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Fast Atom Bombardment Mass Spectra of *N*-Phosphorylated Peptide Analogs

Hua Fu,[1] Zhaolong Li,[1] Jingzun Wang[2] and Yufen Zhao[1]*
[1] Bioorganic Phosphorus Chemistry Laboratory, Department of Chemistry, Tsinghua University, Beijing 100084, China
[2] Beijing Institute of Microchemistry, Beijing 100091, China

A series of *N*-(*N*-alkyl)- and *N*-(*O*-alkyl)phosphopeptide analogs were determined by positive and negative ion fast atom bombardment mass spectrometry and their fragmentation pathways were investigated. Their mass spectra gave abundant $[MH - NH_2R']^+$, $[MH - HOR'']^+$, $[MH - H_2O]^+$ or $[M - H - NH_2R']^-$, $[M - H - HOR'']^-$ ions, which contained pentavalent tricoordinate phosphorus ions through the loss of amine or water. In the positive ion mass spectra, $^+NH_3R'$ and $^+NH_3CHRCONHR'$ were found, while $[MH - NH_2CHRCONHR']^+$ was suppressed. In the negative ion mass spectra, the pseudomolecular ion $[M - H]^-$ was the base peak, and almost all the fragment ions contained a phosphoryl group. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

N-Phosphoamino acids show many interesting bioorganic chemical reactions under mild conditions.[1–6] For example, *N*-phosphoamino acids can self-activate to give peptides and esters, together with the phosphoryl ester-exchanged and phosphoryl group migration products. A mechanism involving a pentacoordinate intramolecular phosphoric–carboxylic mixed anhydride intermediate was suggested, which was proved by the synthesis of a series of pentacoordinate phosphoric–carboxylic mixed anhydrides.[7] Previously, some *N*-dialkyloxyphosphoamino acids and -peptides were studied by positive and negative ion fast atom bombardment mass spectrometry (FABMS),[8–11] and their fragmentation patterns were summarized. It is interesting that by introduction of the *N*-dialkyloxyphosphoryl group into amino acids, the sensitivity of their positive ion FAB mass spectra could be improved by factors of 4–29. The chemical noise derived from the liquid matrix was greatly reduced.[8] For *N*-phosphopeptides, their positive ion FAB mass spectra showed a novel cleavage pattern in which only the *N*-phosphoryl fragment ions gave intense peaks, while their *C*-terminal series ion and the glycerol matrix were suppressed.[10,11] All these advantages make it convenient to identify the structure of amino acids by FABMS and provide a promising approach for molecular mass determination and sequence analysis for peptides. In this work, a series of *N*-(*N*-alkyl)- and *N*-(*O*-alkyl)phosphorylated peptide analogs were studied by positive and negative ion FABMS and their mass spectral fragmentation pathways were elucidated.

* *Correspondence to*: Y. Zhao, Bioorganic Phosphorus Chemistry Laboratory, Department of Chemistry, Tsinghua University, Beijing 100084, China.
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EXPERIMENTAL

Preparation of samples

***N*-(*N*-Alkyl)phosphorylated peptide analogs $2a_1$–h_1.** Under a nitrogen atmosphere, benzylamine or pentylamine was added to a fresh benzene solution of pentacoordinate phosphoric–carboxylic mixed anhydrides **1a–h**[7] at 25 °C and after hydrolysis each product was isolated by high-performance liquid chromatography (HPLC) to give $\mathbf{2a_1}$–$\mathbf{h_1}$ and $\mathbf{2a_2}$–$\mathbf{h_2}$.

***N*-(*O*-Alkyl)phosphorylated peptide analogs 3a–h.** Each of the *N*-(*N*-benzyl)phosphorylated peptide analogs **2a–h** was reacted with an alcohol solution to give the corresponding *N*-(*O*-alkyl)phosphorylated peptide analogs **3a–h**, which were purified by HPLC.

Their structures were characterized by ^1H, ^{13}C and ^{31}P NMR spectroscopy. For example, the ^1H, ^{13}C and ^{31}P NMR data for $\mathbf{2d_1}$ and $\mathbf{3d_1}$ are listed as examples. The ^1H and ^{13}C NMR spectra were recorded on a

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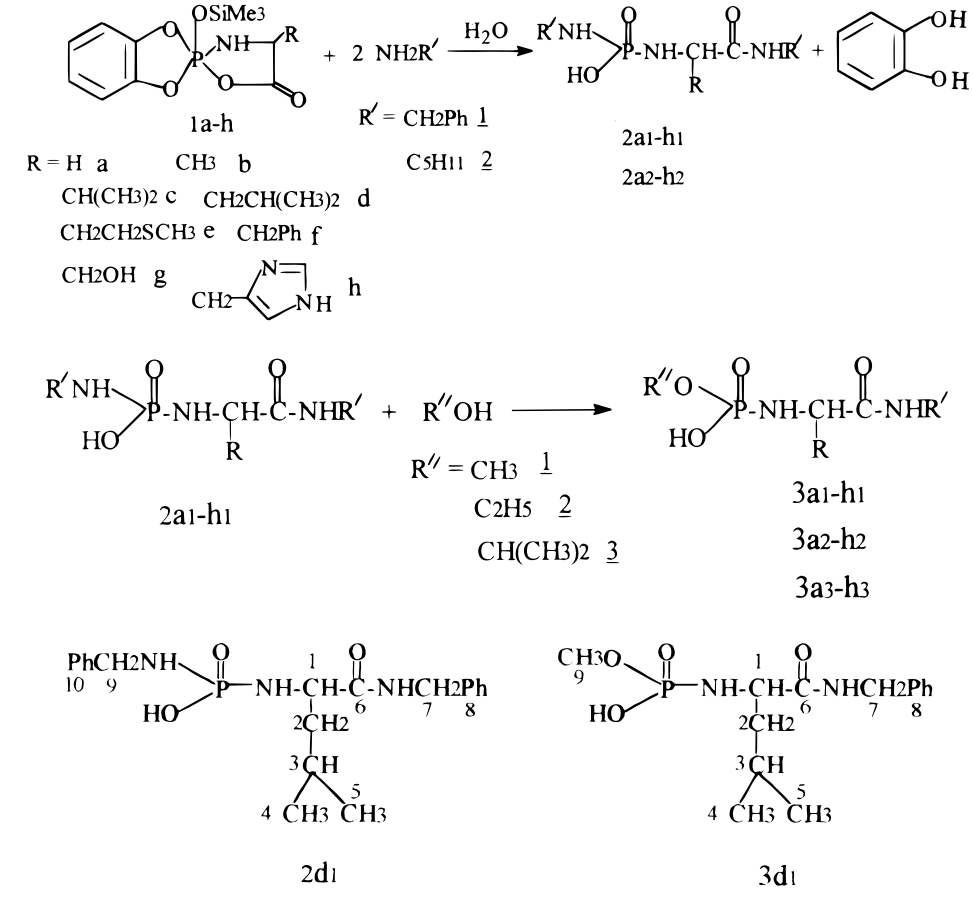


Bruker AM 500 spectrometer at 500 MHz and the ^{31}P NMR spectra on a Bruker AM 200 spectrometer at 200 MHz, with D_2O as solvent.

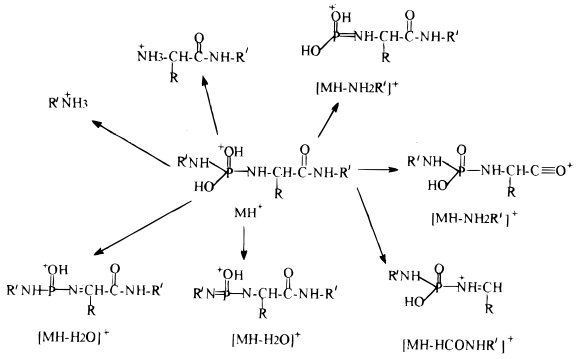
For *N*-(*N*-benzyl)phospholeucine *N*-benzylamide (**2d**$_1$): ^1H NMR, δ 0.94, 1.02 (q, 6H, 4,5-CH_3), 1.76 (m, 1H, 3-CH), 2.42 (m, 2H, 2-CH_2), 3.87 (m, 1H, 1-CH, $^3J_{P,H}$ = 9.1 Hz), 4.24 (s, 2H, 7-CH_2), 4.57 (d, 2H, 9-CH_2, $^3J_{P,H}$ = 6.9 Hz), 7.43–7.71 (m, 10H, 8,10-Ph); ^{13}C NMR, δ 21.7 23.1 (4,5-CH_3), 25.20 (3-CH), 42.86 (2-CH_2), 44.23, 44.61 (7,9-CH_2, $^2J_{P,C}$ = 7.2 Hz), 53.36 (1-CH, $^2J_{P,C}$ = 7.4 Hz), 128.21, 128.26, 128.54, 129.17, 129.94, 130.15, 131.79, 132.33, 133.57, 134.37, 135.33, 136.77 (8,10-Ph), 166.23 (6-CO); ^{31}P NMR, δ 10.8.

For *N*-(*O*-methyl)phospholeucine *N*-benzylamide (**3d**$_1$): ^1H NMR, δ 0.91, 1.02 (q, 6H, 4,5-CH_3), 1.73 (m, 1H, 3-CH), 2.43 (m, 2H, 2-CH_2), 3.63 (s, 2H, 9-CH_2, $^3J_{P,H}$ = 8.2 Hz), 3.87 (m, 1H, 1-CH, $^3J_{P,H}$ = 8.4 Hz), 4.56 (s, 7-CH_2), 7.44–7.50 (m, 5H, 8-Ph); ^{13}C NMR, δ

Table 1. Positive ion FABMS main fragment ions for *N*-(*N*-alkyl)phosphorylated peptide analogs 2a–h [relative intensity (%) in parentheses][a]

2	MH$^+$	[MH − H_2O]$^+$	[MH − NH_2R']$^+$	[MH − HCONHR′]$^+$	[X + H]$^+$	$^+NH_3R'$
a$_1$	334 (15)	316 (13)	227 (100)	199 (14)	165 (16)	108 (21)
a$_2$	294 (13)	276 (12)	207 (100)	179 (15)	145 (12)	88 (16)
b$_1$	348 (16)	330 (14)	241 (100)	213 (16)	179 (18)	108 (23)
b$_2$	308 (13)	290 (12)	221 (100)	193 (14)	159 (15)	88 (26)
c$_1$	376 (14)	358 (8)	269 (100)	241 (12)	207 (18)	108 (20)
c$_2$	336 (12)	318 (7)	249 (100)	221 (5)	187 (34)	88 (12)
d$_1$	390 (14)	372 (16)	283 (100)	255 (10)	221 (16)	108 (10)
d$_2$	350 (12)	332 (10)	263 (100)	235 (5)	201 (10)	88 (8)
e$_1$	408 (18)	390 (16)	301 (100)	273 (8)	239 (21)	108 (23)
e$_2$	368 (14)	350 (15)	281 (100)	253 (8)	219 (21)	88 (27)
f$_1$	424 (19)	406 (19)	317 (100)	289 (12)	255 (20)	108 (30)
f$_2$	384 (18)	366 (20)	297 (100)	269 (11)	235 (22)	88 (24)
g$_1$	364 (10)	346 (18)	257 (100)	229 (8)	195 (18)	108 (19)
g$_2$	324 (8)	306 (17)	237 (100)	209 (9)	175 (16)	88 (23)
h$_1$	414 (28)	396 (3)	307 (100)	279 (4)	245 (30)	108 (44)
h$_2$	374 (15)	356 (3)	287 (100)	259 (3)	225 (42)	88 (32)

[a] X = $H_2NCHRCONHCH_2Ph$; R′ = CH_2Ph (subscript 1) and C_5H_{11} (2).



Scheme 1. Positive ion FAB mass spectral fragmentation pathways for *N*-(*N*-alkyl)phosphorylated peptide analogs **2a–h**.

21.7, 23.1 (4,5-CH_3), 25.18 (3-CH), 42.86 (2-CH_2), 44.32 (7-CH_2), 52.82 (9-CH_3, $^2J_{P,C}$ = 6.3 Hz), 53.85 (1-CH, $^2J_{P,C}$ = 7.5 Hz), 127.57, 128.16, 128.78, 129.18, 129.45, 129.78, (8-Ph), 167.18 (6-CO); ^{31}P NMR, δ 8.2.

Mass spectrometric conditions

Positive and negative ion FAB mass spectra were recorded on a Finnigan MAT 90 double-focusing instrument (Finnigan MAT, Bremen, Germany) of BE geometry. The standard gun was operated at current 5 mA and energy 20 keV using the Cs^+ ion as a bombarding ion. All the samples were measured in a glycerol matrix. Some ions were analyzed using the manufacturer's software for *B*/*E* linked scan in the first field-free region of the instrument. The scan rate was 10 s per decade, and conventional resolution of the instrument was adjusted to 1000 (10% valley definition). The scan mass range was from 50 to 800 u.

RESULTS AND DISCUSSION

Positive ion FAB mass spectra of *N*-(*N*-alkyl)- and *N*-(*O*-alkyl)phosphorylated peptide analogs 2a–h and 3a–h

According to Table 1, for each of the *N*-(*N*-alkyl)phosphorylated peptide analogs **2a–h**, all the protonated molecular ion MH^+ was observed together with a peak due to the loss one molecule of water, $[MH - H_2O]^+$;

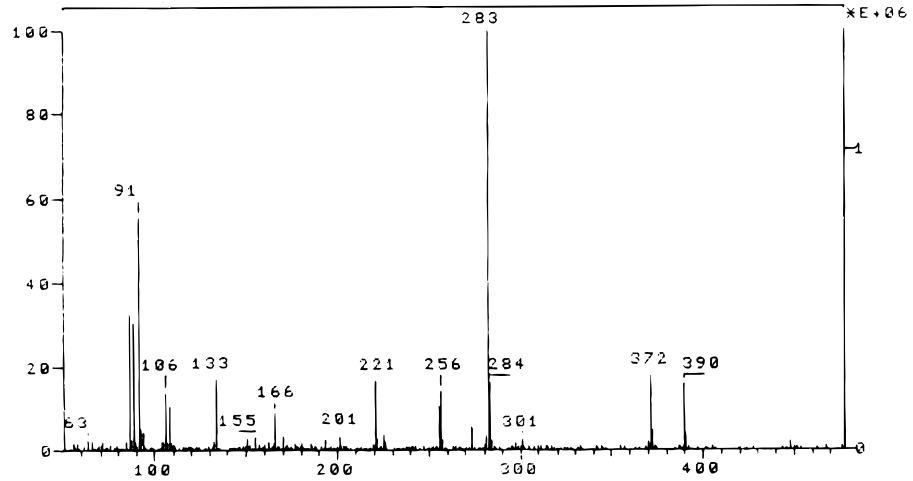


Figure 1. Positive ion FAB mass spectrum of *N*-(*N*-benzyl)phospholeucine *N*-benzylamide (**2d$_1$**).

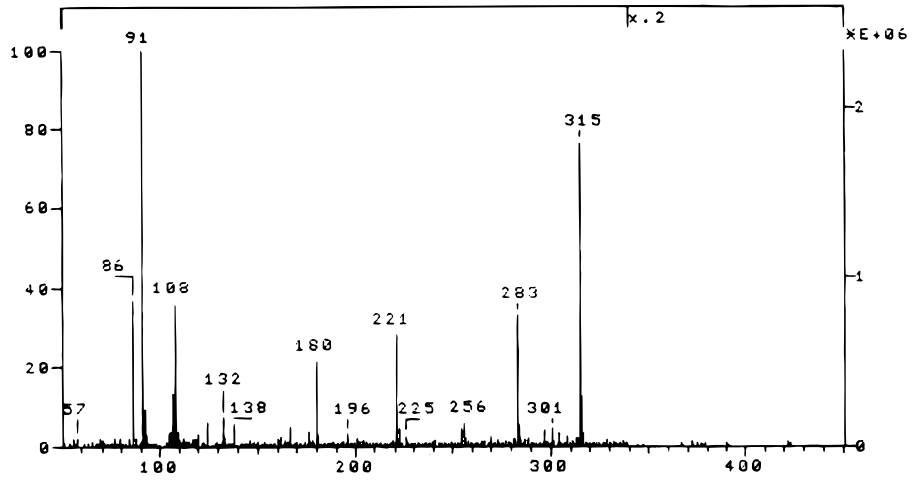
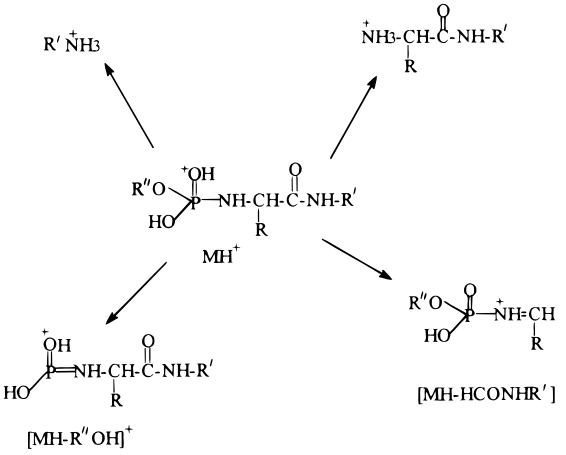


Figure 2. Positive ion FAB mass spectrum of *N*-(*O*-methyl)phospholeucine *N*-benzylamide (**3d$_1$**).



Scheme 2. Positive ion FAB mass spectral fragmentation pathways for *N*-(*O*-alkyl)phosphoamino acid *N*-benzylamides **3a–h**.

the ions $[MH - NH_2R']^+$ showed the base peaks as deduced by either the cleavage of an amide bond, —CO—NH— or by loss of one molecule of amine, —P(O)—NH— bonds. Other ions, $[MH - CONHR']^+$, $^+NH_3R'$ and $[H_2NCHRCONHR' + H]^+$, were observed, similarly to the fragmentation behaviour of a peptide.[12] Their general fragmentation pathways are shown in Scheme 1. For example, the spectrum of *N*-(*N*-benzyl)phospholeucine *N*-benzylamide ($\mathbf{2d_1}$) is shown in Fig. 1. The protonated molecular ion at *m/z* 390 together with the water-loss ion at *m/z* 372 were observed. The base peak at *m/z* 283 was due to the loss of a benzylamine molecule. Ions $^+NH_3R'$ at *m/z* 108 and $^+NH_3CHRCONHR'$ at *m/z* 221 were also observed.

When the *N*-alkyl group on phosphorus in compounds **2a–h** was displaced by *O*-alkyl to give **3a–h**, their mass spectra showed some similar fragmentation pathways, as shown in Table 2, Fig. 2 and Scheme 2, with the difference that $[MH - H_2O]^+$, $[MH - NH_2R')^+$ were suppressed. It has been reported previously that *N*-(*O*-dialkyl)phosphoamino acids and -peptides usually exhibit the peaks due to two successive alkene losses,[8–10] while the *N*-(-*O*-alkyl)phosphorylated peptide analogs **3a–h**, containing a hydroxyl on phosphorus, could lose an alcohol to form pentavalent tricoordinate phosphorus ions.

Negative ion FAB mass spectra of *N*-(*N*-alkyl)- and *N*-(*O*-alkyl)phosphorylated peptide analogs

The negative ion FAB mass spectral data for *N*-(*N*-alkyl)phosphorylated peptide analogs are given in Table 3. It can be seen that all pseudomolecular ions $[M - H]^-$ for **2a–h** were the base peaks, which were correlated with their structures due to a strong acidic hydroxyl group on the phosphorus. The abundant ions $[M - H - NH_2R']^-$ and $[M - H - NH_2CHRCONHR']^-$ were also observed. For example, for *N*-(*N*-alkyl)phospholeucine *N*-benzylamide ($\mathbf{2d_1}$) (Fig. 3), the deprotonated molecular ion at *m/z* 388 showed the base peak and, the peaks at *m/z* 281 and 168 were due to $[M - H - NH_2R']^-$ and $[M - H - NH_2CHRCONHR']^-$. Their fragmentation pathways are shown in Scheme 3. It is worth noting that almost all ions contained a phosphoryl group.

For all the *N*-(*O*-alkyl)phosphorylated peptide analogs **3d–h**, their negative FAB mass spectra showed

Table 2. Positive ion FABMS main fragment ions for *N*-(*O*-alkyl)phosphoamino acid *N*-benzylamides 3a–h [relative intensity (%) in parentheses][a]

3	MH^+	$[MH - R''OH]^+$	$[X + H]^+$	$[MH - HCONHCH_2Ph]^+$	$^+NH_3CH_2Ph$
$\mathbf{a_1}$	259 (62)	227 (36)	165 (23)	124 (12)	108 (47)
$\mathbf{a_2}$	273 (56)	227 (32)	165 (31)	138 (14)	108 (53)
$\mathbf{a_3}$	287 (45)	227 (28)	165 (18)	152 (8)	108 (47)
$\mathbf{b_1}$	273 (68)	241 (31)	179 (32)	138 (6)	108 (37)
$\mathbf{b_2}$	287 (57)	241 (27)	179 (27)	152 (11)	108 (33)
$\mathbf{b_3}$	301 (58)	241 (30)	179 (21)	166 (5)	108 (31)
$\mathbf{c_1}$	301 (70)	269 (42)	207 (31)	166 (9)	108 (47)
$\mathbf{c_2}$	315 (56)	269 (26)	207 (26)	180 (13)	108 (32)
$\mathbf{c_3}$	329 (28)	269 (31)	207 (18)	194 (16)	108 (46)
$\mathbf{d_1}$	315 (74)	283 (30)	221 (28)	180 (20)	108 (35)
$\mathbf{d_2}$	329 (69)	283 (8)	221 (56)	194 (28)	108 (48)
$\mathbf{d_3}$	343 (53)	283 (18)	221 (35)	208 (19)	108 (49)
$\mathbf{e_1}$	333 (45)	301 (18)	239 (22)	198 (23)	108 (40)
$\mathbf{e_2}$	357 (53)	301 (24)	239 (12)	212 (31)	108 (37)
$\mathbf{e_3}$	371 (35)	301 (12)	239 (20)	226 (14)	108 (38)
$\mathbf{f_1}$	349 (38)	317 (16)	255 (13)	214 (12)	108 (41)
$\mathbf{f_2}$	363 (44)	217 (25)	255 (16)	228 (14)	108 (45)
$\mathbf{f_3}$	377 (28)	317 (11)	255 (15)	242 (15)	108 (53)
$\mathbf{g_1}$	389 (22)	257 (8)	195 (12)	154 (8)	108 (41)
$\mathbf{g_2}$	303 (32)	257 (16)	195 (5)	168 (6)	108 (45)
$\mathbf{g_3}$	317 (16)	257 (6)	195 (8)	182 (9)	108 (21)
$\mathbf{h_1}$	339 (18)	307 (12)	245 (42)	204 (15)	108 (32)
$\mathbf{h_2}$	353 (26)	307 (16)	245 (32)	218 (18)	108 (36)
$\mathbf{h_3}$	367 (18)	307 (14)	245 (38)	232 (16)	108 (42)

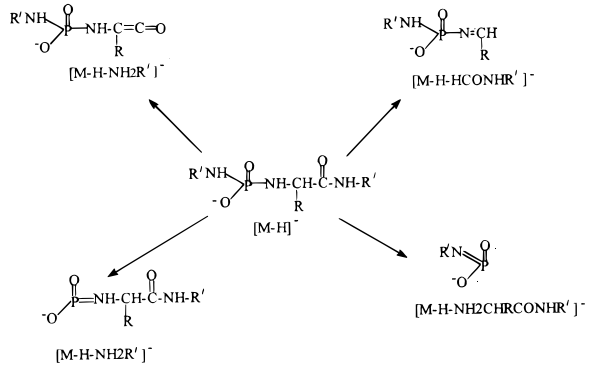
[a] Tropolium ion at *m/z* 91 is the base peak. X = $H_2NCHRCONHCH_2Ph$; R″ = CH_3 (subscript 1), C_2H_5 (2) and $CH(CH_3)_2$ (3).

Table 3. Negative ion FABMS main fragment ions for *N*-(*N*-alkyl)phosphorylated peptide analogs 2a–h [relative intensity (%) in parentheses][a]

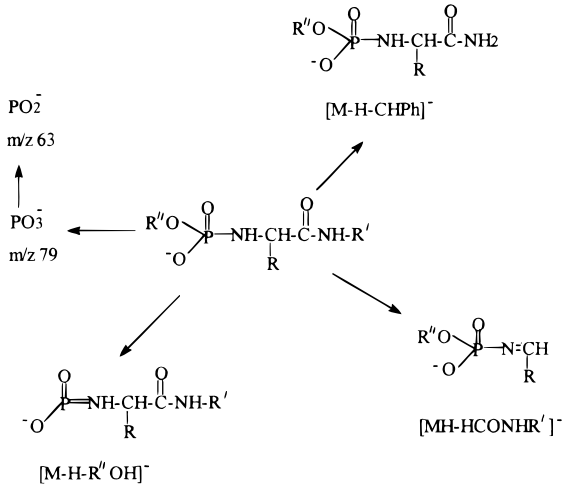
2	$[M-H]^-$	$[M-H-NH_2R']^-$	$[M-H-HCONHR']^-$	$[M-H-NH_2CH\ RCONHR']^-$
a_1	332 (100)	225 (56)	197 (8)	168 (10)
a_2	292 (100)	205 (52)	177 (6)	148 (11)
b_1	346 (100)	239 (48)	211 (10)	168 (12)
b_2	306 (100)	219 (52)	191 (7)	148 (10)
c_1	374 (100)	267 (63)	239 (5)	168 (11)
c_2	334 (100)	247 (88)	219 (7)	148 (11)
d_1	388 (100)	281 (44)	253 (5)	168 (12)
d_2	348 (100)	261 (44)	233 (8)	148 (16)
e_1	406 (100)	299 (51)	271 (8)	168 (15)
e_2	366 (100)	279 (49)	251 (6)	148 (14)
f_1	422 (100)	315 (54)	287 (10)	168 (14)
f_2	382 (100)	295 (43)	267 (9)	148 (12)
g_1	362 (100)	255 (32)	227 (7)	168 (15)
g_2	322 (100)	235 (28)	207 (5)	148 (11)
h_1	412 (100)	305 (66)	277 (13)	168 (20)
h_2	372 (100)	285 (63)	257 (12)	148 (18)

[a] R′ = CH_2Ph (subscript 1) and C_5H_{11} (2).

similar fragmentation behaviour to the above (Table 4, Scheme 4). For example *N*-(*O*-alkyl)phospholeucine *N*-benzylamide (**3d**$_1$) (Fig. 4) showed an abundant deprotonated molecular ion at *m/z* 313. In addition, the ion at *m/z* 281 came from loss of an alcohol. Abundant ions corresponding to PO_2^- at *m/z* 63 and PO_3^- at *m/z* 79 were also found, while ions [M − H



Scheme 3. Negative ion FAB mass spectral fragmentation pathways for *N*-(*N*-alkyl)phosphorylated peptide analogs **2a–h**.



Scheme 4. Negative ion FAB mass spectral fragmentation pathways for *N*-(*O*-alkyl)phosphoamino acid *N*-benzylamides.

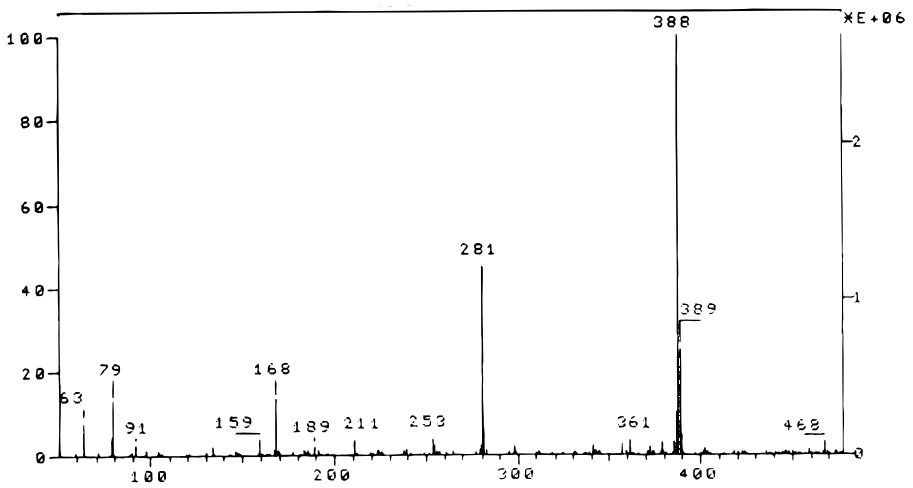


Figure 3. Negative ion FAB mass spectrum of *N*-(*N*-benzyl)phospholeucine *N*-benzylamide (**2d**$_1$).

Table 4. Negative ion FABMS main fragment ions for *N*-(*O*-alkyl)phosphoamino acid *N*-benzylamides 3a–h [relative intensity (%) in parentheses][a]

3	$[M-H]^-$	$[M-H-NH_2R']^-$	$[M-H-HCONHR']^-$	PO_3^-	PO_2^-
a_1	257 (100)	225 (55)	122 (5)	79 (64)	63 (22)
a_2	271 (42)	225 (34)	136 (8)	79 (100)	63 (26)
a_3	285 (23)	225 (21)	150 (12)	79 (100)	63 (21)
b_1	271 (100)	239 (42)	136 (6)	79 (57)	63 (20)
b_2	285 (35)	239 (34)	150 (11)	79 (100)	63 (31)
b_3	299 (31)	239 (21)	164 (15)	79 (100)	63 (27)
c_1	299 (100)	267 (39)	164 (7)	79 (61)	63 (23)
c_2	313 (47)	267 (30)	178 (4)	79 (100)	63 (18)
c_3	327 (40)	267 (21)	192 (9)	79 (100)	63 (16)
d_1	313 (100)	281 (34)	178 (8)	79 (60)	63 (25)
d_2	327 (32)	281 (20)	192 (5)	79 (100)	63 (24)
d_3	341 (10)	281 (9)	206 (1)	79 (100)	63 (15)
e_1	331 (100)	299 (51)	196 (4)	79 (65)	63 (24)
e_2	345 (51)	299 (35)	210 (7)	79 (100)	63 (20)
e_3	359 (28)	299 (19)	224 (8)	79 (100)	63 (13)
f_1	347 (100)	315 (53)	212 (11)	79 (54)	63 (21)
f_2	361 (41)	315 (32)	226 (10)	79 (100)	63 (21)
f_3	375 (26)	315 (27)	240 (7)	79 (100)	63 (12)
g_1	287 (100)	255 (41)	152 (8)	79 (61)	63 (31)
g_2	301 (65)	255 (32)	166 (6)	79 (100)	63 (35)
g_3	315 (39)	255 (27)	180 (4)	79 (100)	63 (32)
h_1	337 (100)	305 (48)	202 (5)	79 (58)	63 (18)
h_2	351 (52)	305 (29)	216 (7)	79 (100)	63 (23)
h_3	365 (37)	305 (33)	230 (4)	79 (100)	63 (28)

[a] R″ = CH_3 (subscript 1), C_2H_5 (2) and $CH(CH_3)_2$ (3).

$-NH_2CHRCONHR']^-$ at *m/z* 95 and $[M-H-NH_2R']^-$ at *m/z* 106 were suppressed.

CONCLUSION

The positive and negative ion FAB mass spectra of *N*-phosphorylated peptide analogs provided abundant structural information and could complement each other. In the positive ion mass spectra, the abundance of the protonated molecular ion was lower, whereas abundant $[MH-NH_2R']^+$ could be observed and $[MH-H_2O]^+$, $^+NH_3R'$ and $^+NH_3CHRCONHR'$ were also found. In the negative ion mass spectra, almost all of the deprotonated molecular ions were base peaks, and fragment ions contained a phosphoryl group. FABMS has the advantages of low discrimination and good detection limits for the analysis of *N*-phosphorylated peptide analogs.

Acknowledgements

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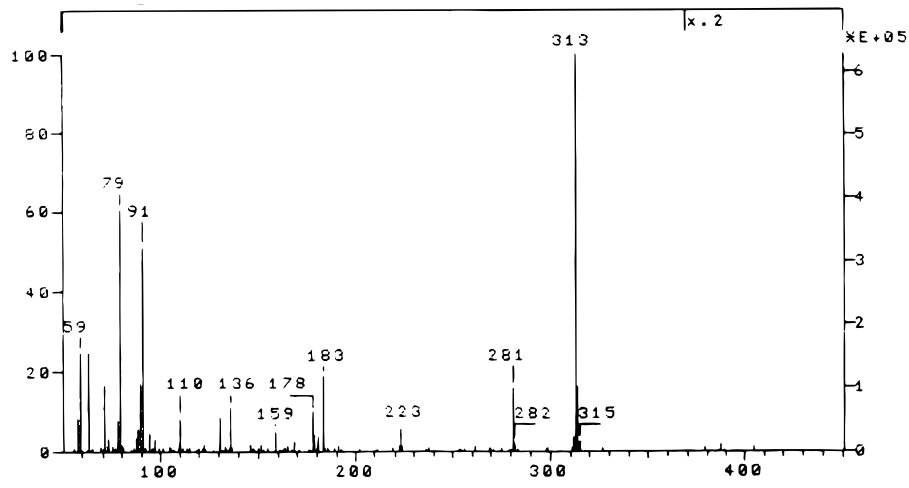

Figure 4. Negative ion FAB mass spectrum of *N*-(*O*-methyl)phospholeucine *N*-benzylamide (**3d$_1$**).

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