Evaluation of a Novel Approach for Peptide Sequencing: Laser-induced Acoustic Desorption Combined with $P(OCH_3)_2^+$ Chemical Ionization and Collision-activated Dissociation in a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

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A novel mass spectrometric method has been developed for obtaining sequence information on small peptides. The peptides are desorbed as intact neutral molecules into a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR) by means of laser-induced acoustic desorption (LIAD). Reactions of the neutral peptides with the dimethoxyphosphenium ion, $P(OCH_3)_2^+$, occur predominantly by addition of the peptide to $P(OCH_3)_2^+$ followed by the loss of two methanol molecules, thus yielding product ions with the composition $(peptide + P - 2H)^+$. Upon sustained off-resonance irradiation for collision-activated dissociation (SORI-CAD), the $(peptide + P - 2H)^+$ ions undergo successive losses of CO and NH=CHR or H_2O, CO, and NH=CHR to yield sequence-related fragment ions in addition to the regular a_n- and b_n-type ions. Under the same conditions, SORI-CAD of the analogous protonated peptides predominantly yields the regular a_n- and b_n-type ions. The mechanisms of the reactions of peptides with $P(OCH_3)_2^+$ and the dissociation of the $(peptide + P - 2H)^+$ ions were examined by using model peptides and molecular orbital calculations. (J Am Soc Mass Spectrom 2007, 18, 525–540) © 2007 American Society for Mass Spectrometry

A major research area in mass spectrometry is the development of better methods for peptide sequencing to facilitate the determination of protein structures. The introduction of fast atom bombardment (FAB) [1] in the early 1980s led the way to techniques that allow nearly simultaneous desorption and ionization of peptides and small proteins in mass spectrometers. The development of matrix-assisted laser desorption ionization (MALDI) [2] and electrospray ionization (ESI) [3], in particular, facilitated the evaporation and ionization of large biomolecules in mass spectrometers. The analytes are ionized by protonation, deprotonation, or attachment of metal ions during the desorption process.

The predominantly used approach for determining sequence information for ionized biopolymers involves collision-activated dissociation (CAD) of mass-selected ions [4]. For peptides with no strongly basic residues, the majority of fragment ions originate from cleavages of the peptide bonds. CAD promotes migration of the added, mobile proton to the N atoms of the peptide bonds [5], thereby weakening them, so that they dissociate to yield sequence-diagnostic b_n and y_n fragment ions. If a sufficient number of b_n and/or y_n ions are observed, the entire sequence is easily deduced. However, this is not always true. For example, collisional activation does not induce fragmentation of many peptide bonds in protonated peptides with strongly basic residues because the basic residue sequesters the added protons. Therefore, alternatives to CAD, such as photon [6] and surface-induced dissociation (SID) [7], electron capture dissociation (ECD) [8], blackbody infrared dissociation (BIRD) [9], and others, have been explored for their potential to provide complementary structural information for ionized peptides.

In recent years, considerable effort has been dedicated toward developing gas-phase ion-molecule reactions for structural characterization of biomolecules ionized and evaporated by ESI or MALDI [10]. Unfortunately, ion-molecule reactions of protonated peptides are mostly limited to proton transfer and hydrogen/deuterium (H/D) exchange reactions. Although a much broader range of reactivity can be expected for neutral peptides, only a few studies have focused on these reactions as an alternative means of obtaining sequence information [11, 12]. One reason for the lack of interest in these reactions is the fact that, until now, only reactions of very small

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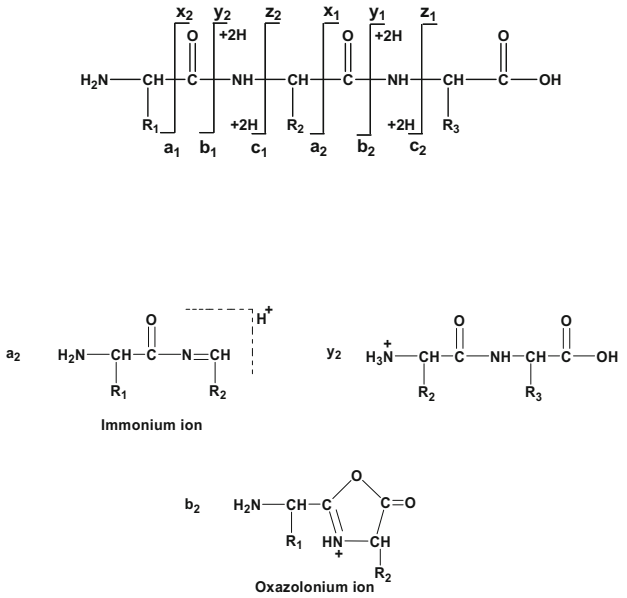


Scheme 1

neutral peptides could be studied because larger peptides cannot be thermally evaporated without decomposition. However, a method now exists for the evaporation of larger peptides (with MW at least up to 1000 Da) as intact neutral molecules into Fourier transform ion cyclotron resonance mass spectrometers: laser-induced acoustic desorption (LIAD) [13].

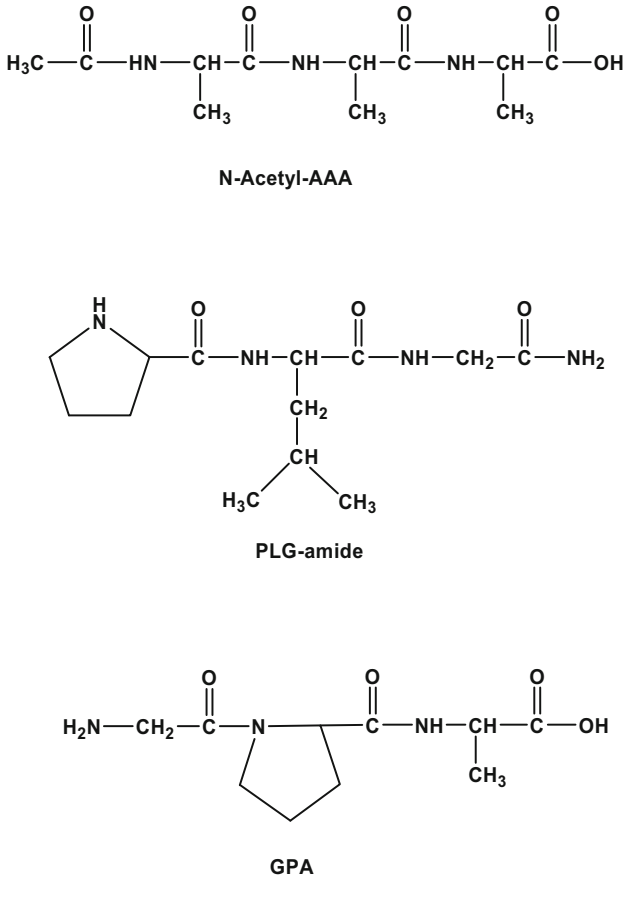
Herein, we report a novel approach, LIAD combined with gas-phase ion-molecule reactions (chemical ionization, CI) and sustained off-resonance irradiation for collision-activated dissociation (SORI-CAD) [14], for obtaining structural information for peptides in a mass spectrometer. LIAD involves the generation of high-amplitude acoustic waves in a thin Ti or Cu foil by firing a laser at the opposite side of the foil from where the sample is deposited [13]. These acoustic waves cause desorption of peptides into a FT-ICR mass spectrometer as intact neutral low-energy molecules. The peptides are then chemically ionized and finally exposed to SORI-CAD to obtain structurally diagnostic fragment ions.

Because peptides contain various nucleophilic sites, reactions with strongly electrophilic reagent ions should be fast. Thus, a phosphenium ion, $P(OCH_3)_2^+$, was selected as the chemical ionization reagent for this study. Phosphenium ions, such as PF_2^+, CH_3PF^+, PCl_2^+, PBr_2^+, PH_2^+, CH_3PH^+, $P(CH_3)_2^+$, $HPOCH_3^+$, and $P(OCH_3)_2^+$, are electrophiles because of the presence of only six valence electrons at the phosphorus center. Most of these ions have been shown to have a singlet electronic ground state [15], that is, they possess a lone pair of electrons and a formally vacant orbital at the phosphorus center. The reactivity of these ions is controlled by their electrophilicity, which is determined by their substituents. Phosphenium ions have been found to undergo proton transfer, hydride abstraction, electron abstraction, insertion into heteroatom-hydrogen bonds, and adduct formation upon reaction with neutral substrates [16]. The strongly electrophilic nature of phosphenium ions and the presence of many nucleophilic sites in peptides lead to fast reactions between these species, as demonstrated by a previous study of the reactions of PCl_2^+ with di- and tripeptides [12]. However, as a result of the very high electrophilicity of PCl_2^+, this ion leads to extensive fragmentation of the peptides and provides very little sequence information. Thus, a less electrophilic phosphenium ion, $P(OCH_3)_2^+$, was chosen for this study.

Experimental

Instrumentation

All chemicals were purchased from the Sigma–Aldrich Company and used as received without further purification. Titanium foil was purchased from Alfa Aesar Company. The experiments were performed in two different dual-cell FT-ICR instruments, an Extrel Model FTMS 2001 and a Nicolet Model FTMS 2000, each equipped with an Odyssey data station. These instruments contain a dual cell consisting of two identical cubic 2-in. cells separated by a conductance limit plate. The conductance limit plate has a 2-mm hole in the center for the transfer of ions from one side into the other. The conductance limit plate and the two end-



Scheme 2

Table 1. Primary product ions and their m/z values for reactions between neutral peptides and $P(OCH_3)_2^+$

Peptide	MW	*m/z*	Product ions
GFA	293	322	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		276	Adduct − $2CH_3OH$ − H_2O − CO
		231	Adduct − $2CH_3OH$ − H_2O − CO − PN
		205	b_2
AAA	231	260	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		214	Adduct − $2CH_3OH$ − H_2O − CO
		169	Adduct − $2CH_3OH$ − H_2O − CO − PN
		143	b_2
AGG	203	232	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		186	Adduct − $2CH_3OH$ − H_2O − CO
		141	Adduct − $2CH_3OH$ − H_2O − CO − PN
		129	b_2
VGG	231	260	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		214	Adduct − $2CH_3OH$ − H_2O − CO
		169	Adduct − $2CH_3OH$ − H_2O − CO − PN
		157	b_2
GGV	231	260	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		214	Adduct − $2CH_3OH$ − H_2O − CO
		169	Adduct − $2CH_3OH$ − H_2O −CO − PN
		118	y_1
PGG	229	258	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		212	Adduct − $2CH_3OH$ − H_2O − CO
		155	b_2
GPA	243	—	No (adduct − $2CH_3OH$) (no (peptide + P − 2H)$^+$)
		304	Adduct − CH_3OH (peptide + $POCH_3$ − H)$^+$
		226	Adduct − $2CH_3OH$ − H_2O − CO
		155	b_2
PLG-amide	284	313	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		267	Adduct − $2CH_3OH$ − H_2O − CO
		211	b_2
N-Acetyl-AAA	273	—	No (adduct − $2CH_3OH$) (no (peptide + P − 2H)$^+$)
		185	b_2
DG	190	219	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		173	Adduct − $2CH_3OH$ − H_2O − CO
GV	206	235	Adduct − CH_3OH (peptide + $POCH_3$ − H)$^+$
		173	Adduct − $2CH_3OH$ (peptide + P − 2H)$^+$
		157	Adduct − $2CH_3OH$ − H_2O − CO

trapping plates were kept at +2.0 V in both instruments unless otherwise stated. In the Extrel FTMS 2001 mass spectrometer, the dual cell is aligned collinearly with the field of a 3 T superconducting magnet operated at about 2.7 T, and it is differentially pumped by two Balzers turbomolecular pumps (330 L/s), each backed by an Alcatel 2012 mechanical pump. A nominal baseline pressure of less than 1×10^{-9} Torr was measured by Bayard–Alpert ionization gauges located on each side of the dual cell. In the Nicolet FTMS 2000 mass spectrometer, the dual cell is aligned collinearly with the magnetic field produced by a 3 T superconducting magnet and it is differentially pumped by two Edwards Diffstak 160 diffusion pumps (700 L/s), each backed by an Alcatel 2012 mechanical pump. A nominal baseline pressure of less than 1×10^{-9} Torr was measured by Bayard–Alpert ionization gauges located on each side of the dual cell. Liquid samples were introduced into the instruments by using batch inlet systems equipped with Andonian leak valves. Ar and He gases were pulsed into the instruments by using general valve corporation solenoid pulsed valves.

LIAD was used to desorb the neutral peptides into the gas phase. A detailed description of LIAD can be found in our previous publications [13]. Slightly different LIAD probes were used in the Extrel [13a, 13b] and Nicolet instruments [13c, 13d]. About 100 nmol of a peptide sample was deposited on the surface of a about 12.7 μm thick titanium foil by using electrospray [17, 18]. A high-intensity laser pulse from a Nd:YAG laser (3 ns pulsewidth, 532 nm wavelength, fluence of 1×10^9 W cm^{-2} at the metal surface) was used to initiate acoustic waves at the back side of the metal foil. The acoustic waves desorb the neutral peptide molecules deposited on the front side of the foil. The area affected by a single laser pulse is about 10^{-3} cm^2. This results in intense mass spectral signals. The lower limit of detection was not examined.

Trimethyl phosphite was ionized by electron ionization (EI) in one side of the dual cell to generate $P(OCH_3)_2^+$. Typical ionization parameters were 40- to 100-ms electron beam time, 45 eV electron energy, and 8.0 μA filament current. Nominal base pressure of the neutral reagent varied between 5.0×10^{-8} and 6.0×10^{-8} Torr, as measured by an ion gauge. All the ions in the other side of the dual cell were removed before ion transfer by changing the remote trapping plate voltage from +2.0 to −2.0 V for 12 ms. The reagent ion, $P(OCH_3)_2^+$, was quadrupolarly axialized (QA) [19] before transferring it into the other cell. $P(OCH_3)_2^+$ was subjected to on-resonance excitation at its cyclotron frequency for about 0.8–1.2 s while pulsing in helium at a nominal pressure of about 10^{-5} Torr. The transfer was achieved by grounding the conductance limit plate for 85–100 μs. The transferred ions were allowed to cool by IR emission [20] and by collisions with Ar present at about 10^{-5} Torr for a period of about 1 s. $P(OCH_3)_2^+$ was isolated by using a stored-waveform inverse Fourier transform (SWIFT) [21, 22] excitation pulse to eject all unwanted ions, and allowed to react with GV, DG, GFA, AAA, AGG, PGG, GPA, VGG, GGV, *N*-acetyl-AAA, and PLG-amide desorbed as neutral molecules into that cell by means of LIAD. The number of laser shots was varied between 50 and 200. Almost all the reactions yield singly charged addition/elimination products, apart from others.

Further structural information on the peptides was obtained by SWIFT isolation of $(peptide + P - 2H)^+$ and subjecting this ion to SORI-CAD for about 1 s while pulsing in about 10^{-5} Torr of Ar as a target gas. The SORI-CAD experiments used off-resonance excitation of the isolated ion at a frequency ±1000 Hz off the cyclotron frequency of the ion.

Protonated peptides were generated by proton transfer from protonated acetone. Protonated acetone was formed by EI of acetone for 100 ms with electron energy of 20 eV and filament current of 8 μA, followed by about 1-s reaction of the fragment ions of ionized acetone with neutral acetone. Nominal base pressure of the reagent varied between 5.0×10^{-8} and 6.0×10^{-8} Torr, as measured by an ion gauge. Protonated acetone was transferred into the other cell by grounding the conductance limit plate for about 76 μs. The transferred ions were allowed to cool by IR emission and by collisions with Ar present at about 10^{-5} Torr for a period of about 1 s. The reagent ion was isolated by SWIFT and allowed to react with peptides that were desorbed as neutral molecules into that cell by means of LIAD. The protonated peptides generated in the above reactions were isolated and subjected to SORI-CAD under the same conditions as the $(peptide + P - 2H)^+$ ions.

After reaction, all ions were excited for detection by using chirp excitation at a bandwidth of 2.7 MHz, amplitude of 124 V_{p-p}, and a sweep rate of 3200 Hz μs^{-1}. The spectra were recorded as 64K data points by using one zero-fill before Fourier transform. All the spectra were background corrected to eliminate product ions arising from reactions of the peptides with ionic impurities in the cell. Background spectra were measured by desorbing the neutral peptides into a reagent-ion free cell and allowing them to react with ionic impurities present in the cell.

Computational Studies

Molecular orbital calculations were performed with the Gaussian 98 suite of programs [23]. Several conformers of each isomer were initially examined at the AM1 level of theory. The most stable conformers were then subjected to higher-level calculations. Geometry optimization and vibrational frequency calculations for the most stable conformers were performed using density functional theory at the B3LYP/6-311+G(d,p) level of theory. All theoretical energies were corrected for the zero-point vibrational energy obtained from the harmonic vibrational frequencies calculated at the same level of theory and are presented at 0 K.

Results and Discussion

Nomenclature System for Peptide Fragments

The system illustrated in Scheme **1** is used to label the peptide fragment ions [24, 25]. The fragment ions containing the N-terminal amino acid residue are labeled with the first few letters of the alphabet (a, b, and c), whereas the fragment ions containing the C-terminal amino acid residue are labeled with the last few letters of the alphabet (x, y, and z).

Reactions of $P(OCH_3)_2^+$ with Peptides

Most reactions of $P(OCH_3)_2^+$ with neutral di- and tripeptides (Scheme **2** illustrates the structures of some of the tripeptides) evaporated by LIAD into an FT-ICR resulted in the formation of a major primary addition/elimination product ion, $(peptide + P - 2H)^+$, by the loss of two methanol molecules from the adduct (Table 1). The minor primary product ions include $(peptide + POCH_3 - H)^+$ formed by loss of one methanol molecule from the adduct, and $(peptide + P - 2H - H_2O - CO)^+$ ions formed by loss of two methanol molecules, H_2O, and CO from the

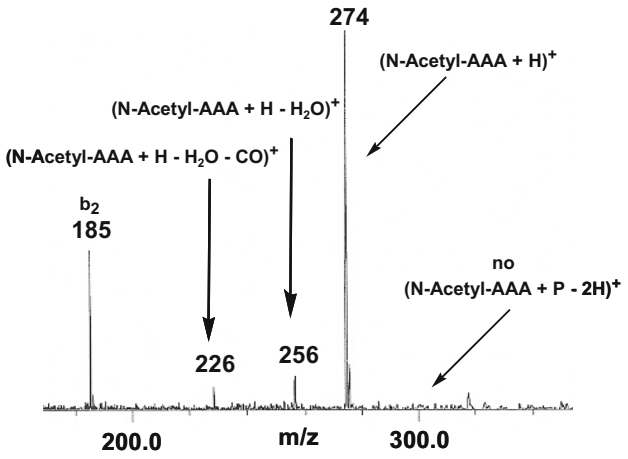


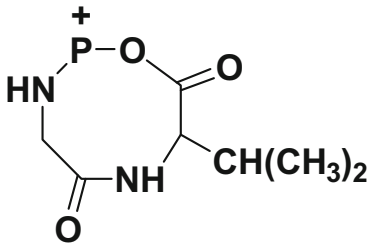
Figure 1. LIAD/$P(OCH_3)_2^+$ mass spectrum of *N*-acetyl-Ala-Ala-Ala (*N*-acetyl-AAA). The only primary product is the b_2 ion (*m/z* 185). The absence of both addition/elimination products is taken as an evidence for the initial attack by the $P(OCH_3)_2^+$ at the free amino terminus.

adduct. The reactions of tripeptides led to the formation of additional primary products, such as $(peptide + P - 2H - H_2O - CO - PN)^+$ by loss of two methanol molecules, H_2O, CO, and PN from the adduct, as well as b_2 or y_1 ions. These reactions are discussed below.

Almost all peptides studied form the primary product $(peptide + P - 2H)^+$ by the addition of the peptide to $P(OCH_3)_2^+$, accompanied by the loss of two methanol molecules. The only exceptions are *N*-acetyl-AAA, which does not produce any addition/elimination products (Figure 1), and GPA, whose adduct either eliminates one methanol molecule or two methanol molecules, H_2O, and CO (Figure 2). In *N*-acetyl-AAA, the amino terminus is blocked by an electron-withdrawing acetyl group that also makes the amino terminus less nucleophilic. Therefore, an adduct of $P(OCH_3)_2^+$, if formed at all (steric hindrance), may not have enough internal energy to fragment by methanol losses. The absence of addition/elimination products for this tripeptide is taken as evidence for the initial attack by $P(OCH_3)_2^+$ at the free N-terminal amino group of the other peptides. On the other hand, the observation that all other tripeptides form an addition/elimination product by loss of two methanol molecules, while GPA shows elimination of either just one methanol molecule or two methanol molecules, H_2O, and CO, indicates that the mechanism for the second methanol elimination is different for this peptide. This would be the case if the second methanol loss for the other peptides was facilitated by proton transfer from the middle amino acid's amido group because this acidic hydrogen is not present in GPA. Finally, the fact that reactions of $P(OCH_3)_2^+$ with the dipeptides GV and DG also lead to the formation of addition/elimination products involving the loss of two methanol molecules (Table 1) is taken as evidence for the C-terminus of the tripeptides not being involved in the formation of the addition/elimination products.

Based on all these observations, three likely mechanisms were considered (Scheme **3**). All three mechanisms involve an initial attack at the N-terminal amino group, intramolecular proton transfer, and methanol loss. The mechanisms differ in the subsequent step that involves addition of either the N-terminal carbonyl group (mechanism 1), the adjacent amido nitrogen (mechanism 3), or the next amino acid's carbonyl group (mechanism 2) to the phosphorus, resulting in the formation of either a five or an eight membered ring. Subsequent proton transfer and elimination of a second methanol molecule yields the $(peptide + P - 2H)^+$ ion. Thus, three possible isomeric structures (isomers A, B, and C) will be considered for the $(peptide + P - 2H)^+$ ion.

The $(peptide + P - 2H)^+$ ion of the dipeptide GV can undergo loss of H_2O and CO (Table 1). This finding rules out isomer B because this isomer cannot readily lose H_2O and CO molecules [the structure of isomer B of $(GV + P - 2H)^+$ is shown below]:



Structure of Isomer B

Thus, MO calculations were performed only for isomers A and C. Several conformers were examined

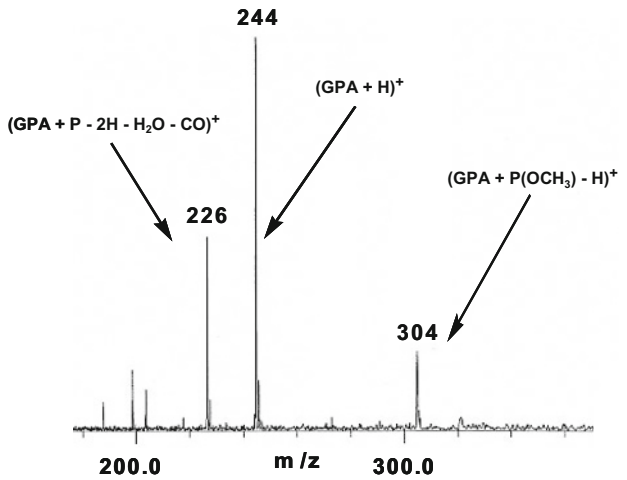
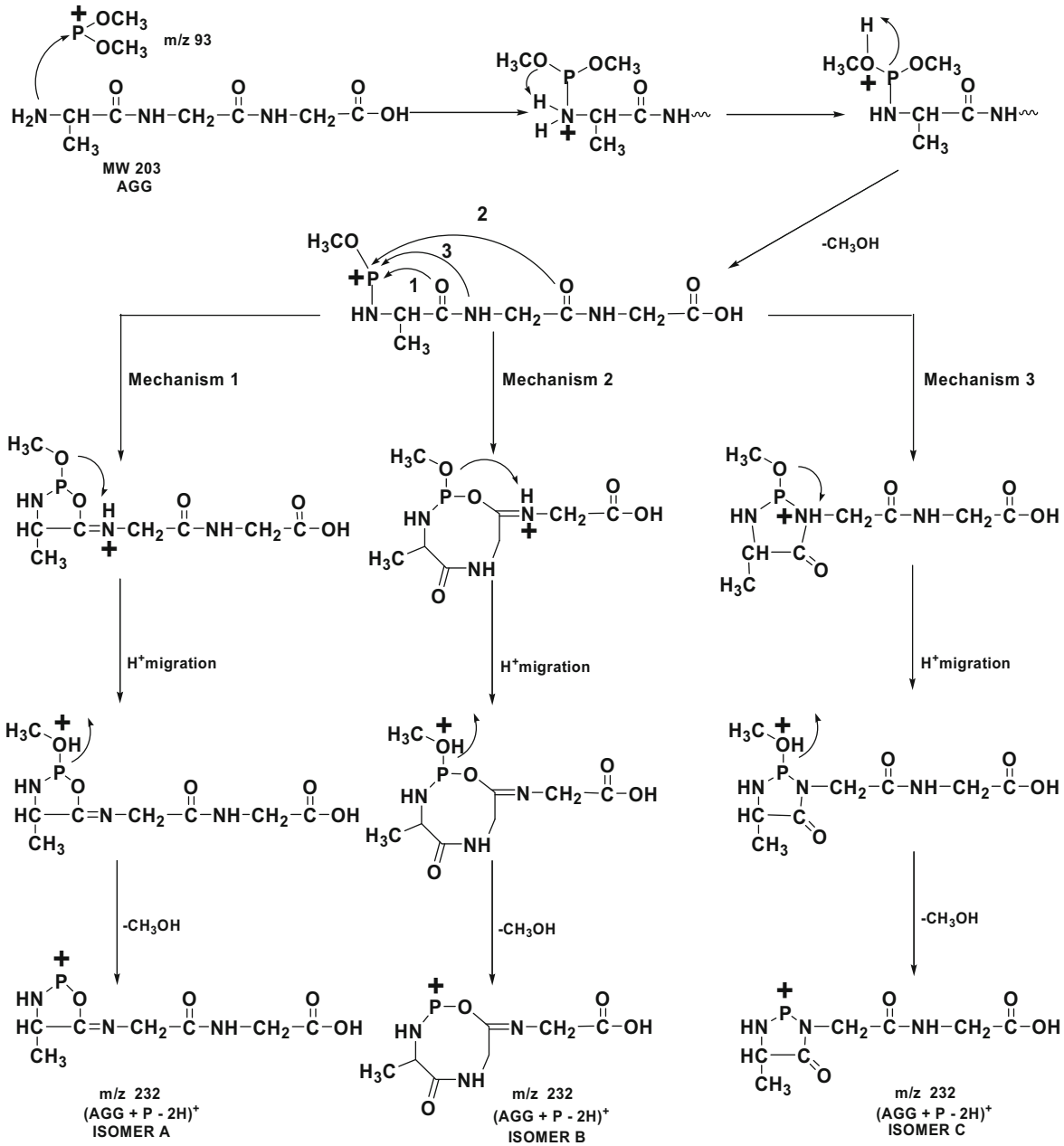


Figure 2. LIAD/$P(OCH_3)_2^+$ mass spectrum of Gly-Pro-Ala (GPA). Two primary products (*m/z* 304 and *m/z* 226) and one secondary product, protonated GPA (*m/z* 244), are indicated.

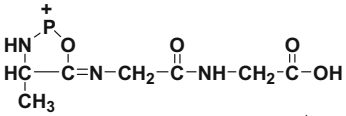
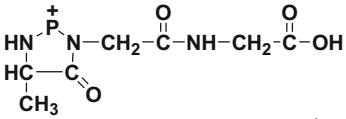
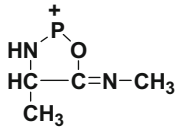
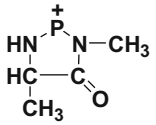


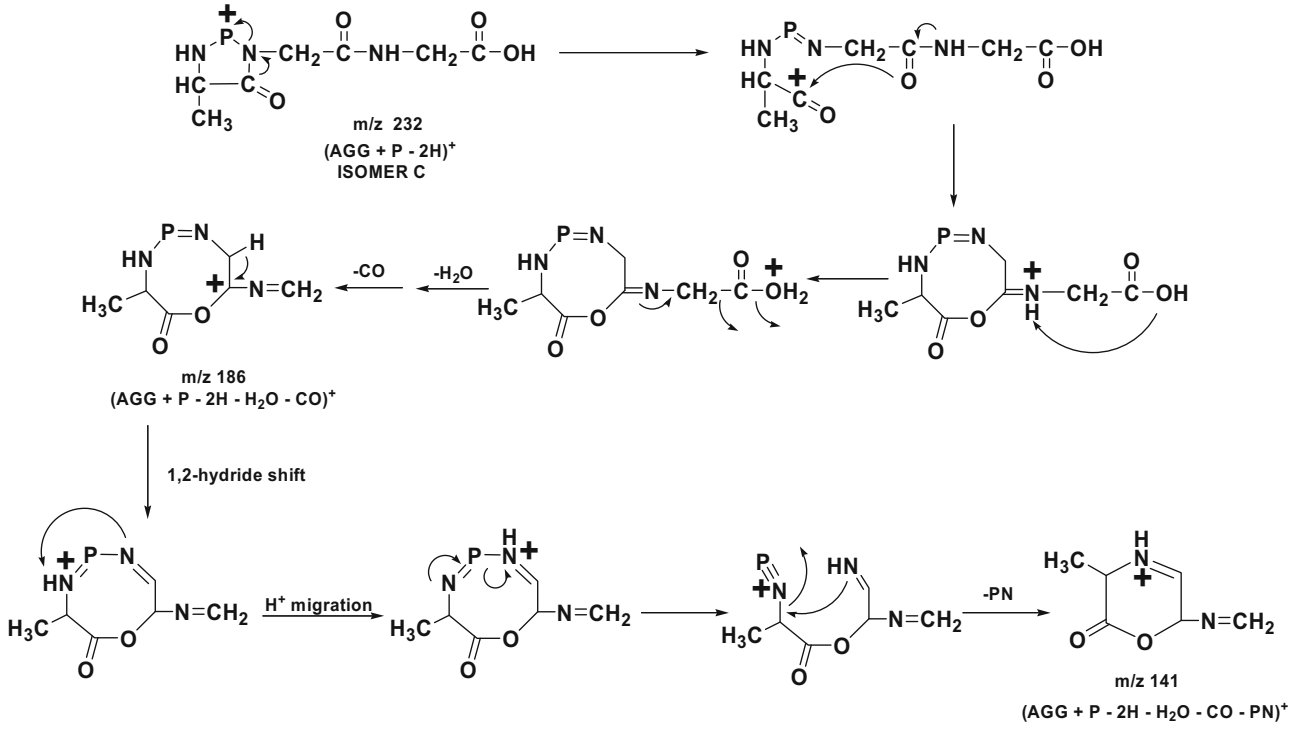
Scheme 3

initially at the AM1 level of theory. The most stable structures were subjected to calculations at the B3LYP/6-311+G(d,p) + ZPVE level of theory. Table 2 gives the relative stabilities of the two isomeric (peptide + P − 2H)$^+$ ions, A and C. Isomer C was found to be about 27 kcal/mol more stable than isomer A. This extra stabilization arises from a more stable ring structure, as revealed by the calculated relative energies of the two isomeric five-membered rings without the peptide side chains (Table 2). Therefore, isomer C is the most likely candidate for the (peptide + P − 2H)$^+$ ion. Experimental evidence in support of this hypothesis is presented later.

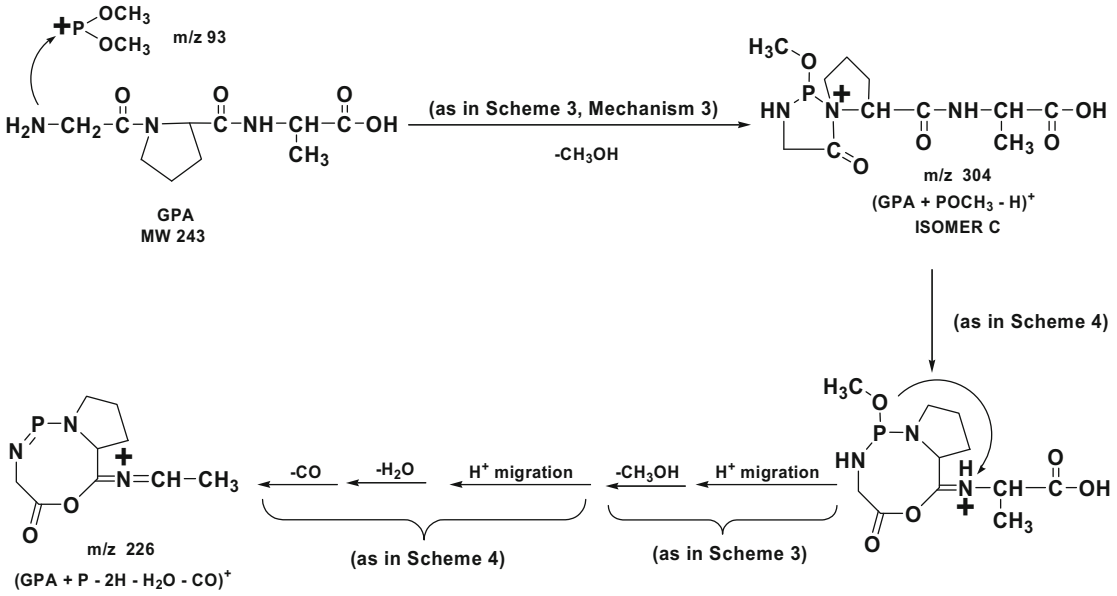
Almost all tripeptides studied form an additional primary product ion by the consecutive loss of H_2O, CO, and PN from (peptide + P − 2H)$^+$. Scheme **4** illustrates a possible mechanism for the loss of H_2O, CO, and PN from isomer C of the (peptide + P − 2H)$^+$ ion of AGG. However, the peptides PGG and PLG-amide do not form this product ion. This finding supports the proposed mechanism wherein the N-terminal amino group's nitrogen is eliminated with the phosphorus atom as PN, given that the N-terminal amino group in PGG and PLG-amide is part of a five-membered ring and cannot be readily eliminated. Finally, the above results indicate that GPA forms a (peptide + P − 2H)$^+$ ion that is unstable toward elimination of H_2O and CO. A possible mechanism for this process is shown in Scheme **5**.

Table 2. Calculated relative energies (at 0 K) of the possible isomeric structures A and C of $(AGG + P - 2H)^+$, and two isomeric five-membered rings

Structure of Isomer	Energy (Hartree)	Relative energy (Hartree)	Relative energy (kcal/mol)
 Isomer A of $(AGG + P - 2H)^+$	−1079.70	0.042405	26.61
 Isomer C of $(AGG + P - 2H)^+$	−1079.74	0	0
 Five membered ring of isomer A without the peptide side chain	−683.06	0.029980	18.81
 Five membered ring of isomer C without the peptide side chain	−683.09	0	0



Scheme 4



Scheme 5

Reactions of Primary Product Ions with Peptides

The ion-molecule reactions between neutral peptides and $P(OCH_3)_2^+$ yield several secondary products, including the protonated peptide and its ionic fragments. The secondary product ions were demonstrated to arise from protonation of the neutral peptide by primary product ions (predominantly those of lower *m/z* values; Table 1). For example, ejection of the ions of *m/z* 141 and *m/z* 186 formed during the reaction between the neutral peptide AGG and $P(OCH_3)_2^+$ resulted in the disappearance of most of the protonated peptide (*m/z* 204) and the fragment ions of *m/z* 44 (a_1) and *m/z* 129 (b_2). Similarly, continuous ejection of the protonated peptide, $(AGG + H)^+$, hindered the formation of the a_1 and most of the b_2 ions, indicating that these ions are formed by dissociation of the protonated peptide (Figure 3). However, the fact that only a partial disappearance of the b_2 ions occurred upon continuous ejection of the protonated peptide suggests that some of the b_2 ions are also formed as a primary product in these reactions.

All the peptides studied thus far form b_2 ions as one of the primary products, except GGV, which yields a y_1 ion. The formation of the b_2 ion can be explained by attack of $P(OCH_3)_2^+$ at the carbonyl oxygen of the C-terminal amino acid (Scheme **6**). For GGV, the bulky valine residue in the C-terminal likely hinders the $P(OCH_3)_2^+$ from approaching the C-terminus. Instead, $P(OCH_3)_2^+$ attacks the carbonyl group adjacent to the C-terminal amino acid, resulting in the formation of the y_1 ion (Scheme **7**). These mechanisms are similar to those proposed for the formation of b_2 and y_1 ions from protonated peptides [26].

Dissociation of (Peptide + P − 2H)$^+$ Ions

$(Peptide + P - 2H)^+$ ions were examined further by subjecting them to sustained off-resonance irradiation collision-activated dissociation (SORI-CAD) [14]. Figure 4 shows a typical SORI-CAD spectrum. SORI-CAD resulted in the generation of several fragment ions (Table 3). The most notable ones are the sequence-related fragment ions, such as a_1, a_2, b_2, $(a_1 + P + O - H)^+$, $(b_1 + P + O - H)^+$, $(b_2 + P - 3H)^+$, $(b_2 + P + O - H)^+$, and $(a_2 + P + O - H)^+$. Most of these fragment ions are likely formed by the initial loss of either H_2O (Pathway 1, Schemes **8**, **9**, **10**) or CO (Pathway 2, Schemes **9**, **10**, **12**), followed by successive losses of CO and NH=CHR (Pathway 1), or two NH=CHR molecules and two CO molecules (Pathway 2), respectively.

Experimental support for the assignment of isomer C and not A to the $(peptide + P - 2H)^+$ ions of AGG is obtained by considering the most likely mechanisms for fragmentation of isomer A by consecutive loss of H_2O (*m/z* 214), CO (*m/z* 186), and NH=CHR (*m/z* 157) (Pathway 1 in Scheme **8**). According to this mechanism, the final loss of NH=CHR leads to $(b_2 + P - 3H)^+$ (*m/z* 157), which should undergo one more CO elimination to yield the ion $(a_2 + P - 3H)^+$ (*m/z* 129). An ion of *m/z* 129 was indeed observed. However, exact mass measurements (measured 129.070 Da) revealed that this ion is the b_2 ion (calculated 129.066 Da) and not the $(a_3 + P - 3H)^+$ ion (calculated 129.021 Da). Similarly for the other peptides, SORI-CAD of the addition/elimination product $(peptide + P - 2H)^+$ yields the b_2 ion and not the $(a_3 + P - 2H)^+$ ion. Thus, the $(b_2 + P - 3H)^+$ ion of *m/z* 157 is concluded not to undergo CO elimination. This result rules out isomer A as a candidate for the $(peptide + P - 2H)^+$ ion. Isomer C is concluded to be the structure of the $(peptide + P - 2H)^+$ ion.

Pathway 1 in Scheme **9** accounts for the successive elimination of H_2O, CO and NH=CHR from isomer C of $(AGG + P - 2H)^+$ to form the ions $(AGG + P - 2H - H_2O)^+$, $(AGG + P - 2H - H_2O - CO)^+$, and $(b_2 +$

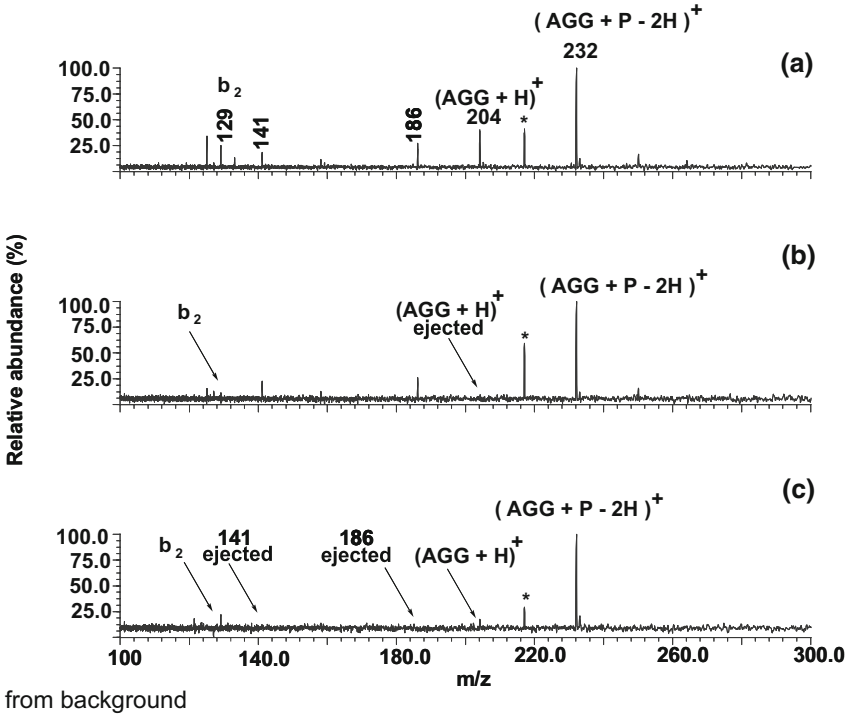
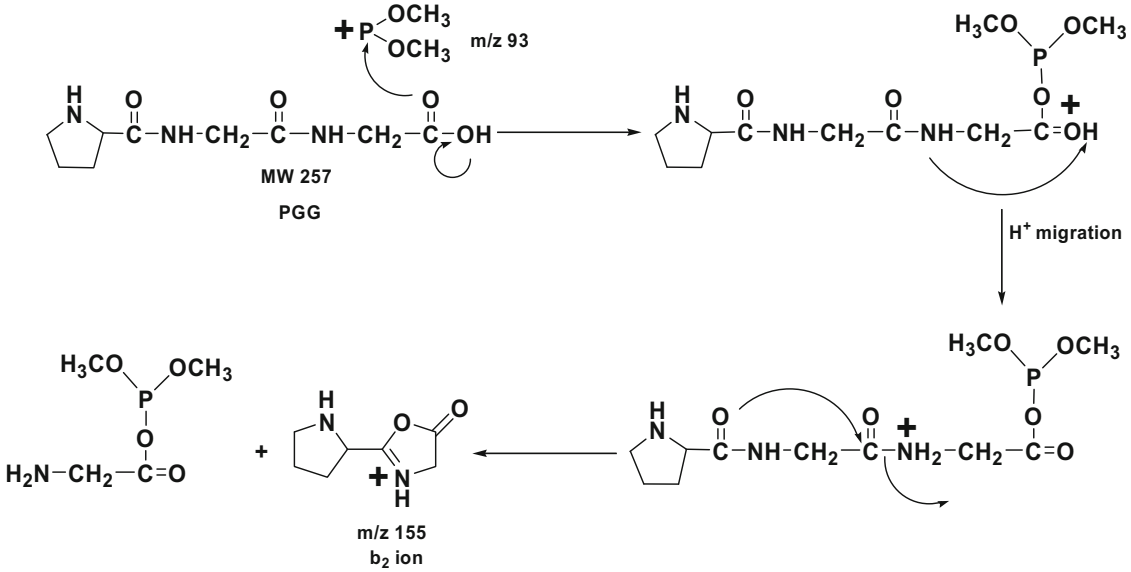
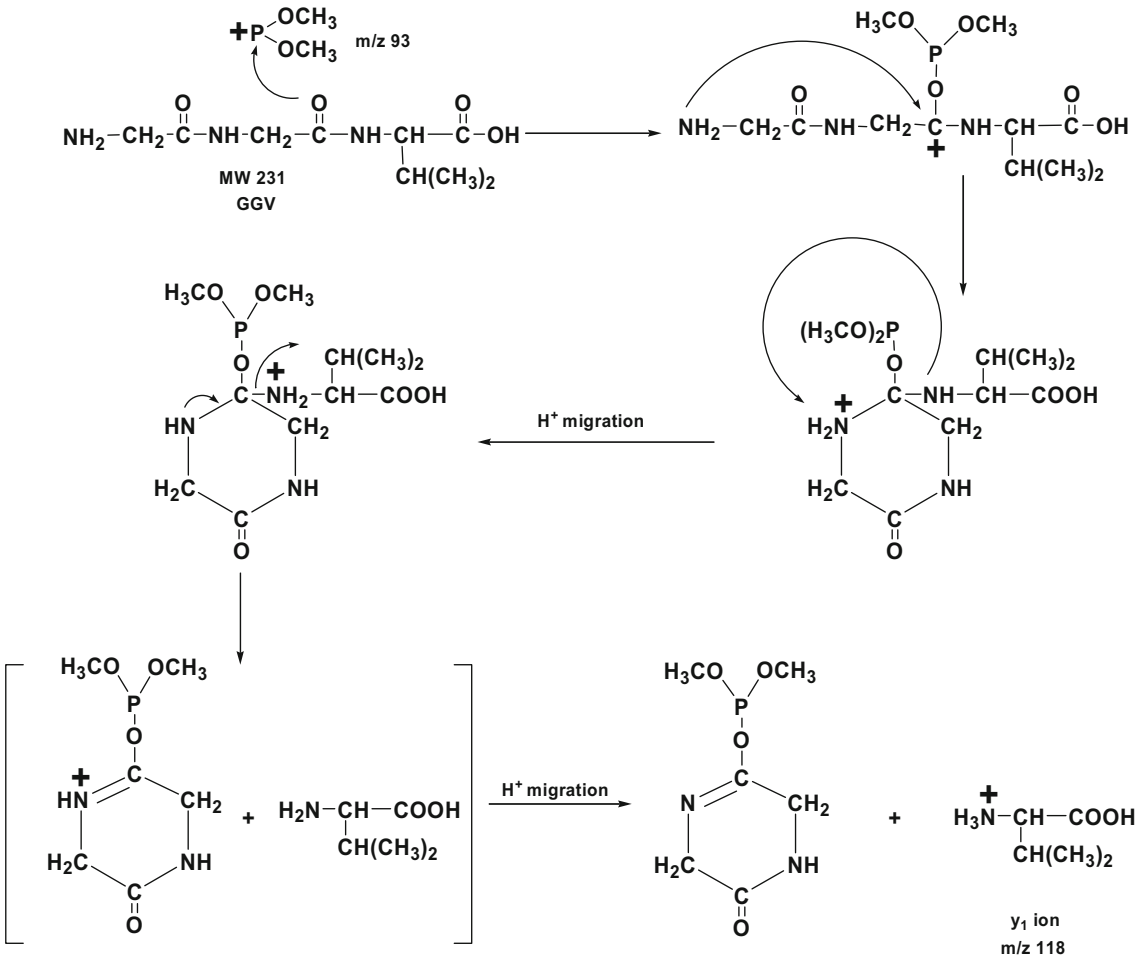


Figure 3. (**a**) A mass spectrum measured for the reaction between Ala-Gly-Gly (AGG) and $P(OCH_3)_2^+$. All the primary products and one secondary product, the protonated peptide, are marked. (**b**) A mass spectrum measured after SWIFT ejection of the protonated peptide $(AGG + H)^+$. This hindered the formation of most of the b_2 (*m/z* 129) ions (also all a_1 ions of *m/z* 44; not shown in the spectrum). (**c**) Mass spectrum measured after SWIFT ejection of the product ions of *m/z* 141 and 186. The disappearance of most of the protonated peptide (*m/z* 204) and b_2 (*m/z* 129) ions (also a_1 of *m/z* 44; not shown) indicates that the protonated peptide is formed predominantly by proton transfer from the ejected ions of *m/z* 141 and 186 to the neutral peptide in the cell, and that the protonated peptide fragments to ions of *m/z* 44 and 129.

$P - 3H)^+$, respectively. Based on this mechanism, the $(b_2 + P - 3H)^+$ ion of *m/z* 157 has a structure that cannot readily undergo further dissociation by CO loss, as opposed to the ion of *m/z* 157 formed from isomer A. This mechanism is supported by the observation that, although SORI-CAD of the (peptide + P $- 2H)^+$ ion of all other tripeptides results in the loss of H_2O, CO, and NH=CHR, a different product re-



Scheme 6



Scheme 7

sulting from the loss of NH_3, CO, and NH=CHR was observed for $(PLG\text{-amide} + P - 2H)^+$ (Table 3) where the C-terminus is blocked by an amido group.

Almost all the above-mentioned fragment ions were observed for all the peptides investigated, with one exception. SORI-CAD of $(PGG + P - 2H)^+$ does not result in the loss of an intact C-terminal imine, NH=CHR, but rather in the loss of the $\cdot N{=}CH_2$

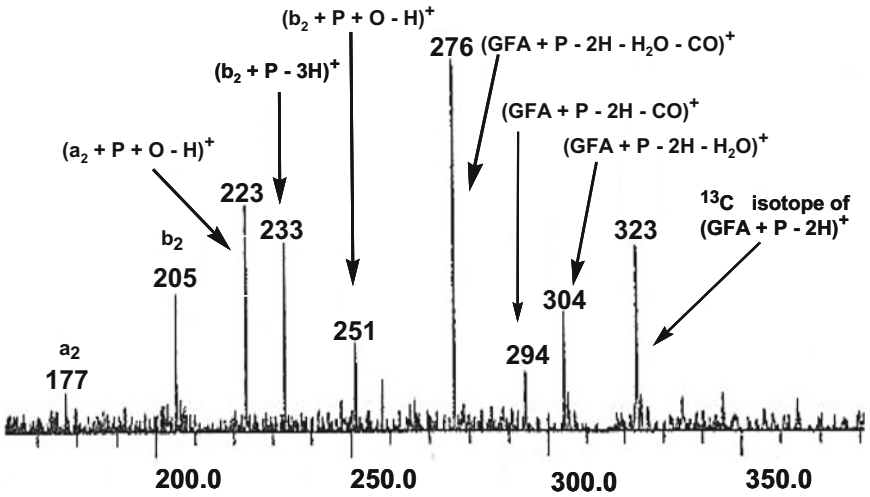
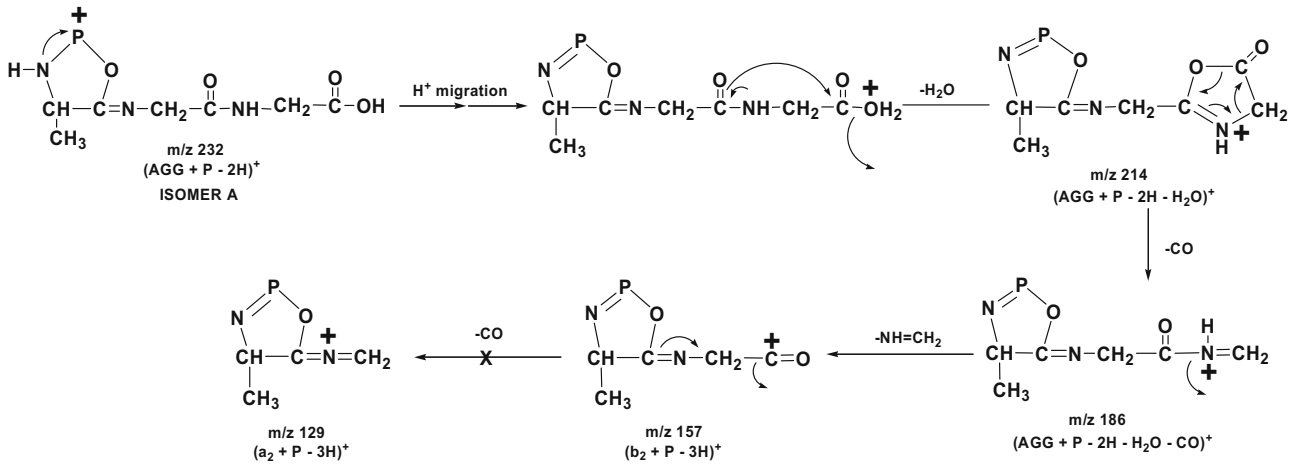


Figure 4. A SORI-CAD mass spectrum of $(GFA + P - 2H)^+$. This spectrum shows the various sequence-related fragment ions that are formed in the dissociation of $(GFA + P - 2H)^+$.

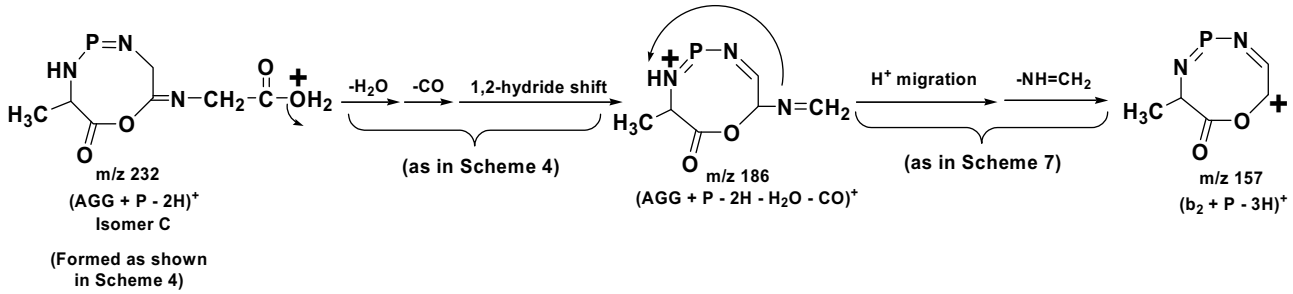
Table 3. Product ions observed upon SORI-CAD of (peptide + P − 2H)$^+$ formed in the reaction of neutral peptides with $P(OCH_3)_2^+$

Fragmenting ion, *m/z*	*m/z*	Product ions	Likely pathway
(VGG + P − 2H)$^+$, 260	242	(VGG + P − 2H − H_2O)$^+$	1
	232	(VGG + P − 2H − CO)$^+$	2
	203	(b_2 + P + O − H)$^+$	2
	185	(b_2 + P − 3H)$^+$	1
	175	(a_2 + P + O − H)$^+$	2
	146	(b_1 + P + O − H)$^+$	2
	118	(a_1 + P + O − H)$^+$	2
	157	b_2	
	72	a_1	
(GFA + P − 2H)$^+$, 322	304	(GFA + P − 2H − H_2O)$^+$	1
	294	(GFA + P − 2H − CO)$^+$	2
	276	(GFA + P − 2H − H_2O − CO)$^+$	1
	251	(b_2 + P + O − H)$^+$	2
	233	(b_2 + P − 3H)$^+$	1
	223	(a_2 + P + O − H)$^+$	2
	205	b_2	
	177	a_2	
(AGG + P − 2H)$^+$, 232	214	(AGG + P − 2H − H_2O)$^+$	1
	204	(AGG + P − 2H − CO)$^+$	2
	186	(AGG + P − 2H − H_2O − CO)$^+$	1
	175	(b_2 + P + O − H)$^+$	2
	157	(b_2 + P − 3H)$^+$	1
	147	(a_2 + P + O − H)$^+$	2
	118	(b_1 + P + O − H)$^+$	2
	90	(a_1 + P + O − H)$^+$	2
	129	b_2	
	44	a_1	
(PGG + P − 2H)$^+$, 258	240	(PGG + P − 2H − H_2O)$^+$	1
	212	(PGG + P − 2H − H_2O − CO)$^+$	1
	201	(b_2 + P + O − H)$^+$	2
	184	(b_2 + P − 2H)$^{+\bullet}$	1
	173	(a_2 + P + O − H)$^+$	2
	144	(b_1 + P + O − H)$^+$	2
	70	a_1	
(AAA + P − 2H)$^+$, 260	242	(AAA + P − 2H − H_2O)$^+$	1
	232	(AAA + P − 2H − CO)$^+$	2
	214	(AAA + P − 2H − H_2O − CO)$^+$	1
	189	(b_2 + P + O − H)$^+$	2
	171	(b_2 + P − 3H)$^+$	1
	161	(a_2 + P + O − H)$^+$	2
	143	b_2	
	115	a_2	
	44	a_1	
(PLG-amide + P − 2H)$^+$, 313	296	(PLG-amide + P − 2H − NH_3)$^+$	1
	295	(PLG-amide + P − 2H − H_2O)$^+$	1
	285	(PLG-amide + P − 2H − CO)$^+$	2
	268	(PLG-amide + P − 2H − NH_3 − CO)$^+$	1
	239	(b_2 + P − 3H)$^+$	1
	229	(a_2 + P + O − H)$^+$	2
	211	b_2	
	70	a_1	

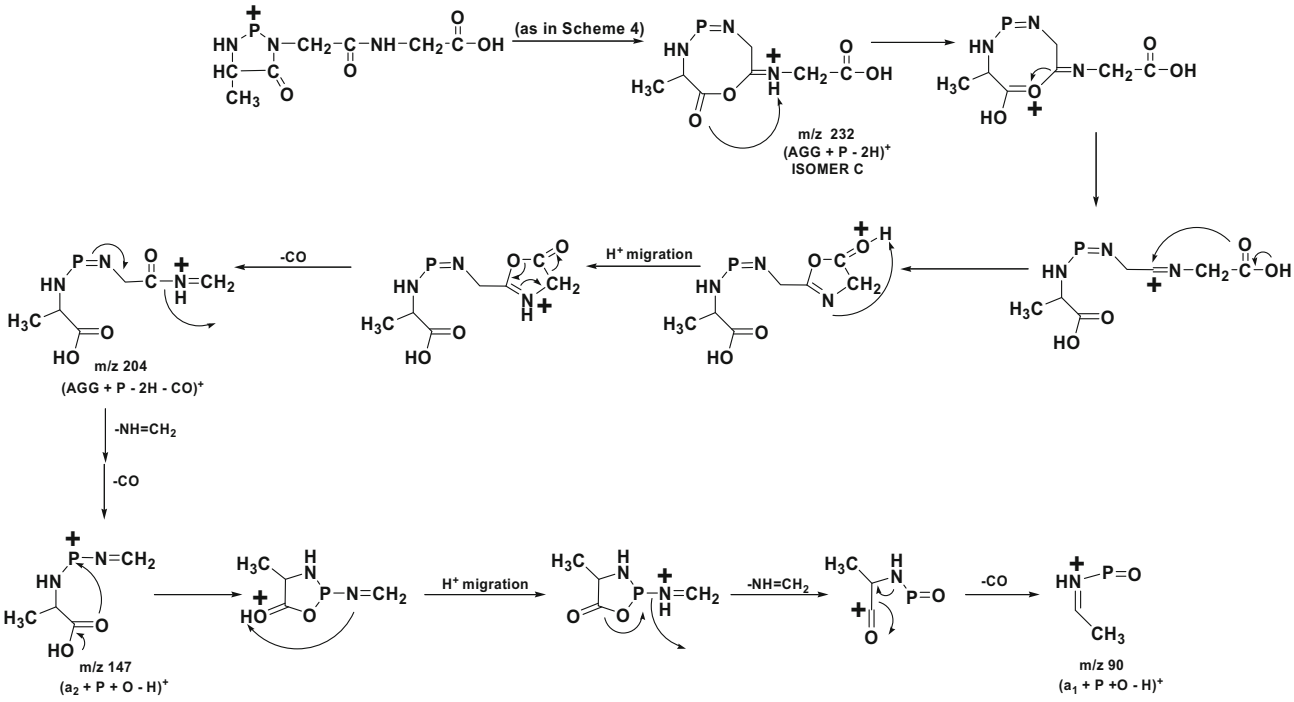
radical with one less hydrogen. This reaction may be explained by the fact that the N-terminal amino acid, proline, possesses only one nitrogen-bound hydrogen atom, unlike the other peptides studied that have two nitrogen-bound hydrogen atoms. For all the peptides, the first N-terminal nitrogen-bound hydrogen is likely abstracted by a methoxy group during the loss of the first methanol molecule (Scheme **3**). The second



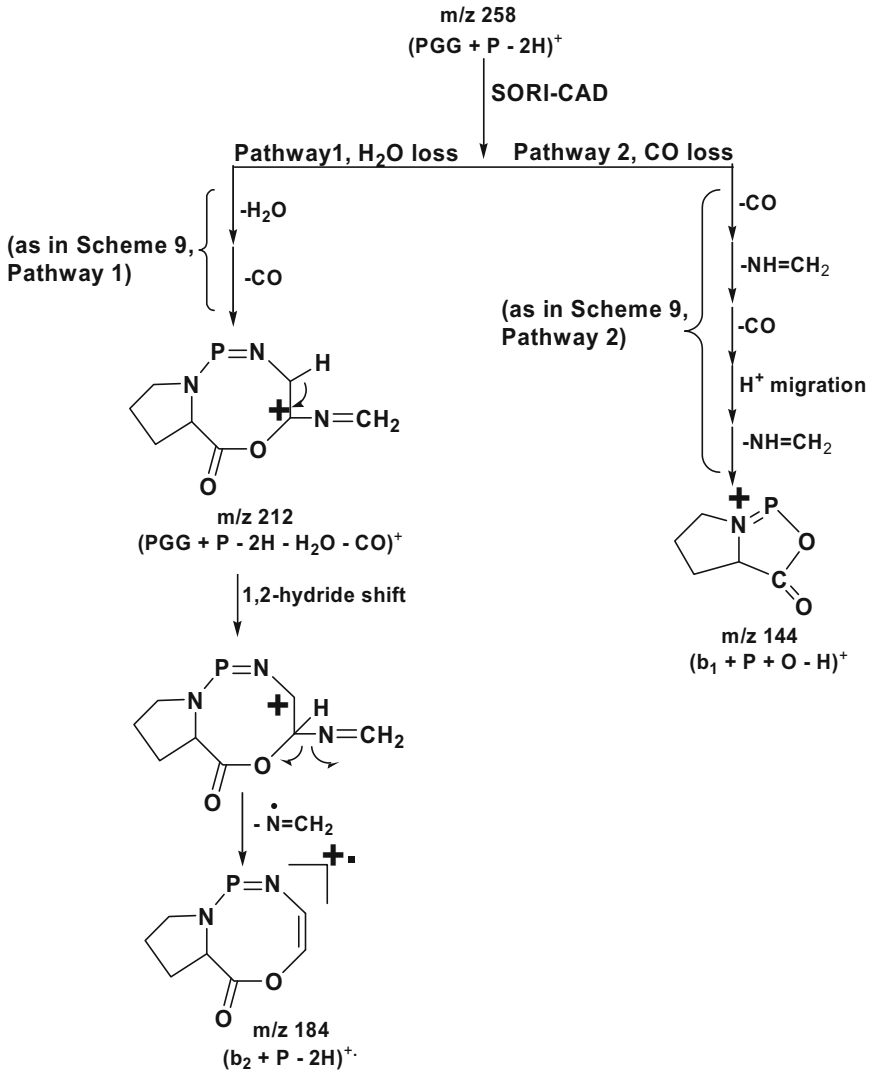
Scheme 8



Scheme 9, Pathway 1



Scheme 9, Pathway 2



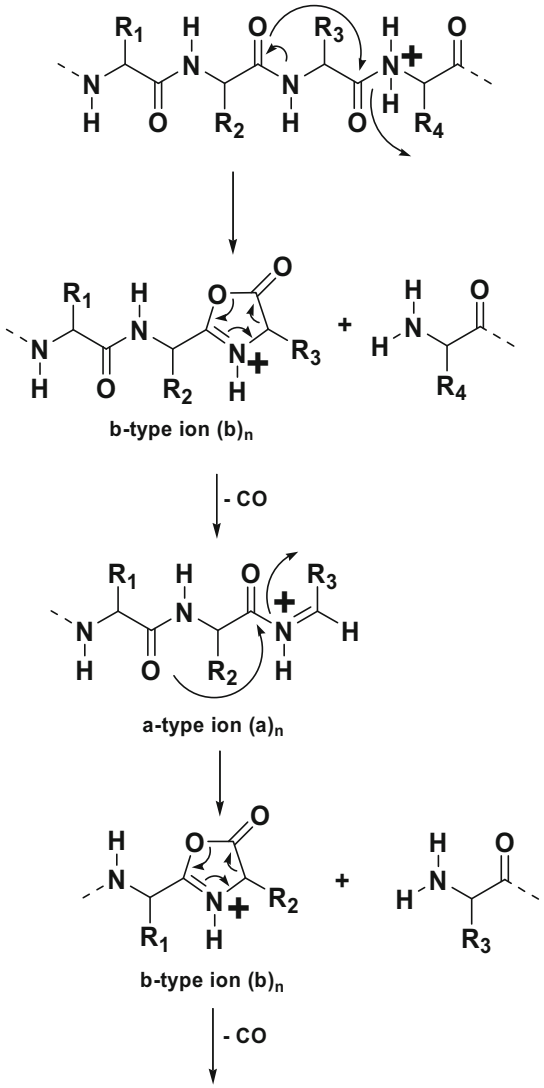
Scheme 10

N-terminal nitrogen-bound hydrogen is thought to be transferred to the RCH=N— group before its cleavage as HN=CHR for all other peptides (Scheme **9**, pathway 1). However, this hydrogen atom does not exist in $(PGG + P - 2H)^+$. Thus, although $(PGG + P - 2H)^+$ can undergo successive losses of H_2O and CO like other peptide's analogous products, the loss of NH=CHR cannot take place. Instead, $\cdot N{=}CH_2$ is lost in a homolytic bond cleavage (Scheme **10**).

Pathway 2 in Scheme **9** accounts for the formation of most of the other sequence-related fragment ions from isomer C of $(AGG + P - 2H)^+$, such as $(AGG + P - 2H - CO)^+$, $(b_2 + P + O - H)^+$, $(a_2 + P + O - H)^+$, $(b_1 + P + O - H)^+$, and $(a_1 + P + O - H)^+$, upon successive losses of several CO and NH=CHR molecules. This pathway is proposed to involve the formation of a protonated oxazolone structure similar to the one proposed [26, 27a, 27b, 28] for b_n ions formed during the dissociation of small protonated peptides (Scheme **11**). Indeed, the steps of pathway 2 resemble those proposed [27, 29] for the formation of b-ions upon low-energy CAD of protonated peptides (Scheme **11**).

Almost all the above-mentioned fragment ions formed in pathway 2 were observed for all the peptides investigated, with a few exceptions. Fragmentation of $(PGG + P - 2H)^+$ stops at the $(b_1 + P + O - H)^+$ ion (Scheme **10**). No further fragmentation of this ion was observed. The reason for this lack of fragmentation is currently under investigation.

Similarly, SORI-CAD of $(GFA + P - 2H)^+$ does not give rise to all the product ions observed for other $(peptide + P - 2H)^+$ ions. $(GFA + P - 2H)^+$ does not form the $(b_1 + P + O - H)^+$ and $(a_1 + P + O - H)^+$ fragments. The lack of further fragmentation of $(a_2 + P + O - H)^+$ to these fragments may arise from its special stability resulting from delocalization of the positive charge into the aromatic ring after a hydride transfer (Scheme **12**). Further, although dissociation of $(peptide + P - 2H)^+$ of AGG, PGG, AAA, VGG, and PLG-amide results in the formation of a_1 ions (an

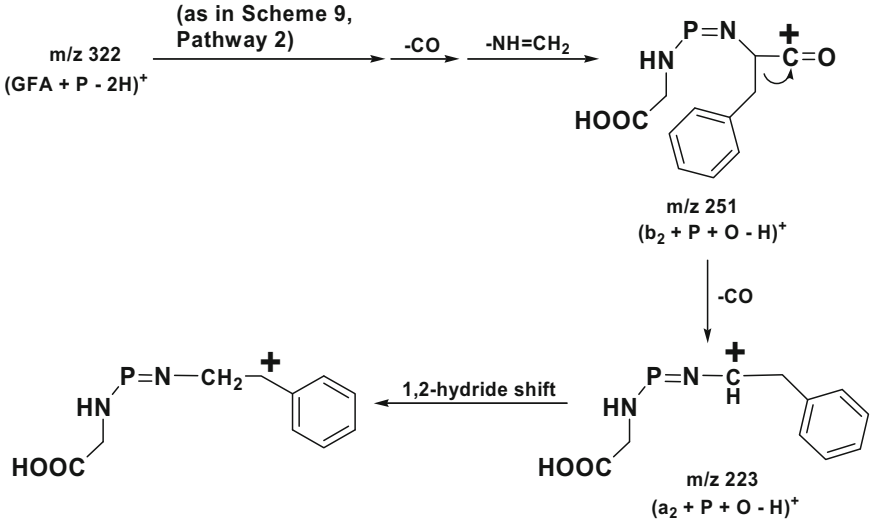


Scheme 11

Table 4. Product ions observed upon SORI-CAD of $(M + H)^+$ formed by the reaction of neutral peptides with $[(CH_3)_2COH]^+$

Fragmenting ion, *m/z*	*m/z*	Product ions
$(VGG + H)^+$, 232	186	$(VGG + H - H_2O - CO)^+$
	157	b_2
	72	a_1
$(GFA + H)^+$, 294	276	$(GFA + H - H_2O)^+$
	205	b_2
	177	a_1
	120	Immonium ion of phenylalanine
$(AGG + H)^+$, 204	129	b_2
$(PGG + H)^+$, 258	155	b_2
$(AAA + H)^+$, 232	214	$(AAA + H - H_2O)^+$
	161	y_2
	143	b_2
	115	a_2
	72	b_1
	44	a_1

immonium ion corresponding to the N-terminal amino acid), the ion of GFA yields an a_2 ion (Table 3). This observation suggests that phenylalanine residue provides extra stabilization to the a_2 ion. Similar observations were made in an earlier study [30] where fragmentation reactions of protonated tri- and tetrapeptides containing phenylalanine were examined. The tripeptides containing phenylalanine as the central residue predominantly fragment by forming the b_2 ion, which fragments further to the a_2 ion. All other protonated tripeptides, including those containing phenylalanine either in the N- or the C-terminus, primarily yield the a_1 ion or either the b_2 ion or the y_1 ion. Another study [27c] carried out to



Scheme 12

examine the pathways leading to the formation of immonium ions (a_n) from protonated peptides revealed that a_1 ions can be formed directly by fragmentation of b_2 ions. This appears to be an important route for the fragmentation of b_2 ions when the a_1 immonium ion is more stable than the a_2 ion formed by loss of CO from the b_2 ion.

Complete sequencing of the peptides studied here can be achieved by considering the fragment ions, especially the $(b_2 + P - 3H)^+$, $(b_2 + P + O - H)^+$, $(a_2 + P + O - H)^+$, $(b_1 + P + O - H)^+$, $(a_1 + P + O - H)^+$, a_1, a_2, and b_2 ions, formed upon SORI-CAD of (peptide + $P - 2H)^+$. The mechanisms for the formation of the regular b_n- and a_n-type ions from the addition/elimination product (peptide + $P - 2H)^+$ during the SORI-CAD event in the current study are currently under investigation.

To compare the amount of structural information that can be obtained from CAD of (peptide + $P - 2H)^+$ with that obtained by CAD of the corresponding protonated peptides, SORI-CAD was carried out for protonated tripeptides under the same conditions as for (peptide + $P - 2H)^+$. The protonated tripeptides undergo a preferential cleavage at the C-terminus, predominantly forming a_n and b_2 ions (Table 4). Similar observations were reported in earlier studies [26, 29, 31]. It is evident from the above results that more sequence-related fragment ions for each amino acid residue are formed upon SORI-CAD of the addition/elimination product than the protonated peptide.

Conclusions

The LIAD/$P(OCH_3)_2^+$/FT-ICR mass spectrometric approach yields abundant ion-molecule reaction products for neutral peptides, including a major primary addition/elimination product, (peptide + P $- 2H)^+$, by loss of two methanol molecules. Various experimental observations suggest that $P(OCH_3)_2^+$ attacks the N-terminal amino group of the peptides to form this product. Molecular orbital calculations as well as experimental observations suggest that an isomer where the P is bonded to the N-terminal amino nitrogen and the adjacent amino acid's amido nitrogen is the likely structure of the (peptide + P $- 2H)^+$ ion. SORI-CAD of the isolated addition/elimination product, (peptide + $P - 2H)^+$, yields diagnostic fragment ions, such as $(b_2 + P - 3H)^+$, $(b_2 + P + O - H)^+$, $(a_2 + P + O - H)^+$, $(b_1 + P + O - H)^+$, and $(a_1 + P + O - H)^+$, as well as regular a_n and b_2 ions, indicating that peptide bonds are cleaved in a specific fashion. The formation of various sequence-related ions can be explained by the successive losses of CO and NH=CHR or H_2O, CO, and NH=CHR. Comparison of the SORI-CAD spectra of (peptide + $P - 2H)^+$ and the analogous protonated peptides shows that CAD of (peptide + $P - 2H)^+$ yields a larger number of sequence-related fragment ions. These findings suggest that the LIAD/$P(OCH_3)_2^+$/SORI-CAD/FT-ICR approach has the potential to provide useful sequence information for peptides.

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