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Lysine Peroxycarbamates: Free Radical-Promoted Peptide Cleavage

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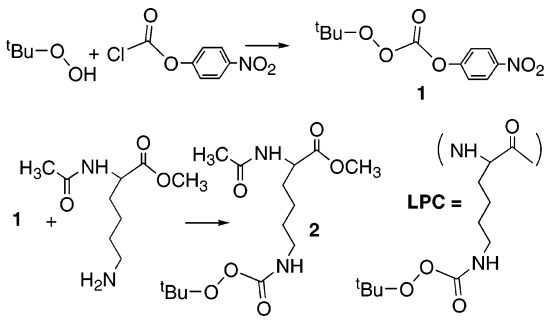
The cornerstone methods used in protein identification are enzymatic proteolysis and analysis of isolated small peptide fragments by mass spectrometry.[1,2] Chemical digestion of proteins has also provided strategies that are helpful in assignment of peptide sequences.[3] Both chemical and enzymatic protein digestion, however, can be cumbersome and time-consuming, and attempts to avoid this step by the use of powerful mass spectrometric "top-down" approaches are being explored to provide protein primary sequences.[4,5]

Strategies that combine in one step a predictable chemical-based protein digestion with mass spectrometry might prove to be convenient for rapid primary peptide sequence analysis. We report here experiments in which lysine residue amino groups in peptides and proteins are modified by reaction with the peroxycarbonate **1**. The resulting lysine peroxycarbamates[6] undergo homolytic fragmentation under conditions of low-energy collision-induced dissociation (CID). One result of this process is predictable peptide fragmentation in the mass spectrometer at or near the modified lysine residues of small peptides.



Reaction of *p*-nitrophenylchloroformate with *tert*-butyl hydroperoxide gave the peroxycarbonate **1**.[7] In basic water or water−acetonitrile mixtures, **1** gave an almost immediate yellow color, indicative of the formation of *p*-nitrophenoxide.[8] In basic aqueous acetonitrile solutions, α*N*-acetyl lysine methyl ester was converted cleanly to **2** on reaction with 10 equiv of **1**. The peroxycarbamate **2** (Ac-LPC-OMe)[9] was stable to chromatography and was fully characterized by spectroscopy and HRMS, see Supporting Information. Analysis of **2** by electrospray showed a parent ion for the H, Li, Na, K, and Ag peroxycarbamate adducts. Collision-induced dissociation (CID) of the parent ion led to loss of −C (O)OO^{t}Bu (m/z = 117) for each of the adducts (Li, Na, K, and Ag gave similar CID results, whereas the H^+ adduct resulted only in loss of H^+). The Li adduct of **2**, for example, gave a parent ion at m/z = 325, while CID on **2** at −15 eV gave a major ion at m/z = 208, see Figure 1. Increasing CID offset energies gave rise to smaller lithium ion complexes at m/z = 192, 150, and 137. CID fragmentation of the lithium adduct of α*N*-acetyl lysine methyl ester, the precursor to **2**, does not give similar ions.

The chemistry associated with the fragmentations of the peroxycarbamate (Li-**2**, m/z = 325) shown in Figure 1 is consistent with an initial free radical dissociation of the weak −O−O− bond

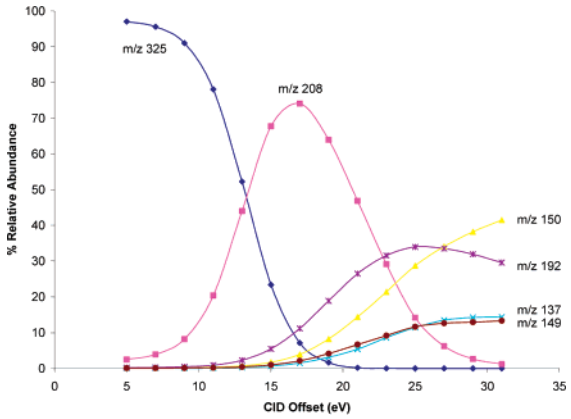
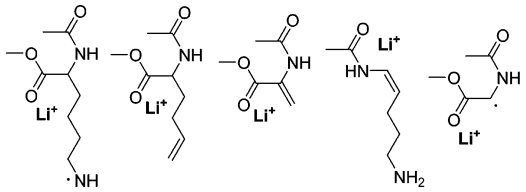


Figure 1. Electrospray collision-induced dissociation ions formed from lithium complex of **2** vs offset energy.

followed by decarboxylation to form an aminyl radical, **3**.[6] shown here as the lithium complex. Other proposed adducts formed by subsequent fragmentation of **3** are shown below. An MS/MS analysis of **3** confirmed that fragments **4**−**7** are daughter ions derived from **3**.



3, m/z 208 **4**, m/z 192 **5**, m/z 150 **6**, m/z 149 **7**, m/z 137

Several peptides were reacted with **1**, and adducts or multiple adducts were observed in every case by electrospray mass spectrometry. Thus, Ac-Gly-Ser-Ala-Lys-Val-Ser-Phe (**Ac-7**; M + 1, m/z = 853.4) gave a product with **1** at m/z = 853.4 + 116, presumably, **Ac-7-LPC** Ac-Gly-Ser-Ala-**LPC**-Val-Ser-Phe. Mass spectral analysis of **Ac-7-LPC** with added LiCl gave monolithium adducts as shown in Scheme 1, E = Li. The formation of each of the species, **9**−**13**, can be understood to be based on generation of an intermediate aminyl radical followed by remote hydrogen abstraction and β-fragmentation of the intermediate carbon radical. Of particular interest is the fact that adducts **9** and **11** result from peptide bond fragmentation. The peptide **14**, Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe gave a peroxycarbamate derivative that also gave peptide cleavage under CID to give a species analogous to **11**, as shown in Scheme 1. The conversion of radical **8** to **11** has ample precedent in the well-known Hofmann−Löffler reaction.[10,11] The carbon radical formed from this sequence, as shown in Scheme 1, gives **11** by a simple β fragmentation, another reaction that is well-known in radical chemistry.

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg (**Ac-15, (M+3)$^{3+}$**, m/z = 712.7) gave products upon reaction with peroxycarbonate **1** in which one, two, or three

10.1021/ja038615u CCC: $27.50

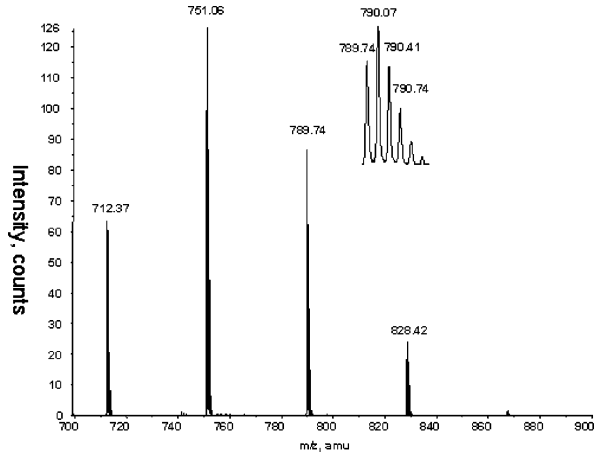
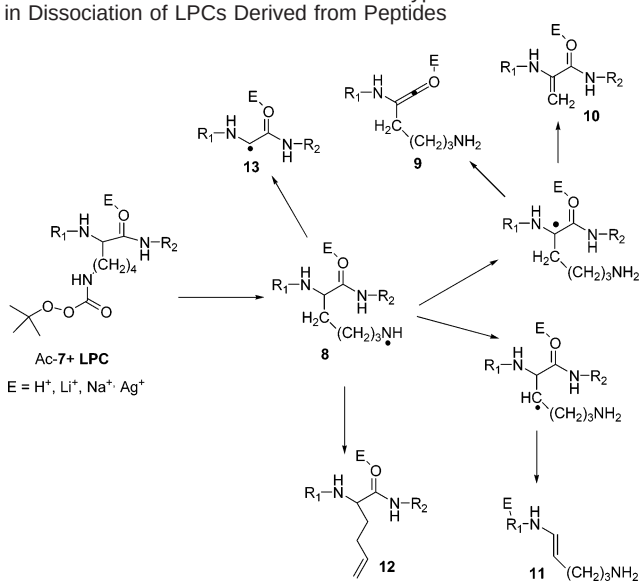


Figure 2. Electrospray mass spectrum $(\mathbf{M}+\mathbf{3})^{3+}$ of peroxycarbamate-modified **Ac-15**. Inset, expansion of $(\mathbf{Ac\text{-}15}+\mathbf{2LPC}+\mathbf{3H})^{3+}$.

Scheme 1. Mechanism for Formation of Typical Products Formed in Dissociation of LPCs Derived from Peptides



free lysine side chain amino groups were converted to peroxycarbamates. Figure 2 shows the electrospray mass spectrum for the **LPC** modified **Ac-15**. The analogous peptide **15**, having a free terminal amine, NH_2-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg, gave products upon reaction with **1** that had up to four amino groups modified. CID of these modified peptides showed, in every case, loss of n $-C(O)OO^tBu$, where n is the number of peroxycarbamates in the peptide species analyzed. Thus, dissociation of $(\mathbf{Ac\text{-}15}+\mathbf{LPC}+\mathbf{3H})^{3+}$ gave $(\mathbf{Ac\text{-}15}+\mathbf{3H})^{3+}$, presumably the radical species analogous to **8**, formed by loss of one peroxycarbamate group while $(\mathbf{Ac\text{-}15}+\mathbf{2LPC}+\mathbf{3H})^{3+}$ lost two $-C(O)OO^tBu$, and so forth.

Matrix assisted laser desorption ionization (MALDI) of the percarbamate-modified peptides also gave backbone fragments formed by loss of the $-C(O)OO^tBu$ groups. The MALDI experiment for **Ac-15** shown in Figure 3 is typical. The spectrum displayed in Figure 3b is for the same mixture of LPC-modified **Ac-15** whose electrospray mass spectrum is shown in Figure 2. By MALDI, no LPC adducts were observed, but a complex set of ions near $m/z = 2135$, that of the parent peptide **Ac-15**, was observed. The LPC-modified peptide clearly did not survive the laser desorption process intact. Fragmentation of the weak peroxide bond presumably leads to aminyl radicals as outlined in Scheme 1. In addition, ions at $m/z = 1423$, 1804, and 1932 were prominent in the LPC-modified compound, while they were not observed for

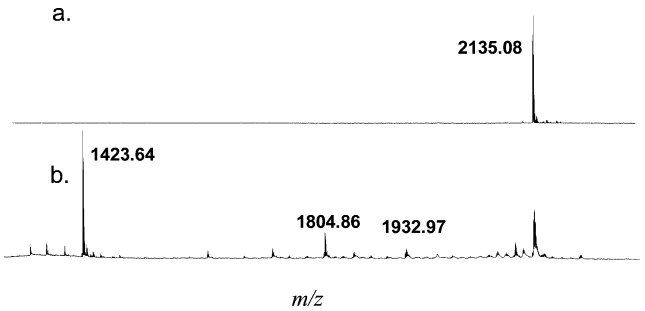


Figure 3. MALDI mass spectrum intensity vs m/z of (a) **Ac-15** and (b) peroxycarbamate-modified **Ac-15** consisting of a mixture of 1, 2, and 3 LPC modifications.

the parent peptide. Each of these ions corresponds to fragmentation of the peptide at one of the three lysine residues in the peptide, with formation of species analogous the enamide **11** with E = H, as shown in Scheme 1. The ion observed at $m/z = 1423$, for example, corresponds to **11** with E = H and R_1 = Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly, while for $m/z = 1804$, R_1 = Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly- and for $m/z = 1932$, R_1 = Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys. Sequencing experiments on the ion with $m/z = 1423$ are consistent with the proposed structure. Loss of $C{=}CH(CH_2)_2{-}NH_2$ from this ion is observed as well as peptide fragmentation expected from the sequence.

We note that proteins having molecular weights of 10000 or greater gave fragmentation patterns by electrospray CID or MALDI that were complex and not readily interpreted. The preliminary results communicated here make it clear, however, that strategies for directed fragmentation of peptides by free radical-promoted processes are feasible[12] and suggest that such strategies might simplify schemes for protein analysis.

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Supporting Information Available: Preparation of percarbonate **1** and full characterization of the peroxycarbamate **2**, MS/MS data for Ac-**7**, and MS/MS data for intermediate **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Usual precautions regarding peroxides were followed for compounds **1** and **2**.
(8) The half-life of **1** in pH 8.2 phosphate buffer (50% MeOH) is approximately 112 s. Details of the kinetics will be published in due course.
(9) For convenience, we adopt here the shorthand **LPC** for lysine peroxycarbamate. The compound **2** is therefore Ac-**LPC**-methyl ester. The peptides use the following shorthand $(\text{peptide}+n\mathbf{LPC}+m\text{E})^{m+}$.
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