359 ($M^+ - C_7H_7$, 20), 91 (C_7H_7, 100). C.I. (isobutane): 451 ($M^+ + 1$, 35).

5,6-Dihydro-2-oxa-4,7-dithiaindan-1,3-dione (11). The dimer **10** (200 mg) was dissolved by heating in a methanolic solution of KOH (2 N). The solvent was evaporated off and the residue was extracted with ethyl acetate and water. The aqueous layer was acidified with 2 N HCl and extracted with ethyl acetate to yield, after the usual workup, 150 mg (71%) of the anhydride **11**; mp 108 °C IR (Nujol) 1835, 1755 (C=O anhydride), 1220 (C—O) cm^{-1}. ^1H NMR ($CDCl_3$) δ 3.38 (4 H, s). ^{13}C NMR ($CDCl_3$) δ 163.8 (s, 2 C), 132.9 (s, 2 C), 26.2 (t, 2 C). MS, *m/e* (rel intensity): 188 (M^+, 75), 144 ($M^+ - CO_2$, 15), 116 ($M^+ - C_2O_3$, 50), 88 (100).

2-(Benzylthio)-7-carbamoyl-2-methoxy-9-phenyl-3,6,8-trithianonanamide (13). Diazomethane was added dropwise to a methanol solution of **4** (500 mg) at −78 °C under nitrogen until a colorless solution was obtained. Following this, 470 mg of $ZnCl_2$ in methanol was added, the temperature was slowly raised, and at −60 °C the first bubbles of nitrogen were observed. When the generation of nitrogen had ceased, the reaction mixture was poured into an aqueous solution affording 450 mg (80%) of **13** by filtration; mp 147 °C. IR (Nujol) 3400, 3200 (NH), 1680 (C=O), 1500 (aromatic rings), 1100 (C—O) cm^{-1}. ^1H NMR (DMSO-d_6) δ 7.29–7.27 (10 H, m), 4.40 (1 H, s), 3.85 (2 H, AB system, J_{AB} = 12 Hz), 3.75 (2 H, AB system, J_{AB} = 12 Hz), 3.38 (3 H, s), 2.84 (4 H, m). ^{13}C NMR (DMSO-d_6) δ 169.7 (s), 168.2 (s), 137.3 (s), 136.6 (s), 128.7 (d, 4 C), 128.2 (d, 4 C), 126.8 (d, 2 C), 98.3 (s), 51.8 (q), 51.8 (d), 34.5 (t), 34.3 (t), 30.0 (t, 2 C). MS, *m/e* (rel intensity): 270 (10), 210 (35), 91 (100).

2-Carbamoyl-4,5-dihydro-3-(methoxycarbonyl)thiophene (14). The adduct **6** (200 mg) was treated with KOH/MeOH (2.5 N) at room temperature with constant stirring for 2 h. The reaction mixture was then quenched with water and extracted with ethyl acetate. The aqueous layer was acidified with HCl (2 N) and extracted with ethyl acetate. The organic layer was concentrated and the residue was esterified with diazomethane to afford after chromatography **14** (20%); mp 73–75 °C. IR (Nujol): 3300 (NH), 1700 (COOMe), 1680 ($CONH_2$) cm^{-1}. ^1H NMR ($CDCl_3$) δ 3.79 (3 H, s), 3.4–3.1 (4 H, m). ^{13}C NMR ($CDCl_3$) δ 164.6 (s), 163.2 (s), 155.1 (s), 123.9 (s), 52.2 (q), 39.6 (t), 28.8 (t). MS, *m/e* (rel intensity): C.I. (isobutane), 188 ($M^+ + 1$, 80), 171 ($M^+ - NH_2$, 60), 129 (($M^+ + 1$) − COOMe, 100).

5-Carbamoyl-7-phenyl-2-oxa-4,6-dithiaheptane (15). Diazomethane was added to a methanol solution of the dithioxamide **4** (300 mg) at −78 °C under nitrogen until the red color had vanished. Two drops of CF_3COOH were added and the reaction mixture was allowed to warm to −50 °C. When the generation of nitrogen had ceased, the reaction was assumed to have finished. The usual workup gave 110 mg of **13** and 150 mg (41%) of **15**, mp 64 °C. IR (Nujol) 3400, 3200 (NH), 1690, 1650 (C=O), 1500 (aromatic ring), 1200 (C—O) cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.25 (5 H, m), 4.72 (2 H, AB system, J_{AB} = 12 Hz), 4.30 (1 H, s), 3.83 (2 H, AB system, J_{AB} = 12 Hz), 3.29 (3 H, s). ^{13}C NMR ($CDCl_3$) δ 171.4 (s), 136.5 (s), 129.0 (d, 2 C), 128.5 (d, 2 C), 127.3 (d), 74.5 (t), 56.3 (q), 50.5 (d), 36.2 (t). MS, *m/e* (rel intensity). 257 (M^+, 20), 226 ($M^+ - OCH_3$, 20), 213 ($M^+ - CONH_2$, 40), 180 ($M^+ - C_2H_5OS$, 35), 134 ($M^+ - SC_7H_7$, 95), 91 (C_7H_7, 100).

Synthesis of Nitroxyl (Aminoxyl) Labeled Probes for Studies of Intracellular Environment by EPR and MRI

George Sosnovsky,*,† Nuti Uma Maheswara Rao,† Shu Wen Li,† and Harold M. Swartz‡

Department of Chemistry, University of Wisconsin—Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201, and College of Medicine at Urbana-Champaign, University of Illinois, Urbana, Illinois 61801

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Syntheses are delineated for the following three classes of nitroxyl (aminoxyl) labeled active esters: (1) phosphoric acid derivatives containing phenyl and *p*-nitrophenyl moieties, (2) EDTA and DTPA containing acetoxymethyl and (pivaloyloxy)methyl as protecting groups, and (3) several amino acid derivatives containing the acetoxymethyl group. These compounds are expected to be of interest as potential probes in studies of intracellular environments by ESR spectroscopy and magnetic resonance imaging (MRI).

Introduction

The nitroxyl radicals originally were developed as biophysical probes for studies of motion in physical and biophysical model systems,[1-6] but now have become employed increasingly in functional biological systems. This development not only has introduced potential new limitations and requirements, especially in regard to solubility and stability, for the properties of nitroxyl probes but also has made possible a broader range of applications of nitroxyl-labeled compounds. In addition to their utilization as probes of motion, nitroxyl radicals also have been employed in biological systems in studies aimed at their use as contrast agents[7-22] for NMR imaging (MRI) and spectroscopy (MRS), agents[23,24] for electron spin resonance imaging (ESRI or EPRI), in vivo ESR spectroscopy,[25] radiosensitizers,[26-28] anticancer agents,[29-35] and probes in metabolism.[20] The latter applications are based on the effect of oxygen on the ESR spectra,[36] on the reduction by cells of nitroxyl radicals to the hydroxylamines[18,37] and the oxidation of hydroxylamines to nitroxyl radicals,[22] including the effect of oxygen on these reactions.

† University of Wisconsin—Milwaukee.
‡ University of Illinois.

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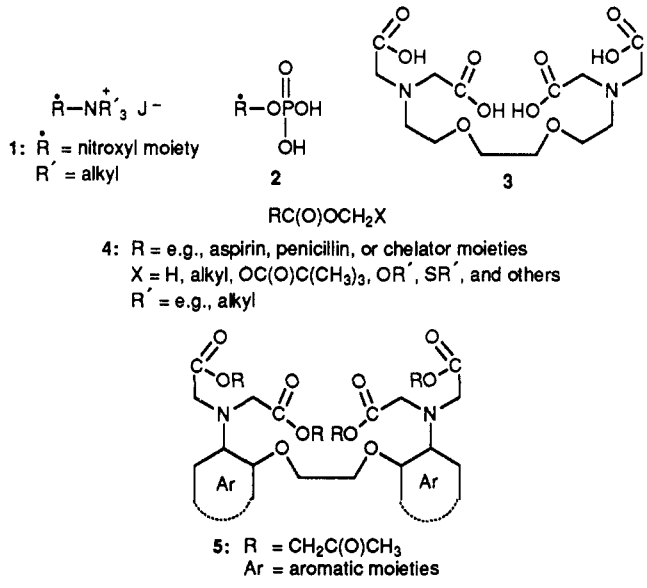
In view of recent developments, especially in areas of MRI, MRS, ESRI, ESRS, and cell-perfusion techniques for magnetic resonance studies, there now is a tremendous potential for the application of nitroxyl probes to detect and characterize diseases associated with hypoxia and/or altered metabolism, such as cancer, inflammation, and ischemia, using the physical and biochemical interactions of nitroxyls with cells. Many of these applications would benefit from the availability of nitroxyl probes that could be localized selectively in the intracellular compartments. The principal metabolism of nitroxyl radicals in vivo occurs intracellularly, and recent studies using techniques that can be applied only in studies of isolated cells have demonstrated the feasibility and potential importance of nitroxyls in measurements of the intracellular concentrations of oxygen.[20,36]

In order to probe the intracellular environment, a number of invasive and noninvasive methodologies have been used.[38] However, the most promising approaches are based on selectively localizing probes on the basis of membrane permeability. It was found[12,15,38,39] that nitroxyl labels with non-ionic or partially ionized monosubstituents, such as amino, hydroxy, keto, amido, and carboxylate functions, equilibrate rapidly through membranes, whereas highly charged species such as quaternary salts (1),[12] phosphoric acid[12] derivatives (2) and polycarboxylate chelators,[39-46] such as EGTA (3), are membrane-impermeant. The polyacids of type 3 can be made membrane-permeant[39-46] by converting them into the so-called active esters (4, 5), which are much more hydrophobic than the parent acids and, consequently, migrate comparatively readily through membranes. These active esters can then be deblocked by intracellular proteases to the corresponding polyacids of type 3, which cannot recross the membranes into the extracellular space.[39-46] The rate of intracellular hydrolysis can vary considerably, depending on the protecting group. Hence, it is possible to design a series of esters ranging from rapidly hydrolyzable (e.g., 4, X = $OC(O)CH_3$), whose acid derivatives would be retained in the intracellular space, to slowly hydrolyzable (e.g., 4, X = CH_3), which could equilibrate through membranes between the intra- and extracellular space.



To date, a number of active esters have been synthesized[46-51] involving important drugs such as aspirin and penicillin. These prodrugs usually contain the readily hydrolyzable moieties of the acylal [4, X = $OC(O)CH_3$, $OC(O)C(CH_3)_3$], acylal acetal (4, X = OR′), or acylal thioacetal (4, X = SR′) type. The most often used protective moiety is the acetoxymethyl group [4, X = $OC(O)CH_3$] derived from bromomethyl acetate.[52] The more readily available protecting moiety the pivaloyloxy group [4, X = $OC(O)C(CH_3)_3$] derived from the commercial chloromethyl pivalate was also proposed,[51] however, apparently not widely used in synthetic design of prodrugs. Initially, this moiety was introduced[53] about two decades ago for the N-protection (aminal–acylal type) in purine syntheses.[53-55]

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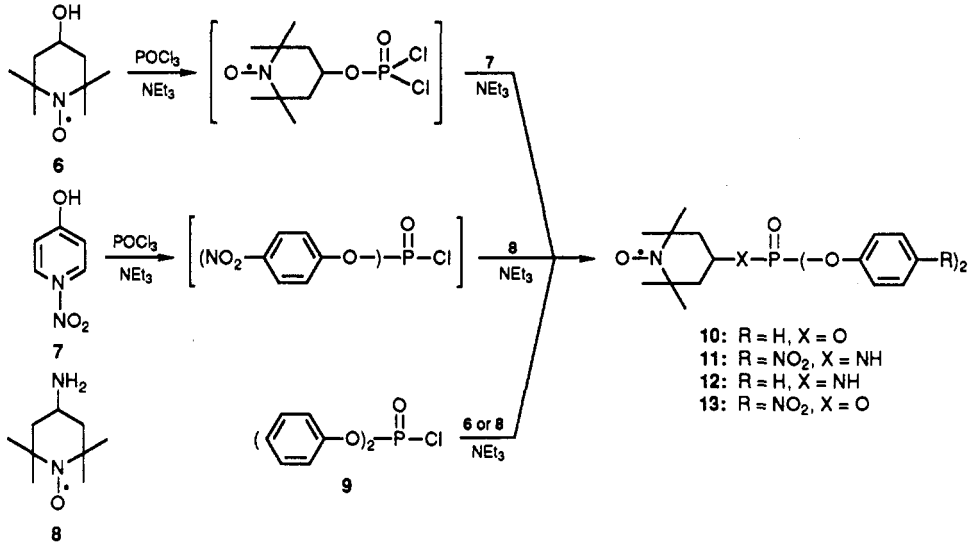
Scheme I



In the last decade, Tsien and co-workers[39-45] and others[46] have synthesized a number of acetoxymethyl esters of membrane-permeant type **5**, based on the structure of the well-known metal chelator EGTA (**3**). In order to follow the intracellular events by fluorescence spectrometry, the EGTA structure was extensively modified by incorporating aromatic nuclei capable of fluorescence. These probes primarily were used for the determination of calcium content in the intracellular space.

Recently, we described[13] several nitroxyl-labeled complexons of EDTA, CDTA, DTPA, TTHA, and their gadolinium complexes, as potential contrast agents for MRI.

We have now designed several classes of nitroxyl-labeled active esters and related derivatives as potential probes in studies of intracellular environments by the ESR spectroscopy and NMR techniques.

The present report shall be restricted to the syntheses of these probes. The biophysical applications of these compounds will be communicated elsewhere.[56]

Results and Discussion

Syntheses. The general plan for the design of nitroxyl-labeled probes for studies of intracellular space was based on the idea that all membrane-impermeant highly charged nitroxyl radicals could be derivatized to cell-membrane-permeant species, which would then be converted intracellularly to cell-membrane-impermeant derivatives. Thus trapped, these spin-labeled species would enable the exploration of the intracellular environment by EPR spectroscopy and MRI.

It was known[12] that the highly charged spin-labeled phosphoric acid derivative **2** is cell-membrane-impermeant. Hence, it was expected that the conversion of **2** into noncharged, more hydrophobic species, such as the aryl esters, would enable these derivatives to migrate into the intracellular space, where they would be readily deblocked to give **2**.

The esters of phosphoric acid derivative **2** containing phenyl and *p*-nitrophenyl moieties **10–13** were prepared as shown in Scheme I. The condensation of either **6** or **7** with phosphoryl chloride in the presence of triethylamine resulted in intermediates, which were further reacted in situ either with **7** or **8** to give compounds **11** and **13**. The reaction of diphenyl chlorophosphate[29] with either **6** or **8** in the presence of either triethylamine or, if necessary, the sterically hindered base *N,N*-diisopropylethylamine yielded compounds **10** and **12**. Compound **10** was previously prepared.[56] This condensation in the presence of triethylamine was unsuccessful. To the best of our knowledge, *p*-nitrophenyl 1-oxy-2,2,6,6-tetramethyl-4-piperidinyl phosphate is the only spin-labeled aryl phosphate that was used in a cell study;[39] however, neither the synthesis nor the authenticity of this compound was indicated.

In the intracellular studies,[39-43] using fluorescence spectroscopy, a number of active esters **5** of suitably substituted polyacids of type **3** have been extensively used. In the present work, considering the projected ESR and NMR studies, several active esters **17** and **23–27** of spin-labeled chelating agents using different synthetic methodology were prepared (Scheme II). The starting materials **18–20** and the EDTA and DTPA anhydrides were synthesized as previously reported.[13] The condensation of **15** with either **8** or **16** yielded compounds **17–20**. The esterification of these compounds (**17–20**) with either bromomethyl acetate (**21**) or chloromethyl pivalate (**22**) in the presence of *N,N*-diisopropylethylamine resulted in active esters of EDTA and DTPA analogues **23–27** (Scheme II).

Although bromomethyl acetate (**21**) has been known since the turn of the century[52] and used on various occasions in the syntheses of active esters, either the preparation of this compound has not been mentioned[47-50,52] or the methods were somewhat cumbersome.[45,57] Thus, in one method,[58] the condensation of paraformaldehyde with acetyl bromide in the presence of anhydrous zinc chloride under nitrogen was prescribed, followed by chromatography at −10 °C of the reaction mixture. Nevertheless, the product contained 1,1′-dibromodimethyl ether as impurity. In a more recent method,[45] the preparation of **21** was recommended starting with trimethylsilyl bromide, zinc chloride, and methylene diacetate, which, in turn, was obtained from formaldehyde and acetic anhydride. Although the product was claimed to be free of the 1,1′-dibromodimethyl ether, no physical data were provided. In the present work, it was found that a quite satisfactory quality of **21** can be attained by the original condensation[52] of paraformaldehyde with acetyl bromide in the presence

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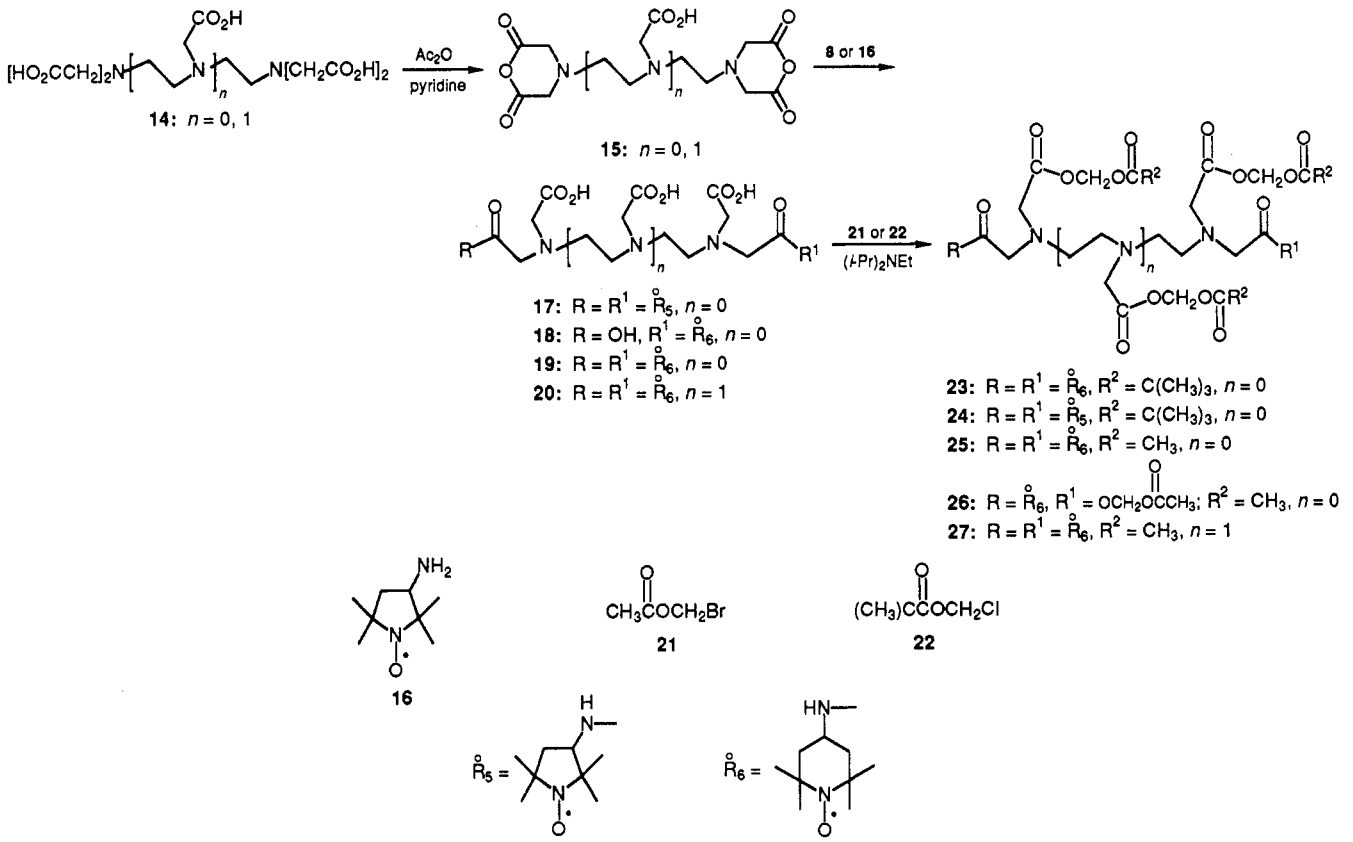
Scheme II



of zinc chloride, whereby neither nitrogen atmosphere nor the low-temperature chromatography was required. Repeated distillation of the product using a short Vigreux headpiece resulted in a 75% yield of **21**, which was entirely satisfactory for use in the syntheses of active esters. Because of volatility, hygroscopicity, and corrosiveness, compound **21** could not be analyzed by microanalysis and mass spectrometry. However, the structure of **21** was unambiguously confirmed by ^1H and ^{13}C NMR spectra, indicating little impurity.

The spin-labeled amino acid derivatives **33–41** were synthesized by a multistep methodology starting from triacetonamine via bromination and the Favorskii ring contraction, as delineated in Scheme III. Thus, compounds **30–32** were prepared from the hydrobromide salt of **29** and the corresponding hydrochloride salts of amino acid ethyl esters in the presence of potassium carbonate. The condensations using free amino acids were unsuccessful. The Favorskii reaction was carried out in a methanol and water medium for **30** and in a tetrahydrofuran and water medium for **31** and **32**. These compounds were then oxidized with a 30% aqueous hydrogen peroxide solution in the presence of sodium tungstate as catalyst to give the corresponding nitroxyl radicals **33**, **36**, and **39**. The synthesis of the spin-labeled glycine ethyl ester **33** with a melting point of 65–66 °C dec was reported earlier[59] using the Favorskii reaction with ethyl glycinate and 3,5-dibromo-2,2,6,6-tetramethylpiperidine-1-oxyl, instead of **29**. In contrast, the synthesis of **33** by two different methods using active esters[60] produced a compound with a melting point of 76–77 °C dec. The compound **33** prepared in the present work had a melting point of 76–77 °C dec.

The hydrolysis of compounds **33**, **36**, and **39**, using methanol and aqueous sodium hydroxide, yielded the corresponding acids **34**, **37**, and **40** (Scheme III). Compound **34** with a melting point of 130–131 °C was previously synthesized[61] by using glycine and 1-(1-oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl)imidazole. More recently, compound **34** with a melting point of 165–167 °C dec was synthesized[62] from glycine and 3-(azidocarbonyl)-2,2,5,5-tetramethylpyrroline-1-oxyl. The compound **34** prepared in the present work had a melting point of 164–167 °C dec. The esterification of compounds **34**, **37**, and **40**, using bromomethyl acetate[18] in the presence of *N*,*N*-diisopropylethylamine, resulted in the spin-labeled acetoxymethyl esters **35**, **38**, and **41**, respectively.

It is assumed that the spin-labeled acids **33**, **36**, and **39** will be cell-membrane-impermeant and the corresponding ethyl esters **33**, **36**, and **39** will be permeant, but will not be readily hydrolyzed in cells and, as a consequence, will equilibrate through cell membranes, whereas the active esters **35**, **38**, and **41** will be permeant and will be readily hydrolyzed in the intracellular space.

Thus, all these compounds should be of interest as potential probes in studies of the intracellular environment by ESR and NMR techniques.

Analyses. The structure of compounds **10–13**, **17**, **23–27**, and **30–41** were ascertained with physical data obtained by microanalyses, mass spectrometry, and where applicable, by ^1H NMR and ^{13}C NMR spectroscopy (Tables I–IV).

The structures of pyrroline derivatives of amino acids **30–32** were confirmed by the coupled ^1H NMR and the decoupled ^{13}C NMR spectra (Table III). The chemical

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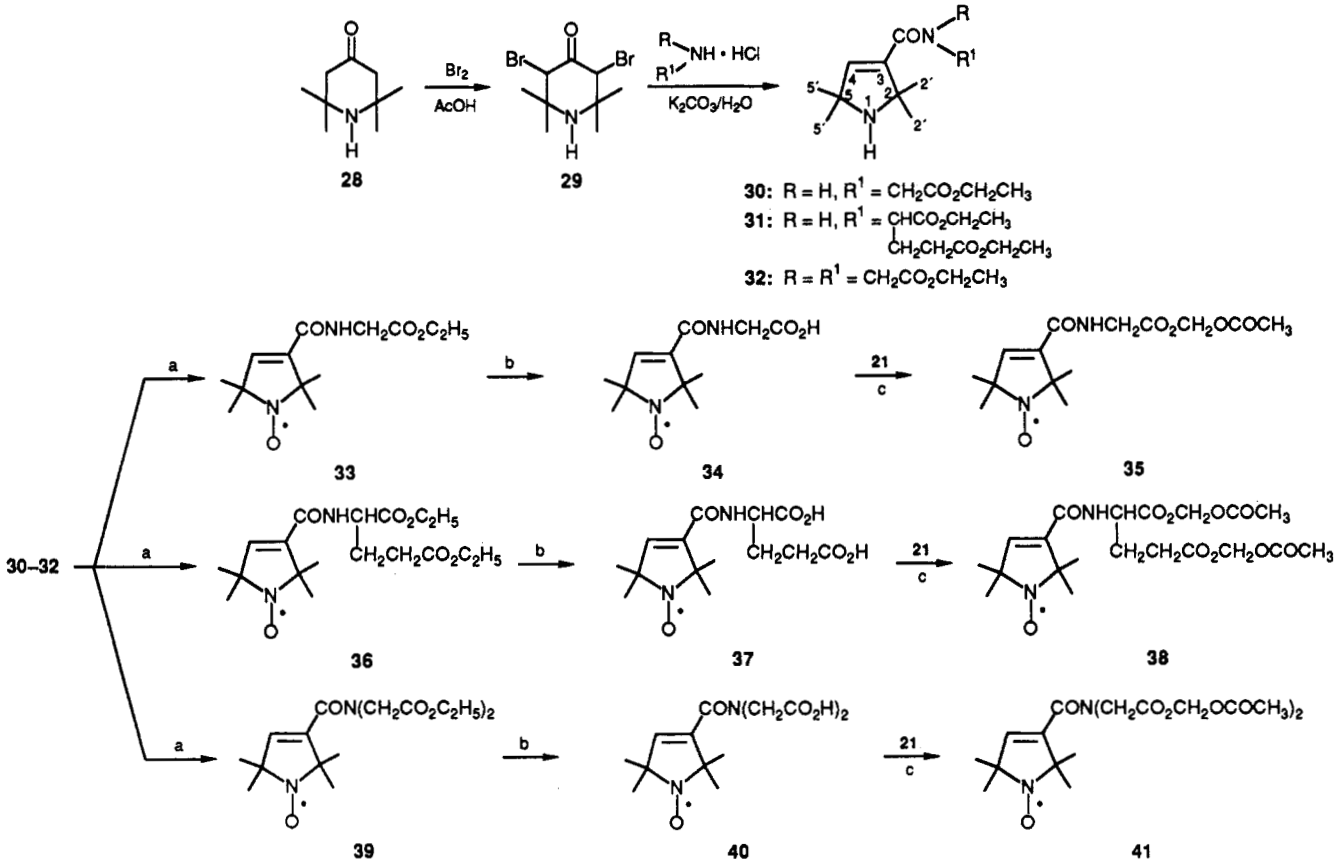
Scheme III[a]



[a] (a) H_2O_2/Na_2WO_4 (30%); (b) $CH_3OH/NaOH$; (c) $(i\text{-}C_3H_7)_2NC_2H_5$.

Table I. Physical Properties of Spin-Labeled Phosphoric Acid Derivatives

compd	molecular formula[a]	yield, %	mp, °C	MS,[b] *m/e*
10	$C_{21}H_{27}NO_5P$ (404.42)	60	44–46[c]	405 (M^+ + 1, 3), 251 (M^+ – 154, 100), 124 (M^+ – 275, 4), 109 (M^+ – 300, 14)
11	$C_{21}H_{26}N_4O_8P$ (493.45)	65	169–170	495 (M^+ + 1, 1), 479 (M^+ – 16, 2), 208 (M^+ – 287, 6), 168 (M^+ – 327, 6), 140 (M^+ – 353, 100)
12	$C_{21}H_{28}N_2O_4P$ (403.42)	67	139–141	404 (M^+ + 1, 100), 403 (M^+, 27), 389 (M^+ – 143, 35), 250 (M^+ – 153, 2)
13	$C_{21}H_{25}N_3O_9P$ (494.42)	69	117–119	495 (M^+ + 1, 4), 478 (M^+ – 163, 58), 154 (M^+ – 340, 58), 140 (M^+ – 354, 100)

[a] The microanalyses (C, H, and N) were in satisfactory agreement with calculated values within ±0.3%. [b] Relative percent intensities of the peaks. Mass spectra were obtained by using chemical ionization. [c] Literature[57] mp 52–54 °C.

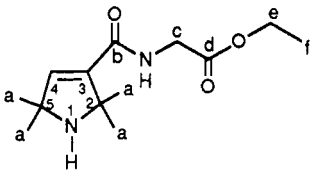
Table II. Physical Properties of Spin-Labeled EDTA and DTPA Derivatives

compd	molecular formula[a]	yield, %	mp, °C	MS,[d] *m/e*
17	$C_{26}H_{46}N_6O_8$[b] (570.68)	49	105 dec	480 (M^+ – 90, 14), 465 (M^+ – 105, 100), 355 (M^+ – 215, 49), 297 (M^+ – 273, 42), 231 (M^+ – 339, 74), 171 (M^+ – 399, 43), 125 (M^+ – 445, 68)
23	$C_{40}H_{70}N_6O_{12}$ (827.01)	59	oil	464 (M^+ – 363, 32), 362 (M^+ – 465, 22), 360 (M^+ – 467, 16), 291 (M^+ – 536, 38), 233 (M^+ – 594, 34), 219 (M^+ – 608, 25), 124 (M^+ – 703, 100)
24	$C_{38}H_{66}N_6O_{12}$ (798.97)	43	oil	389 (M^+ – 409, 65), 274 (M^+ – 524, 29), 243 (M^+ – 555, 16), 216 (M^+ – 582, 100), 160 (M^+ – 638, 54)
25	$C_{34}H_{58}N_6O_{12}$ (742.86)	33	oil	183 (M^+ – 559, 28), 169 (M^+ – 573, 26), 153 (M^+ – 589, 26), 130 (M^+ – 612, 42), 124 (M^+ – 618, 100)
26	$C_{28}H_{46}N_4O_{14}$ (661.69)	45	oil	581 (M^+ – 80, 36), 545 (M^+ – 116, 9), 509 (M^+ – 152, 19), 479 (M^+ – 182, 54), 427 (M^+ – 234, 27), 361 (M^+ – 300, 33)
27	$C_{41}H_{69}N_7O_{16}$[c] (915.84)	28	oil	198 (M^+ – 717, 4), 168 (M^+ – 747, 7), 140 (M^+ – 775, 19), 138 (M^+ – 777, 24), 124 (M^+ – 791, 100)

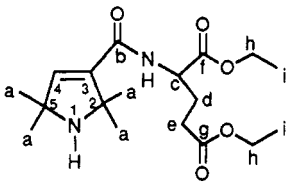
[a] The microanalyses (C, H, and N) were in satisfactory agreement with calculated values within ±0.3%, except for **17** and **26**. [b] Calculated: C, 54.70; H, 8.10; N, 16.70. Found: C, 54.10; H, 8.31; N, 14.64. [c] Calculated: C, 53.72; H, 7.53; N, 10.70. Found: C, 53.77; H, 7.83; N, 11.81. [d] Relative percent intensities of the peaks. Mass spectra were obtained by using the electron-impact (15 eV) method.

shift data of the magnetically equivalent nuclei in ^1H NMR and ^{13}C NMR are listed in Table III. In general, paramagnetic species, such as nitroxyl-labeled compounds, tend to have very broad lines in nuclear magnetic resonance. Since the electron has a much larger magnetic moment than the nuclear magnetic moment, the motion of the paramagnetic component will produce intense fluctuating fields which result in greatly spin–lattice relaxation times of the nuclei and, consequently, increases of line width in the NMR spectra. Therefore, the broadening of lines makes the interpretation of high-resolution NMR spectra of spin-labeled compounds very difficult, and therefore, the use of mass spectrometry for the characterization of these compounds becomes particularly important.

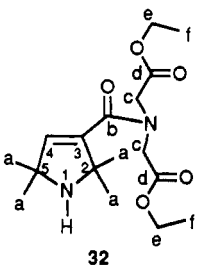
Table III. Physical Properties of Pyrroline Derivatives of Amino Acids



30



31



32

compd	molecular formula[a]	yield, %	mp, °C	MS,[b] *m/e*	^{1}H NMR:[c] δ, ppm	^{13}C NMR:[c] δ, ppm
30	$C_{13}H_{22}N_2O_3$ (254.33)	70	68–69	256 (M^+ + 2, 15.6), 255 (M^+ + 1, 100), 241 (M^+ – 13, 15), 239 (M^+ – 15, 22), 152 (M^+ – 102, 37)	1.200 (s, 3 H), 1.236 (t, 3 H), 1.261 (s, 3 H), 1.383 (s, 6 H), 2.05 (s, NH), 4.168 (dd, 2 H), 6.137 (s, 1 H)	14.07 (C_f), 30.07 (C_a), 41.25 (C_e), 61.51 (C_c), 63.62 (C_5), 67.00 (C_2), 141.16 (C_4), 142.57 (C_3), 164.91 (C_d), 170.04 (C_b)
31	$C_{18}H_{30}N_2O_5$ (354.44)	69	oil	355 (M^+ + 1, 100), 341 (M^+ – 13, 13), 339 (M^+ – 15, 17), 152 (M^+ – 202, 3)	1.197 (t, 3 H), 1.226 (t, 3 H), 1.257 (s, 3 H), 1.264 (s, 3 H), 1.380 (s, 3 H), 1.400 (s, 3 H), 4.118 (m, 8 H), 6.132 (s, 1 H)	14.11 (C_i), 24.80 (C_d), 29.92 (C_a), 30148 (C_e), 51.73 (C_h), 61.61 (C_c), 63.71 (C_5), 67.08 (C_2), 141.04 (C_4), 142.50 (C_3), 164.63 (C_g), 171.95 (C_f), 172.95 (C_b)
32	$C_{17}H_{28}N_2O_5$ (360.41)	39	54.5–55	342 (M^+ + 2, 20), 341 (M^+ + 1, 100), 328 (M^+ – 12, 18), 327 (M^+ – 13, 92), 325 (M^+ – 15, 25), 311 (M^+ – 29, 15), 295 (M^+ – 45, 12)	1.228 (t, 3 H), 1.257 (t, 3 H), 1.264 (s, 3 H), 1.278 (s, 3 H), 1.284 (s, 3 H), 1.381 (s, 3 H), 1.88 (s, NH), 4.183 (m, 4 H), 5.688 (s, 1 H)	14.17 (C_f), 3023 (C_a), 47.23 (C_c), 51.40 (C_e), 64.88 (C_c), 68174 (C_2), 137.85 (C_4), 139.96 (C_3), 168.69 (C_d), 169.31 (C_b)

[a] The microanalyses (C, H, and N) were in satisfactory agreement with the calculated values within ±0.3%. [b] Relative percent intensities of the peaks. Mass spectra were obtained by using chemical ionization. [c] All spectra were recorded by usig deuteriochloroform ($CDCl_3$) solutions with tetramethylsilane (Me_4Si) as the internal standard.

It is expected that the presence of a moiety with an unpaired electron would impart some unusual properties to the spin-labeled molecules and that either the chemical ionization (CI) or the initial ionization using electron bombardment (EI) would result in excited species which are very different from the conventional radical-ion species produced by the excitation of nonradical organic molecules. The CI and EI induced fragmentation pattern of some simple substituted piperidine, pyrrolidine, and pyrroline nitroxyls were previously investigated,[63-67] and their fundamental fragmentation pattern M^+ + 1, M^+ – 15 and other fragments were proposed.[63-66] However, no fragmentation pattern for the more complex compounds which were synthesized in the present work have been reported. In the present study, for the spin-labeled phosphoric acid derivatives **10–13**, for the pyrroline derivatives **30–32**, and for the spin-labeled amino acid derivatives **33–41**, the mass spectra were obtained by the CI method using methane as the reactant gas, and for the EDTA and DTPA derivatives **17**, **23–27**, the mass spectra were obtained by the EI (15 eV) method (Tables I–IV).

In the mass spectra of spin-labeled phosphoric acid esters **11–13**, the fundamental fragments M^+ + 1 and M^+ – 15 of low to medium intensity were observed. However, in the case of the analogue **10**, a low-intensity peak M^+ + 1 was found. Similarly, the loss of phosphorylated hydrocarbon fragments $(X\text{–}P(O)C_6H_4\text{–}R)^+$ was observed in compounds **11–13** (Scheme I, Table I). The unconventional M^+ – 14 peak of low to medium intensity was found in all these compounds (**10–13**). This unconventional peak can be attributed to a loss of the methyl radical from the $(M + 1)^{\bullet+}$ moiety.

The mass spectra of the spin-labeled EDTA and DTPA derivatives **17** and **23–27**, obtained in the EI mode, indicated predominantly the hydrocarbon fragmentation rather than the characteristic nitroxyl pattern of M^+ and M^+ – 15. This result could be rationalized on the basis of the presence of an excess of hydrocarbon moieties in comparison to the nitroxyl moiety. Thus, in the case of **17**, the loss of two carboxyl groups (CO_2H) resulted in a low-intensity fragment M^+ – 90. The successive loss of methyl radical resulted in the predominant M^+ – 105 fragment. In compound **23**, the successive loss of two active ester moieties $CO_2CH_2OC(O)R^2$ and the concomitant loss of two methyl radical moieties gave the predominant M^+ – 703 fragment. Similarly, in **24**, **25**, **26**, and **27**, the major fragments M^+ – 582, M^+ – 618, M^+ – 300, and M^+ – 791 were observed (Table II).

The fragmentation of pyrroline analogues of amino acids (**30–32**) resulted in the usual high-intensity M^+ + 1 peak, the M^+ – 15 peak, and the unconventional M^+ – 14 peak. Furthermore, in the case of **30** a medium-intensity peak M^+ – 102 was recorded, which was ascribed to a loss of the $(NHCH_2CO_2CH_2CH_3)^+$ ion. In the case of compound **31**, a fragment corresponding to a low-intensity peak M^+ – 202 caused by a loss of the $(NCH_2CO_2CH_2CH_3)^+$ ion was found, while in **32**, the successive loss of $(CH_2CH_3)^+$ and $(OCH_2CH_3)^+$ ions led to the low-intensity peaks M^+ – 29 and M^+ – 45, respectively (Table III).

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(64) Kaliska, V.; Toma, S.; Lesko, J. *Collect. Czech. Chem. Commun.* **1987**, *52*, 2266 and references therein.
(65) Davies, A. P.; Morrison, A.; Barratt, M. D. *Org. Mass Spectrom.* **1974**, *8*, 43 and references therein.
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(67) Carmichael, P. J.; Gowenlock, B. G.; Johnson, C. A. F. *J. Chem. Soc., Perkin Trans. 2* **1973**, *14*, 1853.

Table IV. Physical Properties of Spin-Labeled Amino Acid Analogues

compd	molecular formula[a]	yield, %	mp, °C	MS,[b] m/e
33	$C_{13}H_{21}N_2O_4$ (269.3)	41	75–77[c]	270 (M^+ + 1, 55), 269 (M^+, 60), 256 (M^+ – 13, 45), 255 (M^+ – 14, 100), 241 (M^+ – 25, 46)
34	$C_{11}H_{17}N_2O_4$ (241.27)	72	164–167[d]	262 (M^+ + 1, 16), 190 (M^+ – 51, 100)
35	$C_{14}H_{21}N_2O_6$ (313.33)	50	oil	314 (M^+ + 1, 69), 313 (M^+, 56), 299 (M^+ – 14, 100)
36	$C_{18}H_{29}N_2O_6$ (369.44)	57	oil	371 (M^+ + 2, 51), 370 (M^+ + 1, 85), 369 (M^+, 42), 356 (M^+ – 13, 32), 355 (M^+ – 14, 100)
37	$C_{14}H_{21}N_2O_6$ (313.33)	52	77–79	315 (M^+ + 2, 37), 314 (M^+ + 1, 21), 313 (M^+, 17), 29 (M^+ – 141, 65), 185 (M^+ – 128, 55), 184 (M^+ – 129, 60), 170 (M^+ – 143, 100)
38	$C_{20}H_{29}N_2O_{10}$ (457.36)	58	oil	459 (M^+ + 2, 78), 458 (M^+ + 1, 100), 457 (M^+, 48), 443 (M^+ – 14, 82)
39	$C_{17}H_{27}N_2O_6$ (355.41)	38	91–92	352 (M^+ + 2, 33), 356 (M+ + 1, 85), 355 (M^+, 59), 343 (M^+ – 12, 10), 342 (M^+ – 13, 61), 341 (M^+ – 14, 100)
40	$C_{13}H_{19}N_2O_6$ (299.41)	70	172–173	300 (M^+ + 1, 16), 257 (M^+ – 22, 19), 256 (M^+ – 23, 100)
41	$C_{19}H_{27}N_2O_{10}$ (443.38)	55	73–75	445 (M^+ + 2, 59), 444 (M^+ + 1, 37), 443 (M^+, 34), 429 (M^+ – 14, 74), 355 (M^+ – 88, 100)

[a] The microanalyses (C, H, and N) were in satisfactory agreement with the calculated values within ±0.3%. [b] Relative percent intensities of the peaks. Mass spectra were obtained by using chemical ionization. [c] Literature[61,62] mp 130–131 °C and 164–168 °C. [d] Literature[59] mp 74–76 °C.

In the fragmentation of the spin-labeled amino acids **33–41**, again, the usual pattern with $M^+ + 1$, $M^+ - 15$, and the unconventional $M^+ - 14$ peaks were found. In the case of ethyl ester derivatives **33**, **36**, and **39**, the unconventional $M^+ - 14$ peak of high intensity was observed (Table IV). The fragmentation of the acid intermediates **34**, **37**, and **40** resulted in high-intensity moieties $M^+ - 51$, $M^+ - 143$, and $M^+ - 23$, respectively (Table IV). Fragmentation of the active esters **35**, **38**, and **41** consistently produced the high-intensity $M^+ - 14$ peak. Furthermore, the loss of two active ester moieties $(OCH_2OC(O)CH_3)^+$ yielded the $M^+ - 88$ fragment of high intensity (Table IV).

In conclusion, the fragmentation pattern of various nitroxyl-labeled probes which were elucidated in the present work not only has provided confirmation of structural features of these compounds during the syntheses but also will be uniquely valuable in identifying enzymatic degradation products in subsequent studies of the intracellular space where only tiny amounts of degradation products will be available.

Experimental Section

Materials. All commercial starting materials were of the best quality available and were used without further purification. Acetyl bromide, diisopropylethylamine, diphenyl chlorophosphate (**9**), and chloromethyl pivalate (**22**) were purchased from Aldrich Chemical Company (Milwaukee, WI). Solvents were always dried by using standard procedures. 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**8**), 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (**16**), and 3,5-dibromo-2,2,6,6-tetramethyl-4-piperidinone hydrobromide (**29**) were prepared according to the literature methods.[1]

Analytical Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus, Model 6406-K, using a calibrated thermometer. Mass spectra were recorded on a Hewlett-Packard mass spectrometer, Model 5985 GS, using (unless specified otherwise) methane as the reactant gas. ^1H NMR and ^{13}C NMR for compounds **21**, **30**, **31**, and **33** were recorded on a 250-MHz Brucker NMR spectrometer, Model WM-250, using tetramethylsilane and D_2O lock. Microanalyses were performed on a Perkin-Elmer elemental analyzer, Model 240C. The column chromatography was performed by using the flash chromatography technique[68] on silica gel 60 (Fluka) finer than 230 mesh. The purity control by TLC analysis was performed on silica gel 60 F_{254} precoated sheets (EM reagents), layer thickness 0.2 mm with visualization by using UV light and/or iodine chamber. All compounds were uniform by TLC (one spot).

Preparation of Diphenyl 1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl Phosphate (10) and Diphenyl 1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl Phosphoramidate (12). To a solution of diphenyl chlorophosphate (**9**, 0.41 mL, 2 mmol) in dry methylene chloride (10 mL) was added at 5–6 °C a solution of either 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**6**, 340 mg, 2 mmol) or 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**8**, 340 mg, 2 mmol) and triethylamine (0.2 mL, 2 mmol) in benzene (10 mL). The reaction mixture was stirred at 25 °C overnight and concentrated at 60 °C/20 Torr. The residue was purified by flash chromatography on silica gel using methylene chloride and methanol (9.5:0.5 v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/30 Torr gave either compound **10** or **12**. The purity of compounds **10** and **12** was checked by TLC using a solvent system composed of methylene chloride and methanol (9.5:0.5 v/v). The analytical data are presented in Table I.

Preparation of Bis(*p*-nitrophenyl) 1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl Phosphoramidate (11) and Bis(*p*-nitrophenyl) 1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl Phosphate (13). A solution of either nitrophenol (**7**, 556 mg, 4 mmol) and triethylamine (0.4 mL, 4 mmol) in methylene chloride (10 mL) or 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**6**, 340 mg, 2 mmol) and triethylamine (0.2 mL, 2 mmol) in benzene (10 mL) was added at 5–8 °C to a solution of phosphoryl chloride (0.2 mL, 2.15 mmol) in methylene chloride (10 mL). The reaction mixture was stirred at 25 °C for 4 h and then cooled to 5–8 °C. To this mixture was added a solution of either 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (**8**, 340 mg, 2 mmol) and triethylamine (0.25 mL, 2.5 mmol) in methylene chloride (10 mL) or nitrophenol (**7**, 556 mg, 4 mmol) and triethylamine (0.4 mL, 4 mmol) in benzene (10 mL). The mixture was stirred overnight at 25 °C and then concentrated on a rotating evaporator at 25 °C/20 Torr. The residue was purified by flash chromatography using methylene chloride and methanol (9.4:0.6 v/v) as eluant. Concentrations of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave compounds **11** and **13**. The purity of compounds **11** and **13** was checked by TLC using a solvent system composed of methylene chloride/methanol (9.4:0.6 v/v). The analytical data are presented in Table I.

Preparation of EDTA Bis(1-oxy-2,2,5,5-tetramethylpyrrolidine-3-amide) (17). A solution of ethylenediaminetetraacetic dianhydride[14] (**15**, 382 mg, 1.49 mmol) and 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (**8**, 466 mg, 2.97 mmol) in dry dimethylformamide (4 mL) was heated at 60 °C for 6 h. The solvent was then removed in vacuo at 50 °C/20 Torr. The residue was purified by flash chromatography using methylene chloride/methanol (1:1 v/v) as eluant. Concentrations of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave the compound **17**. The purity was checked by using a solvent system composed of methylene chloride/methanol (1:1 v/v). The analytical data are presented in Table II.

Preparation of EDTA Bis(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide) Bis[(dipivaloyloxy)methyl ester] (23), EDTA Bis(1-oxy-2,2,5,5-tetramethylpyrrolidinyl-4-amide) Bis[(pivaloyloxy)methyl ester] (24), EDTA Bis(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide) Bis[(acetyloxy)methyl ester] (25), EDTA Mono(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide) Tris[(acetyloxy)methyl ester] (26), and DTPA Bis(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide)

(68) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

Tris[(acetyloxy)methyl ester] (27). To a solution of either EDTA bis(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide)[14] (**19**, 580 mg, 0.87 mmol), EDTA bis(1-oxy-2,2,5,5-tetramethylpyrrolidinyl-3-amide)[26] (**17**, 200 mg, 0.35 mmol), EDTA mono(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide)[14] (**18**, 480 mg, 1 mmol), or DTPA bis(1-oxy-2,2,6,6-tetramethylpiperidinyl-4-amide)[14] (**20**, 697 mg, 1 mmol) in dimethylformamide (10 mL) were added at 10 °C *N,N*-diisopropylethylamine (0.70 mL, 3.86 mmol) and either (pivaloyloxy)methyl chloride (**22**, 0.42 mL, 2.93 mmol) or bromomethyl acetate (**21**, 0.5 mL, 5 mmol). The reaction mixture was stirred for 48 h at 25 °C. Removal of the solvent in vacuo at 35 °/5 Torr gave an oily residue, which was purified by flash chromatography on silica gel using methylene chloride and methanol (9.5:0.5 v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave compounds **23–27**. The purity was checked by TLC using a solvent system composed of methylene chloride/methanol (9.5:0.5 v/v). The analytical data are presented in Table II.

Preparation of Bromomethyl Acetate (21). Anhydrous zinc chloride (200 mg, 1.67 mmol) was added at 25 °C to a solution of acetyl bromide (23.45 g, 19 mmol) in methylene chloride (20 mL). Paraformaldehyde (6 g, 200 mmol) was then added to the ice-cooled mixture. The reaction mixture was stirred overnight at 25 °C. Distillation of the mixture at 750 Torr using a Vigreux headpiece (35 cm) gave first the unreacted acetyl bromide and methylene chloride followed by the crude product **21** boiling at 120–140 °C/750 Torr. Repeated distillations at 750 Torr gave 23 g (75%) of compound **21**, bp 132–134 °C (lit.[52] bp 130–133 °C). Microanalysis and mass spectrum could not be obtained because of volatility, hygroscopicity, and corrosiveness of compound **21**: ^{1}H NMR (CD_3Cl/TMS) δ 1.907 (s, 3 H), 5.585 (s, 2 H); ^{13}C NMR (CD_3Cl/TMS) δ 19.92 (CH_3), 56.91 (CH_2), 167.98 (>C=O).

Preparation of Ethyl *N*-[2,2,5,5-Tetramethylpyrroline-3-carbonyl]glycinate (30), Diethyl *N*-[2,2,5,5-Tetramethylpyrroline-3-carbonyl]glutamate (31), and Diethyl *N*-[2,2,5,5-Tetramethylpyrroline-3-carbonyl]iminodiacetate (32). To a solution of either ethyl glycinate hydrochloride (5.00 g, 35.80 mmol), diethyl glutamate hydrochloride (8.58 g, 35.80 mmol), or diethyl iminodiacetate hydrochloride (8.07 g, 35.80 mmol) in methanol and deionized water (2:1 v/v, 60 mL) was added with stirring at 25 °C potassium carbonate (23.70 g, 170.0 mmol) followed by the addition of 3,5-dibromo-2,2,6,6-tetramethyl-4-piperidinone hydrobromide (**29**); 12.0 g, 30.5 mmol) over a period of 30 min. The reaction mixture was stirred for a further period of 5 h at 25 °C and then extracted with ethyl acetate (3 × 30 mL). The combined ethyl acetate extracts were washed with a saturated sodium chloride solution (2 × 5 mL), dried over anhydrous magnesium sulfate, and filtered. The filtrate was evaporated to dryness on a rotating evaporator at 40 °C/20 Torr. The residue was purified by flash chromatography on silica gel using chloroform and methanol (9:1 v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave either **30**, **31**, or **32**. Compounds **30** and **32** were repeatedly recrystallized by using ethyl acetate. The purity of compounds **30–32** was checked by TLC using a solvent system composed of chloroform and methane (9:1 v/v). The analytical data are presented in Table III.

Preparation of Ethyl *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]glycinate (33), Diethyl *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]glutamate (36), and Diethyl *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]iminodiacetate (39). To a solution of either **30** (1.0 g, 3.9 mmol), **31** (1.38 g, 3.9 mmol), or **33** (1.33 g, 3.9 mmol) and sodium tungstate (0.15 g) in aqueous methanol (30 mL, 2:1 v/v) was added an aqueous solution of 30% hydrogen peroxide (5 mL). The reaction mixture was left at 25 °C for 24 h and then extracted with chloroform (4 × 25 mL). The combined chloroform extracts were washed with a saturated sodium chloride solution (2 × 25 mL), dried with anhydrous magnesium sulfate, and filtered. The filtrate was concentrated on a rotating evaporator at 40 °C/20 Torr. The resulting residue was purified by flash chromatography on silica gel using chloroform and methanol (9:1 v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave either **33**, **36**, or **39**. Compounds **33** and **39** were repeatedly recrystallized by using ethyl acetate. The purity of compounds **33**, **36**, and **39** was checked by TLC using a solvent system composed of chloroform and methanol (9:1 v/v). The analytical data are presented in Table IV.

Preparation of *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]glycine (34), *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]glutamic Acid (37), and *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]iminodiacetic Acid (40). To a solution of either **33** (0.51 g, 1.89 mmol), **36** (0.70 g, 1.89 mmol), or **39** (0.67 g, 1.89 mmol) in methanol (10 mL) was added a 4 N solution of sodium hydroxide (5 mL). The reaction mixture was stirred at 55–60 °C for 4 h and then extracted with ether (5 mL), and the ether extract was discarded. The pH of the aqueous layer was adjusted with 2 N hydrochloric acid (2 mL) to 3–4 and then extracted with ethyl acetate (4 × 10 mL). The combined ethyl acetate extracts were washed with sodium chloride solution (2 × 2 mL), dried over anhydrous magnesium sulfate, and filtered. The filtrate was concentrated on a rotating evaporator at 40 °C/20 Torr. The resulting residue was purified by flash chromatography on silica gel using chloroform and methanol (9.5:0.5 v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave either **34**, **37**, or **40**. Compounds **34** and **40** were repeatedly recrystallized by using ethyl acetate. The purity of compounds **36**, **37**, and **40** was checked by TLC in a solvent system composed of chloroform and methanol (9:1 v/v). The analytical data are presented in Table IV.

Preparation of Acetoxymethyl *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]glycinate (35), Acetoxymethyl *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]glutamate (38), and Acetoxymethyl *N*-[1-Oxy-2,2,5,5-tetramethylpyrroline-3-carbonyl]iminodiacetate (41). To a solution of either **34** (0.085 g, 0.35 mmol), **37** (0.109 g, 0.35 mmol), or **40** (0.140 g, 0.35 mmol) in dimethylformamide (10 mL) were added at 10 °C *N,N*-diisopropylethylamine (0.50 mL, 2.90 mmol) and then bromomethyl acetate (0.23 mL, 2.47 mmol). The reaction mixture was stirred for 48 h at 25 °C. The solvent was removed in vacuo at 25 °/2 Torr. The oily residue was purified by flash chromatography on silica gel using dichloromethane and methanol (9.5:0.5 v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 Torr gave either **35**, **38**, or **41**. Compound **41** was repeatedly recrystallized by using ethyl acetate. The purity of compounds **35**, **38**, and **41** was checked by TLC in a solvent system composed of chloroform and methanol (9:1 v/v). The analytical data are presented in Table IV.

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