A novel fragmentation rearrangement reaction with a carboxyl oxygen negative charge migration was observed in the N-terminal protected amino acids including Fmoc-protected phosphoserine, phosphothreonine, and phosphotyrosine and their analogues using the electrospray ionization tandem mass spectrometry (ESI-MS/MS). The possible mechanism of a five-membered ring transition state was proposed and supported by the further experiments. It was found that the tendency of the rearrangement was determined by the blocking status of its C-terminal and the reaction was proved to be independent of the N-terminal and side-chain protecting groups of the amino acids.

Keywords: Terminal-protected amino acids, ESI-MS/MS, Electronic migration, Rearrangement

Ganem et al.\textsuperscript{1,2} first demonstrated that electrospray ionization (ESI) mass spectrometry (MS) could be used to study non-covalent receptor-ligand complexes and an extensive review on the subject was written by Loo\textsuperscript{3}. In the recent years, electrospray ionization tandem mass spectrometry (ESI-MS/MS), a technique especially well suited for mixture analysis and sequence determination of peptides with blocked N-terminal, ragged C-terminal or post-translational modifications, has progressed rapidly as an alternative for analysis of peptides with great efficiency\textsuperscript{4-6}. For instance, this technique is becoming a method of choice for the identification of phosphorylation sites, due to its high reliability, sensitivity and speed\textsuperscript{7-20}.

Earlier, we analyzed phosphorylated amino acids and peptides using several kinds of mass spectrometer\textsuperscript{21-25}. Some oligo-phosphopeptides prepared from Fmoc-protected phosphorylated amino acids such as Fmoc-protected phosphoserine (Fmoc-pSer-OH), phosphothreonine (Fmoc-pThr-OH) and phosphotyrosine (Fmoc-pTyr-OH) are found to produce a kindred ion fragment, which may be attributed to the same rearrangement mechanism for these amino acids.

In this paper, we report a unique rearrangement reaction for the N-terminal protected amino acids and their analogues including amino acids with phosphorylated or non-phosphorylated side-chain based on the results from ESI-MS/MS. For comparison, two phosphorylated amino acids with blocked C-terminal (Table 1, compounds 4 and 5), some other Fmoc-protected amino acids (FPAA) with non-phosphorus side-chain protecting groups (such as tBu, Trt) and Boc-protected amino acids (BPAAs) were also studied using ESI-MS/MS and the potential rearrangement mechanism for these studied amino acids was explored.

Experimental Section

All samples were purchased from Sigma Chemical Co., USA and used without further purification. 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 flow rate 4 L/min. The nebulizer gas for pressure was 7 psi. The electrosopy 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 600 in negative-ion mode. The Fmoc-protected amino acids and their derivatives (Table 1) were analyzed by multi-stage ESI-MS.

Results and Discussion

The structure of Fmoc-protected phosphorylated amino acids (1-3) is shown in Fig. 1 and the fragment
Table 1—Fragment ions observed in the tandem mass spectra of the tested compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Precursor ion</th>
<th>Fragment ions (ms/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>497.4</td>
<td>496.1</td>
<td>496, <strong>299.8</strong>, 187</td>
</tr>
<tr>
<td>2</td>
<td>511.5</td>
<td>510.5</td>
<td>510, <strong>313.9</strong>, 287.9, 187</td>
</tr>
<tr>
<td>3</td>
<td>483.4</td>
<td>482.8</td>
<td>482, 303.7, <strong>288</strong>, 262</td>
</tr>
<tr>
<td>4</td>
<td>615.6</td>
<td>614.2</td>
<td>614, 392.7, 187</td>
</tr>
<tr>
<td>5</td>
<td>511.4</td>
<td>510.2</td>
<td>510, 290, 287.187</td>
</tr>
<tr>
<td>6</td>
<td>383.4</td>
<td>382.2</td>
<td>382.2, <strong>186.5</strong>, 160.5</td>
</tr>
<tr>
<td>7</td>
<td>397.5</td>
<td>396.4</td>
<td>396.2, <strong>200.4</strong>, 174.4</td>
</tr>
<tr>
<td>8</td>
<td>459.5</td>
<td>458.1</td>
<td>458, <strong>262</strong>, 236</td>
</tr>
<tr>
<td>9</td>
<td>387.4</td>
<td>386.1</td>
<td>386.1, <strong>190.2</strong>, 164.4</td>
</tr>
<tr>
<td>10</td>
<td>295.3</td>
<td>294</td>
<td>294, <strong>220.3</strong></td>
</tr>
<tr>
<td>11</td>
<td>371.4</td>
<td>370.1</td>
<td>370.1, <strong>296.9</strong></td>
</tr>
</tbody>
</table>

a: 1: Fmoc-protected phosphoserine (Fmoc-pSer-OH); 2: Fmoc-protected phosphothreonine (Fmoc-pThr-OH); 3: Fmoc-protected phosphotyrosine (Fmoc-pTyr-OH); 4: Fmoc-pSer-OCH₂COPh; 5: Fmoc-pSer-OCH₃; 6: Fmoc-Ser(tBu)-OH; 7: Fmoc-Thr(tBu)-OH; 8: Fmoc-Tyr(tBu)-OH; 9: Fmoc-Phe-OH; 10: Boc-Ser(Bzl)-OH; 11: Boc-Tyr(Bzl)-OH

b: Measured at negative mode

c: The values in bold and italic are for [M-H-196]⁻ (for Fmoc-protected amino acids) and [M-H-74]⁻ (for Boc-protected amino acids) fragments

![Fig. 1—Structures of three Fmoc-protected phosphorylated amino acids](image)

Scheme 1—Proposed rearrangement mechanism for the Fmoc-protected amino acids without blocked C-terminal.

ions of all compounds are listed in Table 1. In negative-ion ESI-MS/MS spectra, the molecular ions detected in all the compounds were in [M-H]⁻ mode. Comparing the spectra of these compounds, 187 Da fragment ion corresponding to a phosphoryl group (Fig. 2a, b) was found in compounds 1 and 2. Interestingly, a common fragment corresponding to [M-H-196]⁻ was also observed. For example, 299.8 Da, 313.9 Da and 288 Da for compound 1, 2 and 3, respectively (Fig. 2a, b, c). The 195 Da fragment
could be clearly assigned as the Fmoc-group with its carbonyl cleaved off (Scheme 1). But, how the extrusion of [M-H-196] Da was produced and the 196 Da fragment was migrated from the molecular ion are not yet clear. Although, there is no report available on the above-mentioned issues, some results on the rearrangement reaction were obtained in our laboratory with ESI-MS/MS experiments. Hence, the possible mechanism of fragmentation rearrangement pathway is proposed as follows (Scheme 1).

As mentioned above, in negative-ion mode for ESI-MS, precursor ions exist in [M-H]⁻ mode. Along with the removal of active H⁺ ion from the molecular ion,
the oxygen atom of carboxyl takes a negative charge. As indicated in Scheme 1, if the negative charge on the oxygen atom of carboxyl migrates to the carbonyl of Fmoc-group via a five-membered ring transition state, it would lead to the elimination of 196 Da (Fmoc-moietiy) and subsequently generate [M-H-196]⁻ ions. To determine possible role of the negative charge, a phosphorylated Fmoc-protected serine derivative Fmoc-pSer-OCH₃ (5), with the C-terminal blocked with methyl ester was designed. As no negative charge exists on the C-terminal of this compound, there would be no charge migration and the [M-H-196]⁻ ion would not be generated. Thus, according to the proposed rearrangement mechanism (Scheme 1), [M-H-196]⁻ ion, namely 315 Da for compound 5 was not found in the mass spectra (Fig. 2e). The same result was obtained with another ester derivative Fmoc-pSer-OCH₂COPh (4) and there was also no [M-H-196]⁻ ion, namely 412 Da for this compound (Fig. 2d). These experiments strongly supported the proposed rearrangement mechanism (Scheme 1).

To figure out whether the proposed rearrangement reaction (Scheme 1) was specific for the phosphorylated Fmoc-protected amino acids, a series of Fmoc-protected amino acids (FPAA) with other side-chain protecting groups, Fmoc-Ser(tBu)-OH (6), Fmoc-Thr(tBu)-OH (7), Fmoc-Tyr(tBu)-OH (8) and Fmoc-Phe-OH (9) were subsequently studied. The carboxyl group of C-terminal of FPAA takes a negative charge in negative-ion mode ESI-MS spectra and the [M-H-196]⁻ ions were been observed (Table 1). Furthermore, it was also reported that when the C-terminal carboxyl of compound Fmoc-Ser(tBu)-OH (7) was blocked with methyl group, no [M-H-196]⁻ ion was observed either. These experiments further confirmed the proposed mechanism (Scheme 1) and indicated that the side-chain protecting groups of these amino acids did not affect the rearrangement reaction mechanism.

Finally, Boc-protected amino acids 10 and 11 were analyzed and the fragment ions are shown in Table 1. As shown in Table 1, 220.3 Da fragment for compound 10 and 296.9 Da for compound 11 corresponding to [M-H-74]⁻, and 74 Da (as shown in Scheme 2) could be assigned as the migrated Boc-moietiy with its carbonyl group cleaved off, in a manner similar to the above-mentioned Fmoc-protected amino acids. So, this process similarly involved a five-membered ring rearrangement intermediate (Scheme 2) and the N-terminal protecting groups had no effect on the rearrangement reaction.

**Conclusion**

The negative-ion mode ESI-MS experiment was carried out on a number of Fmoc- and Boc-protected amino acids and a new rearrangement mechanism was proposed. Results confirmed that the negative charge on the C-terminal carboxyl via a five-membered ring rearrangement transition state migrated to the carbonyl of the N-terminal protecting group (Fmoc- or Boc-), when the C-terminal of the protected amino acids was not blocked. And this five-membered ring rearrangement was proved to be independent of the N-terminal protecting and side-chain groups of these amino acids.
Acknowledgement

The authors would like to thank the financial support from the National Natural Science Foundation of China (No. 20272032, NSFCBIC20320130046), the Teaching and Research Award Program for Outstanding Young Teachers in Higher Education Institutions of MOE, P.R.C. (TRAPOYT), and the Specialized Research Fund for the Doctoral Program of Higher Education (SRFDP) (No. 20030003049).

References