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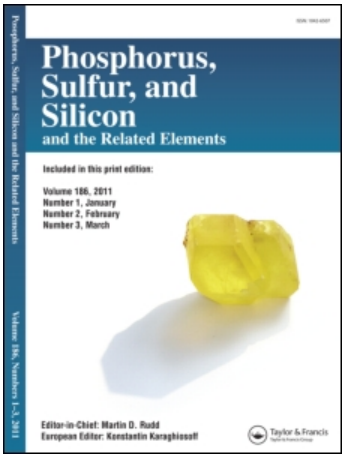

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Preliminary ESI-MS and MALDI-TOF Analysis on Phosphorylated Tetrapeptides with Xaa-Pro Motif

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To cite this Article Zhu, Zhen-Tai , Li, Yan-Mei , Guo, Yan-Ting , Sun, Ming and Zhao, Yu-Fen(2007) 'Preliminary ESI-MS and MALDI-TOF Analysis on Phosphorylated Tetrapeptides with Xaa-Pro Motif', Phosphorus, Sulfur, and Silicon and the Related Elements, 182: 4, 825 — 834
To link to this Article: DOI: 10.1080/10426500601059482
URL: http://dx.doi.org/10.1080/10426500601059482

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Phosphorus, Sulfur, and Silicon, 182:825–834, 2007
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ISSN: 1042-6507 print / 1563-5325 online
DOI: 10.1080/10426500601059482

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To investigate the side-chain effects of O-phosphorylation on the peptidyl-prolyl amide bond cis/trans *isomerization, a series of model phosphopeptides with Xaa-Pro motif were designed and synthesized. Electrospray Ionization Mass Spectrometry (ESI-MS) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) experiments were performed to study the differences between these two methods in their fragmentation behaviors on the phosphopeptides. It was found that the loss of fragment at m/z 98 was observed not only in the ESI-MS spectra but also in the MALDI spectra, which was due to the loss of phosphate derivatives. The loss of small molecules such as ammonia and water was easier in ESI-MS spectrum. Moreover, the peptidyl-prolyl amide bonds (Xaa-Pro) in the phosphopeptides were prone to cleave in both ESI-MS and MALDI-TOF-MS spectra. The results presented here indicated that ESI-MS was more suitable for small molecule analysis while MALDI-MS was more effective in detection of peptides backbone.*

Keywords Assisted laser desorption/ionization time-of-flight mass spectrometry; electrosprey ionization mass spectrometry; matrix; phosphopeptide; Xaa-Pro motif

Received February 15, 2006; accepted September 11, 2006.

The authors thank financial support from the National Natural Science Foundation of China (No. 20472041, 20532020, NSFCBIC20320130046), the Teaching and Research Award Program for Outstanding Young Teachers in Higher Education Institutions of Ministry of Education, People's Republic of China (TRAPOYT), and the Specialized research Fund for the Doctoral Program of Higher Education (SRFDP) (No. 20030003049).

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INTRODUCTION

Mass spectrometry (MS) has become a powerful tool for the identification of peptides and proteins. It can be used to measure the molecular weight of a full-length polypeptide, determine the characteristics of peptide, and predict the structure of peptide. Furthermore, in 1991 Ganem et al.[1,2] reported that a non-covalent complex could be studied by Electrospray Ionization Mass Spectrometry (ESI-MS), and in 1997 Loo[3] wrote an extensive review on this subject. Since then, the structure analysis of proteins and peptides by ESI-MS has progressed rapidly as an alternative with high efficiency.[4] Especially ESI-MS has been used for mixture analysis and peptides sequence determination or posttranslational modification sites in peptides and proteins.[5] Similarly, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) with great improvements in resolving power, sensitivity, and versatility was introduced by Karas and Hillemkamp;[6] this made the analytical methodology highly competitive.[7–9] The recent combination of these two methods in probing phosphopeptides, especially in detecting the phosphorylation site, has been widely used in peptide and protein structure analysis.

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Phosphorylation is one of the well-known posttranslational modifications on proteins. It is probably the most common and important reversible intracellular event related to signal transduction.[10] Understanding the protein function regulated by phosphorylation/dephosphorylation is very important to many fields of biomedicine, such as cell cycle regulation, enzyme activation/de-activation, and protein-protein interaction. Side-chain phosphorylation normally occurs at the Xaa-Pro motif (Xaa = Ser/Thr/Tyr) of proteins and peptides, which affects the peptidyl-prolyl amide bond *cis*/*trans* isomerization and introduces a molecular switch into the peptide backbone to control the cellular biochemical processes (Figure 1). In order to study the effect of phosphorylation on the fragmentation of peptides, many phosphorylated amino acids and peptides have been analyzed by using different kinds of methods.[11–15]

In this work, model compounds, Ac-Ala-Xaa-Pro-Lys-NH-Np, were designed based on the Fmoc-strategy,[16] and a series of phosphorylated/nonphosphorylated tetrapeptides were subsequently synthesized with the solid-phase method. All model compounds were analyzed by both ESI-MS and MALDI-TOF-MS. Differences in the rule of fragmentation between these two methods were also discussed. It was found that the ESI-MS was more suitable for small molecule analysis, while MALDI-TOF-MS was more effective in backbone detection of peptides.

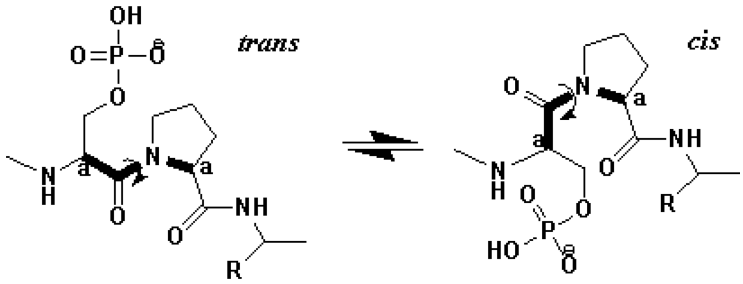

FIGURE 1 Peptidyl-prolyl conformation of *cis*/*trans* isomers.

RESULTS AND DISCUSSION

ESI-MS Study of Phosphopeptides

Nine phosphorylated tetrapeptides were analyzed using ESI-MS, and the results are listed in Table I. All phosphorylated peptides lose a common moiety of m/z 98 and 80 in ion trap mass spectrometry.[17] The peptides that contain phosphoserine and phosphothroenine residues generally lose phosphoric acid (m/z 98) and phosphoric ester derivatives through a β-elimination reaction. The peptides that contain tyrosine residue could not undergo the similar β-elimination reaction because of the bulky benzene group. In general, they lose a neutral fragment of m/z 98. For example, Ac-Ala-Thr($PO(OH)_2$)-Pro-Lys-NH-Np showed peaks at m/z 560.3 and 578, which correspond to ions $[M+H\text{-}H_3PO_4]^+$ and $[M+H\text{-}HPO_3]^+$ (Figure 2). On the other hand, the backbone

TABLE I Phosphopeptides Ac-Ala-Xaa-Pro-Lys-NH-Np for Mass Detection

Entity	Xaa	Molecular Weight (Da)	Precursor ion	
			ESI$(M+H)^+$	MALDI$(M+H)^+$
1	Ser($PO(OH)_2$)	643.60	644.3	644.1
2	Ser(PO(OH)(OMe))	657.62	658.5	658.3
3	Ser($PO(OMe)_2$)	671.65	672.4	672.3
4	Thr($PO(OH)_2$)	657.62	658.3	658.3
5	Thr(PO(OH)(OMe))	671.65	672.1	672.3
6	Thr($PO(OMe)_2$)	685.67	686.1	686.3
7	Tyr($PO(OH)_2$)	719.70	720	720.2
8	Tyr(PO(OH)(OMe))	733.73	734.2	734.2
9	Tyr($PO(OMe)_2$)	747.75	748.1	748.3

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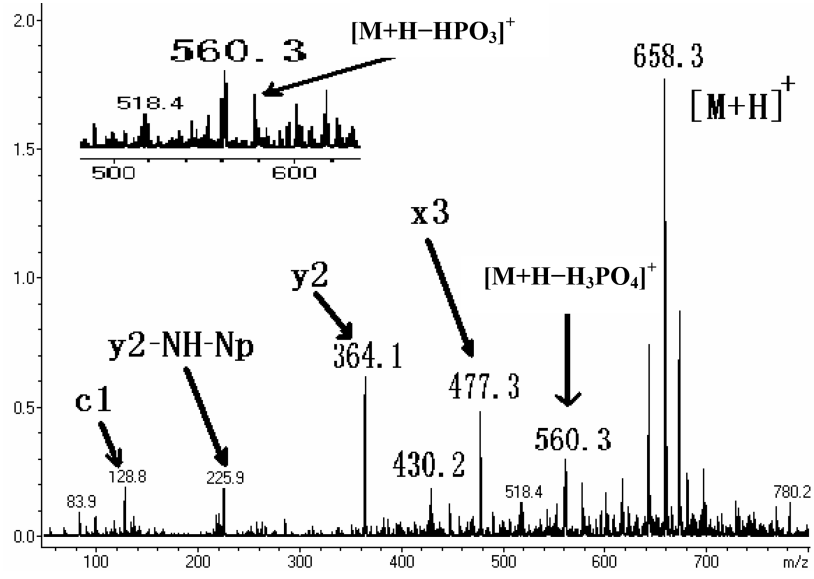

FIGURE 2 ESI-MS spectrum of peptide Ac-Ala-Thr(PO(OH)$_2$)-Pro-Lys-NH-Np.

fragments could also be detected. According to the definition of Siuzkak,[18] the majority of backbone fragments was a *y*-type fragment (Figure 3). For example, peaks of m/z 225.9 and m/z 364.1 were attributed to the signals of *y*-type fragments.

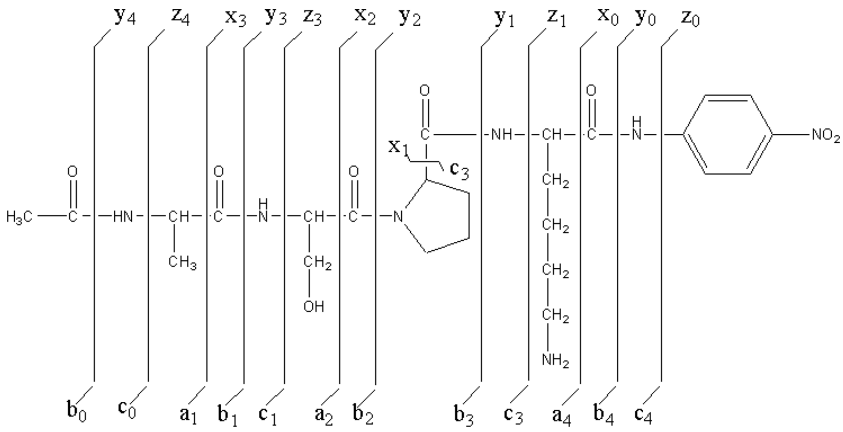

FIGURE 3 Potential backbone fragmentation of peptide Ac-Xaa-Ser-Pro-Lys-NH-Np.

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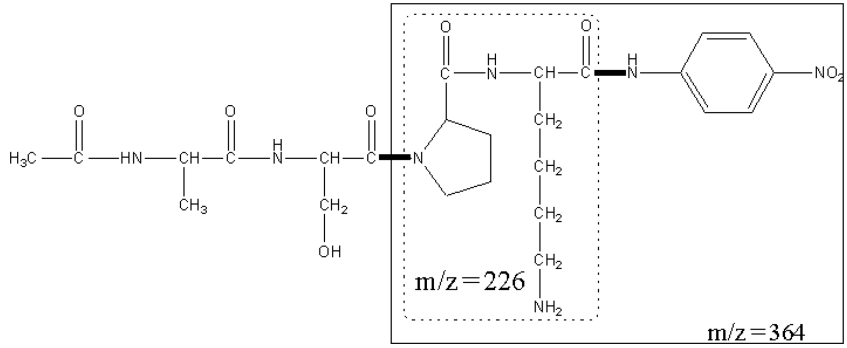

FIGURE 4 Two positions of backbone of peptides that are broken and the fragment ions.

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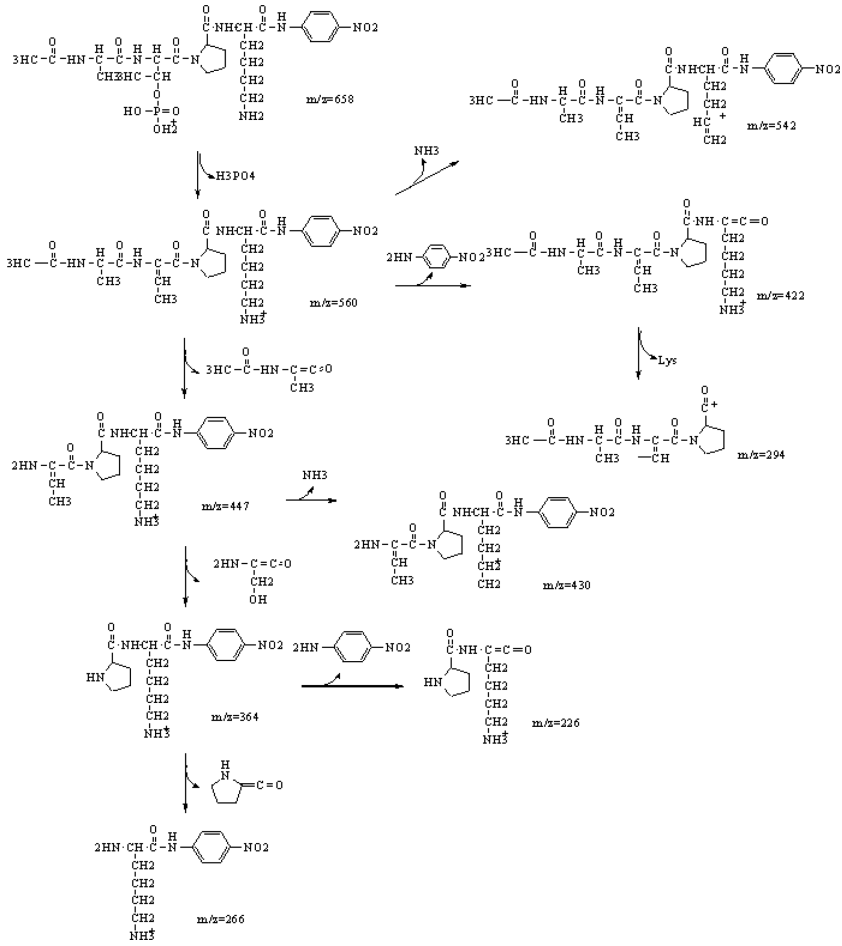
It was found that the loss of a small molecule, such as ammonia (17Da) and water (18Da), is easier in the phosphorylated peptide. As shown in Figure 2, an ammonia molecule cleaved from the backbone fragments at m/z 447, and 560 leds to fragments at m/z 430.2 and 542, respectively.

At last, there are common fragments at m/z 364 and 226 in the mass spectra of almost every compound, as illustrated in Figure 4. This result indicated that the peptidyl-prolyl amide bond between the proline and the residue preceding proline (denoted in the dash box) was prone to cleave in ion trap mass spectrometer. Another position easy to be cleaved was the bond (denoted in the bold box) between C-terminal of Lys and 4-nitroanilide group (NH-Np). Considering all the fragmentation rules deduced from the mass data, as an example, the fragmentation pathway of Ac-Ala-Thr($PO(OH)_2$)-Pro-Lys-NH-Np was proposed, as illustrated in Scheme 1.

MALDI-MS Study of Phosphopeptides

As we know, MALDI is usually used to analyze the structure of larger proteins and peptides. Generally, the lowest molecular weight of the peptide tested by MALDI is 700–800 Da. However, we obtained the data of these phosphopeptides from the MALDI analysis with lower molecular weight.

As depicted in Figure 5, the MALDI spectra were much more complicated than the ESI spectra because of the large number of signals. The loss of phosphoric acid or phosphoric esters was the main fragmentation pattern for the peptides with phosphorylation on serine and threonine.



SCHEME 1 Possible fragmentation mechanism in ESI-MS for peptide Ac-Ala-Thr(PO(OH)$_2$)-Pro-Lys-NH-Np.

Except for the fragments of lost phosphoric acid or phosphoric ester, the backbone fragments also take a large part of the spectra and others are the backbone fragment ions losing little molecule, such as ammonia and water. Generally there are peaks of *b* and *y* fragment ions in Post Source Decay (PSD) spectrum (Figure 6). In MALDI spectrum, almost all types of fragment ions could be found. To vary the degree, PSD fragmentations of phosphorylated peptides were similar to those of its ESI fragmentation, but there are still some differences. In ESI spectrum, *y*-type fragment ions take a large proportion, and only little *b*-type

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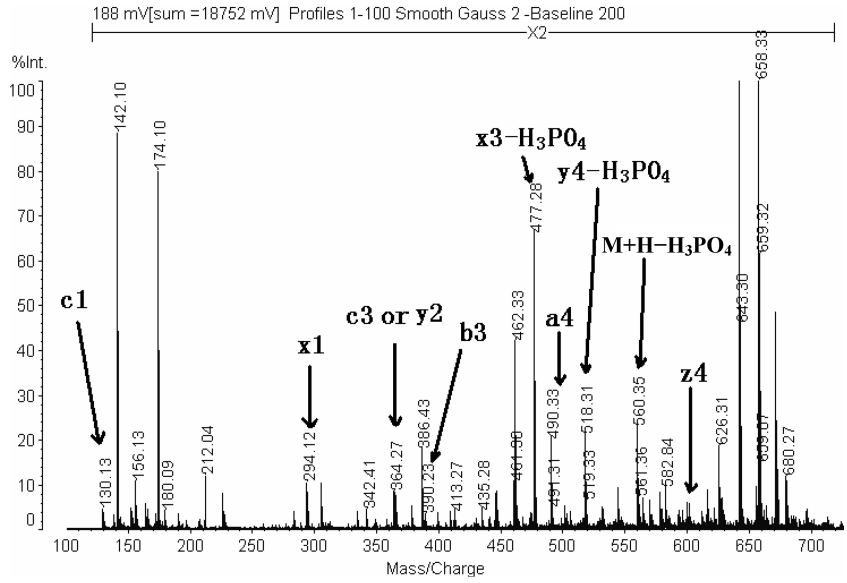

FIGURE 5 MALDI-TOF-MS spectrum of peptide Ac-Ala-Thr(PO(OH)$_2$)-Pro-Lys-NH-Np.

fragment ions are observed. But in PSD spectrum, there are more *b*-type fragment ions, and not all of the *y*-type fragment ions can be found. Hence, *b*-type fragmentation is easier to occur in PSD spectrum than in ESI spectrum.

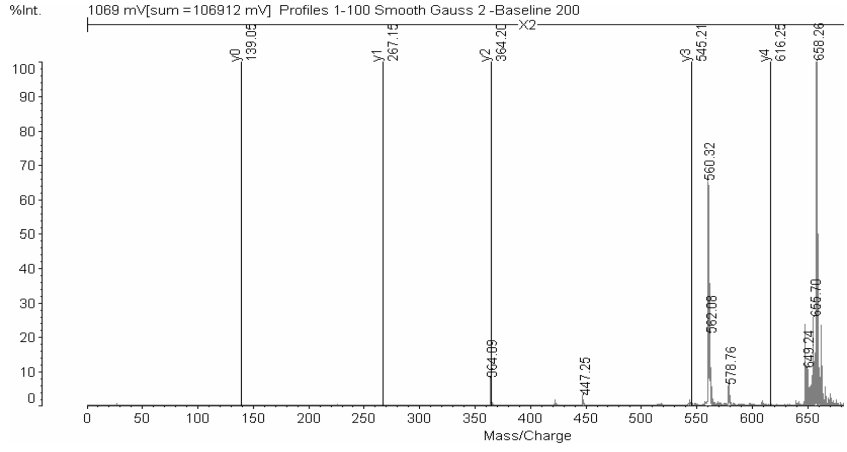

FIGURE 6 PSD spectrum of peptide Ac-Ala-Thr(PO(OH)$_2$)-Pro-Lys-NH-Np.

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Comparison between MALDI and ESI Spectra of these Phosphopeptides

From the MALDI-TOF and ESI mass spectra, several points could be summarized about the advantages and disadvantages of these model peptides, which will help us to select a better way to analyze the properties of the short peptides.

There are peaks of $[M+H]^+$ and $[M+Na]^+$ in the MALDI-MS spectra, while they are invisible in ESI-MS. Besides, there are only peaks of $[M+H]^+$ in the ESI-MS spectra, which means it is easier to determine the molecular weight. The second difference is the fragments of molecular ions losing water or phosphoric acid. These kind of fragments are more significant in the ESI-MS spectra, and they are the main sort of fragment ions in its MS/MS tandem mass spectrometry. Moreover, the peaks of molecular ions fragments in the MALDI-MS spectrum caused by losing water or phosphoric acid are not as clear as those in the ESI-MS one. In the PSD spectrum, these fragment ions are the main part but not as much as those in ESI-MS one, either. Third, the backbone fragments in the ESI-MS spectrum are mainly *y* type, and almost all of the *y* fragment ions can be found. Therefore it was convenient to analyze the small peptide structure and mapping phosphorylation sites. In the MALDI-MS spectrum, there are multiple kinds of fragment signals, which makes the spectrum more complicated. Because of the simpler backbone fragments, PSD is a good tool and will provide more help to analyze the structure of large molecules if appropriate power is executed.

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CONCLUSION

Preliminary ESI-MS and MALDI-TOF analysis on phosphorylated tetrapeptides with Xaa-Pro motif were studied in this work. It was found that the loss of fragment at m/z 98 was observed not only in the ESI-MS spectra but also in the MALDI spectra, which is due to the loss of phosphate derivatives. The loss of small molecules such as ammonia and water was easier in ESI-MS spectrum. The peptidyl-prolyl amide bond in a peptide-containing Xaa-Pro motif was easier to be broken. Moreover, all mass data presented here indicated that ESI-MS was more suitable for small molecule analysis, while MALDI-MS was more effective in detection of peptides backbone.

EXPERIMENTAL

Peptide Synthesis and Purification

The peptides were synthesized with the global phosphorylation method by using solid-phase Fmoc-strategy.[16] Added amino acids

(1.5 mmol) were pre-activated with HOBt (1-hydroxybenzotriazole, 1.5 mmol), HBTU (*O*-benzotriazole-*N*,*N*-*N*′-*N*′-tetramethyluronium hexafluorophosphate, 1.5 mmol), and DIEA (N,N′-diisopropyl ethylamine, 3.0 mmol) in DMF (dimethyl formamide, 4.0 mL) for 5 min, and couplings were run for 1.5–2.0 h. The Fmoc group was removed before each coupling step by reaction with 20% piperidine in DMF for 5 and 20 min. The whole peptide was conjugated with the phosphorylation reagents followed by oxidation to obtain the crude phosphopeptide, which was purified by semi-preparative high performance liquid chromatography (HPLC) for further use.

ESI-MS

The mass spectra were obtained using a Bruker ESQUIRE-LCTM ESI ion trap spectrometer equipped with a gas nebulizer probe. Nitrogen was used as drying gas with a flow rate of 4 L/min. The nebulizer gas for pressure was 7 psi. The electroscopy capillary was typically held at 4 kV. The samples dissolved in methanol were ionized by electrospray ionization. The scan ranges were from m/z 50 to 800 in positive-ion mode.

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MALDI-TOF-MS

MALDI-TOF-MS experiments were performed in positive ion mode on an AXIMA instrument (Shimadzu, Kyoto, Japan). All MALDI-PSD (post-source decay) fragment spectra were measured under the following conditions: nitrogen laser, 337 nm; positive-mode detection; and reflectron mode. α-cyano-4-hydroxycinnamic acid was used as matrix without further purification.

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