-MS/MS of *O*-phosphoryl amino acids, the main fragmentation pattern of $[M-H]^-$ ions was the ion at *m/z* 181, *m/z* 97 or *m/z* 79 corresponding to $(i\text{-PrO})_2P(O)O^-$, $(HO)_2P(O)O^-$ or PO_3^-. Simultaneously, there are fragmentations that were formed by elimination of one water molecule from *O*-phospho-AA or one propylene molecule from *O*-DIPP-AA (Table 2, Scheme 3). The MS/MS spectra of *O*-DIPP-Thr and *O*-phospho-Thr in negative mode are shown in Figure 2. From Figure 2, it can be obtained that the ions at *m/z* 181 and *m/z* 97 or *m/z* 79 can be a mark for DIPP-AA or phospho-AA similarly. However, for tyrosine the existence of phenyl group leads to the absence of the fragment ions at *m/z* 181 or 97, for the bond between the hydroxyl oxygen atom and phenyl group is not easy to dissociate. This is also the reason why the $[O\text{-DIPPAA}+H]^+$ ion can not produce the fragment ion at *m/z* 183.

Conclusions

Positive and negative ESI-MS/MS spectra of *O*-phosphoryl amino acids and *N*-phosphoryl amino

Scheme 3 Fragmentation pathways of deprotonated phosphoryl amino acids (*: data from MS/MS)

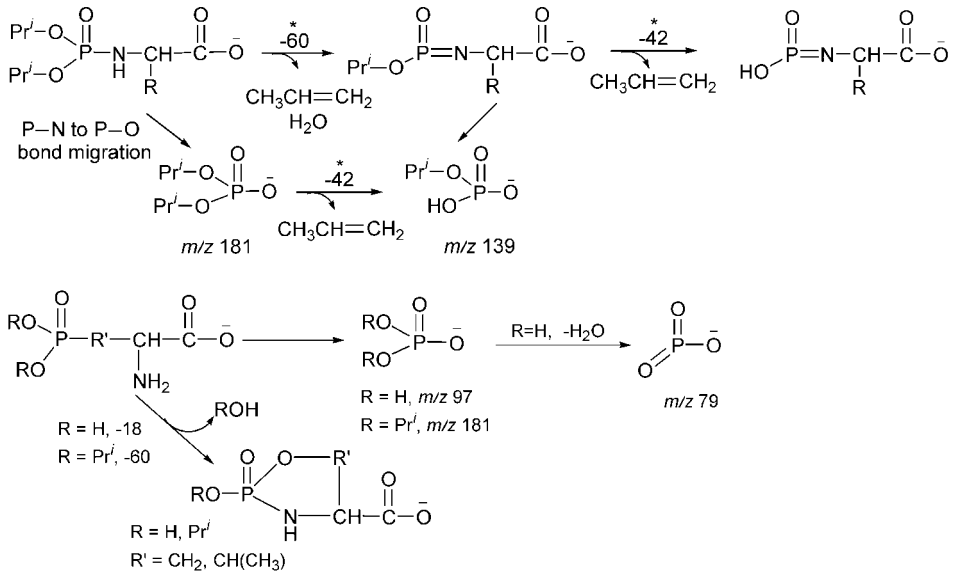


Table 2 ESI-MS/MS negative-ion data for phosphoryl amino acids [*m/z* (relative abundance, %)]

Compd.	Precursor ion $[M-H]^-$	Fragment ion
1a	268 (34)	208 (7), **181** (100), 139 (6), 178 (5)
1b	282 (35)	**181** (100), 139 (5), 238 (6), 220 (3), 178 (9)
1c	344 (71)	284 (100), 256 (11), 242 (7), 214 (7), 164 (8)
2a	268 (70)	**181** (100), 139 (2)
2b	282 (76)	222 (3), **181** (100), 139 (3)
2c	344 (26)	284 (100), 256 (7)
3a	184 (67)	166 (8), **97** (100), **79** (7)
3b	198 (49)	180 (32), **97** (100), **79** (14)
3c	260 (35)	242 (3), **79** (100)

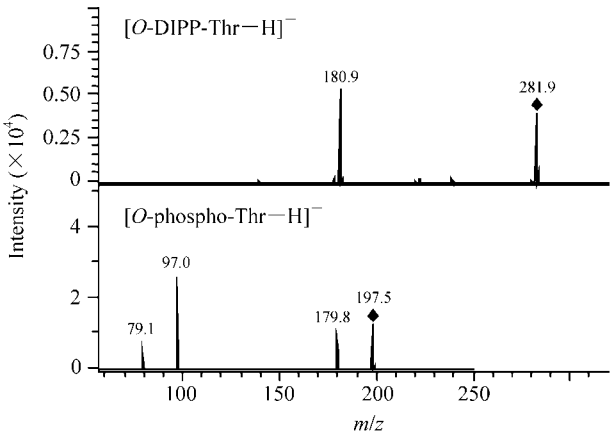


Figure 2 MS/MS spectra of deprotonated *N*-DIPP-Thr and *O*-phospho-Thr.

acids have been investigated and rationalized. Fragmentation patterns of $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ ions of phosphoryl amino acids were compared to each other. It was also found that *N*-DIPP-AA and *O*-DIPP-AA could be distinguished by their ESI-MS characteristics. The ions at *m/z* 181 and *m/z* 97 or *m/z* 79 in negative mode were proposed to be a mark for identification of phosphoryl amino acids. The observation may have some potential applications in the interpretation of the fragment ion spectra of phosphoryl compounds.

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