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New Amino-Nitroxide Spin Labels

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Abstract—Stable free mono- and diradicals containing reactive primary or secondary amino groups in the side-chain have been synthesized by transesterification of amino-substituted esters with paramagnetic alcohols or from spin-labeled acid derivatives and amines. In the second approach the new radical **18** (1-oxyl-3-(2-bromoethoxycarbonyl)-2,2,5,5-tetramethylpyrroline) is proposed as an efficient alkylating species. The nitroxides described are pH-sensitive spin probes and spin labels potentially useful for a diversity of ESR applications in chemistry and biology. New spin-labeled tyramine **16** (*N*-(1-oxyl-3-carbonyl-2,2,5,5-tetramethylpyrroline)tyramine) was successfully employed in a novel assay of protein oxidative damage. Copyright © 1996 Elsevier Science Ltd

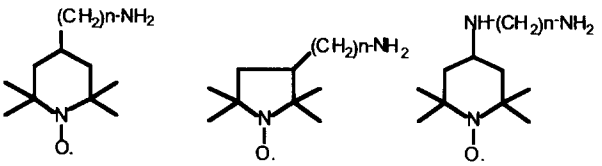
Introduction

Paramagnetic piperidinic, pyrrolinic, pyrrolidinic or imidazolinic amines, reputed to be efficient labeling reagents, have found wide application in ESR studies in chemistry and biology.[1–6] Due to the free amino group(s), stable nitroxide radicals of this class can: (i) bind to appropriate sites in biological substrates and, through ESR spectra of the adduct, provide information on those particular locations; (ii) be used for measurements of light-induced pH gradients across biological membranes;[7] and (iii) serve as reactive starting materials for diradicals containing identical or different paramagnetic moieties. Such diradicals are also the object of many studies on spin–spin interactions[8] or protonation–deprotonation equilibria[6] that are pK_a dependent.

In addition, diradicals offer promise for ESR detection of free radical centers in macromolecules and macromolecular aggregates. Indeed, C-centered macroradicals generated in biological systems or in synthetic polymers react with stable nitroxides to give the corresponding *N*-alkoxy derivatives.[9] When using a nitroxide diradical, only one of its NO· groups binds covalently to the macroradical, while the other gives rise to the easily detectable, immobilized ESR spectrum of the adduct. The success of studies based on spin-labeled amines motivates continued interest in developing new compounds in this class. In this article two approaches are elaborated: in the first, nitroxides with free amino groups are obtained by a procedure based on a transesterification reaction; the second introduces a new spin-labeled haloderivative as the building block for an array of mono- and diradicals.

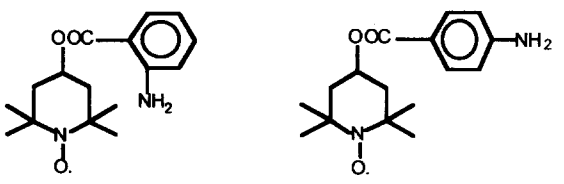
Results and Discussion

With the exception of Tempamine, synthesis of most common spin-labeled primary amines (**1–3**) involves reduction or Hofmann degradation of the parent amide that acts as the key intermediate.[10] More complex structures have been obtained mainly by alkylation of these basic amines. Synthetic routes to nitroxides bearing amino groups described in this paper are based on other acid derivatives (esters or salts), and not amides, as intermediates.



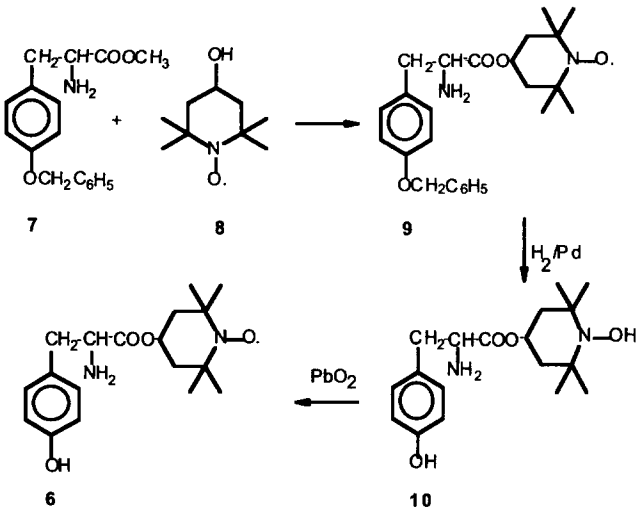
1 n = 0-2 **2** n = 0, 1 **3** n = 2

Paramagnetic esters of aminoaromatic acids have not been reported previously, probably because of the difficulties in preparing the respective acid chlorides. None the less, such esters (e.g. **4** and **5**) can be synthesized easily from commercial spin-labeled alcohols (e.g. Tempol, **8**) and the appropriate, readily available, methyl esters (Table 1). Our general transesterification procedure calls for the reactants to be refluxed in toluene for 3–4 h in the presence of sodium methoxide as catalyst.[11]



4 **5**

The procedure was extended to obtain spin probes of biological importance, including paramagnetic

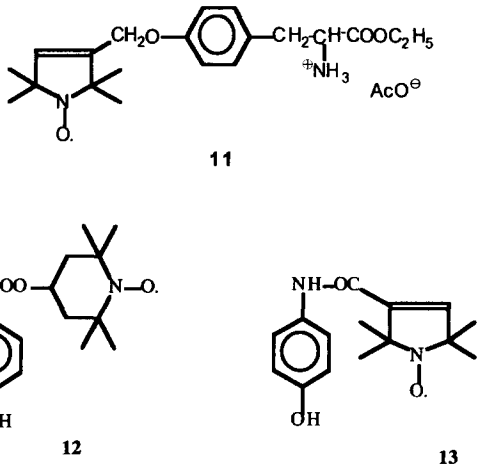


Scheme 1.

analogues of hydroxy acids[11,12] and amino acids. In the case of the new spin-labeled tyrosine **6**, protection of the hydroxy group is necessary prior to transesterification; the benzyl protective group is then easily removed by hydrogenolysis, followed by oxidative regeneration of the radical center (Scheme 1).

The reaction sequence leading to **6** does not imply substitution at the chiral center so that (in principle, at least) **6** should have the same configuration as *S*-tyrosine, used as starting material for ester **7**. However, both benzylation of *S*-tyrosine (first step in synthesis of **7**)[13] and the transesterification reaction leading to **9** require the presence of strong bases (NaOH and MeONa, respectively) that could change configuration at the asymmetric carbon, through intermediate carbanion formation. Using several methods for enantiomer analysis it was shown that compounds **7**, **9** and **6** belong to series *S* (see Experimental).

In the previously reported labeled tyrosine **11**,[14] the paramagnetic moiety is attached through the phenolic group. The availability of spin probes such as **6** in which the OH is free to interact with other phenols or with phenolic residues in proteins is important for studies on damage (through 'dityrosine formation') during enzymatic oxidation of phenols[15] and hydrogen peroxide oxidation of heme-binding proteins.[16] Our approach was to first do a wide-scale search for the best suited phenolic nitroxides to serve as modifying agents of proteins (myoglobin and hemoglobin) subjected to oxidation, and then detect the covalently modified tyrosine residues by monitoring the ESR signal of the incorporated nitroxide. This novel assay can contribute to elucidation of a major, irreversible mechanism of protein crosslinking involving tyrosyl radicals.[16,17]



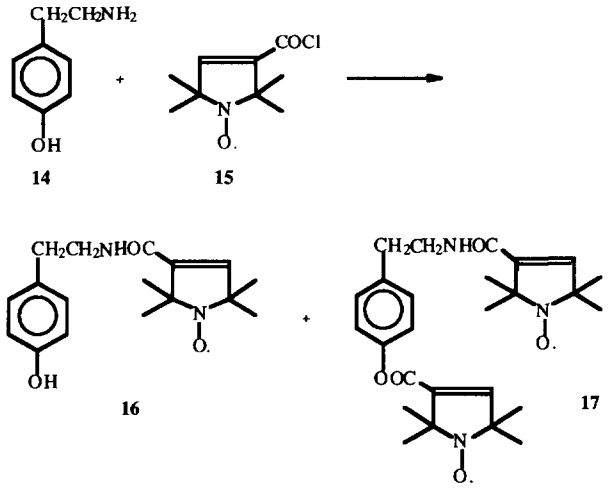
Of the so far synthesized and tested labels, i.e. derivatives of salicylic acid,[11] *p*-hydroxybenzoic acid (**12**),[12] *p*-aminophenol (**13**),[12] tyrosine (**6**), and tyramine (**16**; Scheme 2), the first three were proved to be poor substrates for horseradish peroxidase, whereas **16** gave encouraging results.[12] Two of the conclusions obtained in HRP oxidations are of relevance for the performance of phenolic spin-labels as protein modifying agents: (i) accessibility of the probe at the iron catalytic centre is directly related to its solubility in the bulk solution; (ii) *p*-substituted phenols perform better than their *o*-counterparts, in agreement with the established coupling in *ortho* position[18] leading to dimers and more extensively oxidized polymers. Based on these conclusions, as well as on the enhanced reactivity of **16** in HRP catalysis and the recognized similitude between mechanisms operating in oxidations catalysed by peroxidases and heme, tyramine **16** was also used in studies on the H_2O_2–myoglobin system.

Synthesis of spin-labeled tyramine **16** (Scheme 2) succeeded from tyramine (**14**) and acid chloride **15**[10a] through a conventional Schotten–Baumann procedure; probe **16** and diradical **17** (a by-product resulting from acylation of both reactive groups in tyramine), as well the excess of acid chloride **15** and the parent acid **20** resulting from accompanying hydrolysis of **15**, could be separated from the reaction mixture taking advantage of their different solubility characteristics. Attempts to obtain probe **16** from the same starting materials but in anhydrous solvents (e.g. benzene, 1,2-dimethoxyethane, ethyl acetate) and in the presence of organic bases (pyridine, excess tyramine) failed, confirming literature data on acylation of other aliphatic amines.[19]

The water solution of **16** ('PECU-tyramine') exhibits, in the presence of myoglobin, the expected three-line spectrum with the height of the third line slightly declining (Fig. 1, control trace 1). When probe **16** and equine myoglobin were incubated (8 min, 20 °C) with hydrogen peroxide, an immobilized ESR signal tentatively assigned to the spin-labeled protein was recorded (Fig. 1, trace 2).

Concerning the diradical amines treated in the second part of this paper, it is worthwhile noting that many

members of this comparatively less studied class were derived from labels **1** or **2** and cyanuryl chloride.[20] They, therefore, contain two identical paramagnetic moieties. To produce a diversified array of diradicals a new paramagnetic alkylating agent (**18**) has been prepared. Nitroxide **18** is obtained by a shorter reaction sequence than other commonly used spin-labeled haloderivatives (e.g. 3-bromomethyl-2,2,5,5-tetramethylpyrroline- and -pyrrolidine-*N*-oxyls, 4-bromoethyl-2,2,6,6-tetramethyl-piperidine-1-oxyl);[21] in addition, **18** can be more readily purified and stored safely for 2 years.



Scheme 2.

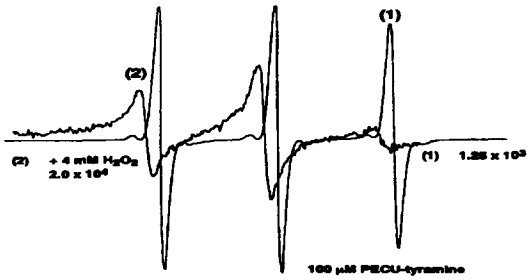
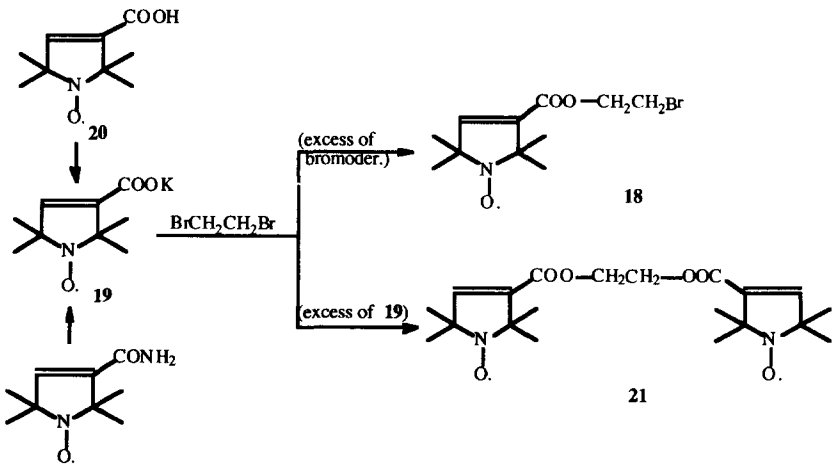


Figure 1. Myoglobin oxidation in the presence of spin-labeled tyramine **16**. (1) ESR spectrum of **16** ('PECU-tyramine'; 100 µmol) solubilized in the presence of myoglobin (4 mmol). (2) ESR spectrum of the myoglobin-bound species generated in system (1) upon hydrogen peroxide addition (4 mmol).

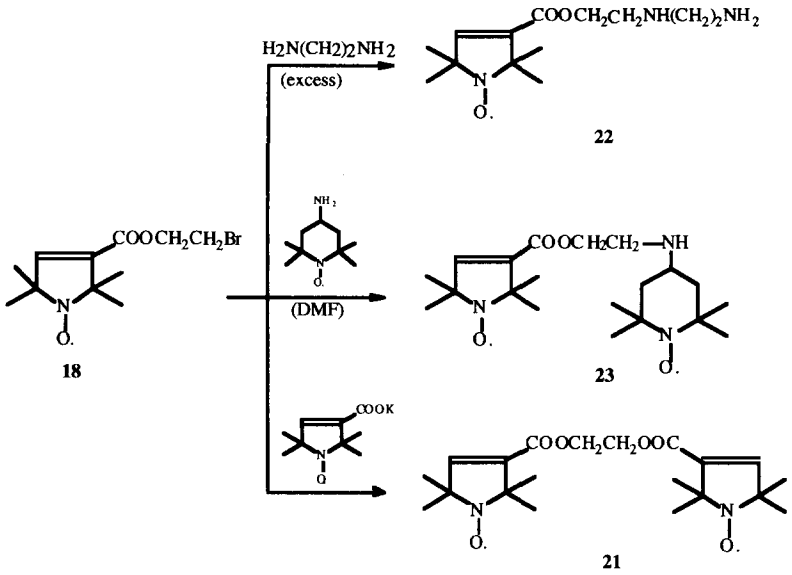
The precursor of the reactive ester **18** is the potassium salt (**19**) of 1-oxyl-3-carboxy-2,2,5,5-tetramethylpyrroline (**20**). Preparation of **19** from acid **20**[10a] and potassium hydroxide succeeded in hot DMF, but not in water or ethanol (even on prolonged boiling), because of the low solubility of the acid in the latter two solvents. Salt **19** could also be obtained by alkaline hydrolysis of the corresponding amide.[10a] Azeotropic distillation with benzene, followed by removal of the solvent, afforded **19** as a dry solid. When heated in dimethylformamide, salt **19** and an excess of 1,2-dibromoethane yielded monoradical **18.** Reversal of the molar ratios of the reactants led only to isolation of the diradical **21** (Scheme 3).

Condensation of compound **18** (in DMF) with an excess of ordinary diamines (ethylenediamine and hexamethylenediamine) produces amino monoradicals (e.g. **22**); diradical amines (e.g. **23**) result from reaction of **18** with equimolar amounts of spin-labeled amines (e.g. Tempamine; Scheme 4). ESR spectra are indicative of the monoradical (three lines) or diradical (five lines) nature of the product. The procedure promises to be useful for spin-labeling of amino acids at the NH_2 group and thereby completes the transesterification method described above affording amino acids spin-labeled at the carboxyl group.

Depending on the reaction partner selected for **18** the general two-step synthesis of diradicals proposed in Scheme 4 can lead either to symmetric or asymmetric structures in which the paramagnetic center is incorporated in both five- or six-membered rings. This contrasts with the mostly symmetric diradicals reported previously.[22] Hyperfine splitting constants a_N (in benzene: ca. 14 G for pyrroline radicals; ca. 15.4 G for



Scheme 3.



Scheme 4.

piperidine radicals; intermediate values for mixed heterocycle diradicals) provide support for structure determination. The availability of different nitroxide moieties in a diradical could be an important feature in biological applications as a result of recognized differences in in vivo reduction rates at radical centers included in various heterocycles.[23]

Experimental

Methyl *o*- and *p*-aminobenzoates were prepared from the respective commercial acids by standard esterification with methanol catalysed by sulphuric acid. The methyl ester of *O*-benzyl-(*S*)-tyrosine (**7**) was obtained from its chlorohydrate[13] using the general procedure described by Fischer.[24] Starting spin labels [Tempol (**8**), 1-oxyl-2,2,5,5-tetramethyl-3-carboxypyrroline (**20**) and 1-oxyl-2,2,6,6-tetramethyl-4-aminopiperidine (Tempamine)] were synthesized according to the literature.[10a]

Spin-labeled amino esters 4, 5, and 9. The calculated amount (Table 1) of catalyst (MeONa) was admitted prior to the reactants as an 8% methanolic solution. It was turned into a fine suspension (in toluene; 50 mL) by distillation of the azeotrope methanol–toluene. To this suspension a solution of methyl aminobenzoate (or ester **7**; 0.01 mol) and the appropriate amount (Table 1) of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinol in toluene (50 mL) was rapidly added and the mixture refluxed with stirring for 3 h with removal of the reaction methanol by azeotropic distillation. Water was then added to the dark-red reaction mixture, layers separated and the organic layer repeatedly washed with water, dried over magnesium sulfate and evaporated under reduced pressure. The crude product was purified as shown in Table 1. Elemental analyses and spectra are given in Tables 2 and 3, respectively.

Ester **9** has specific rotation $[\alpha]_D^{22}+4.6\pm0.4°$ (*c* 1.15, $CHCl_3$). ^1H NMR of **9** ($CDCl_3$, δ (ppm); after phenylhydrazine reduction): 1.2 (s) and 1.27 (s) (12H, CH_3), 3.58 [t(t); $J=7.5$ Hz] (1H, CH—NH_2), 5.1 (s) (2H, $OCH_2C_6H_5$).

(*S*)-Tyrosine, (1-hydroxy-2,2,6,6-tetramethylpiperidinyl) ester (10). To a solution of purified ester **9** (0.60 g; Table 1) in anhydrous ethanol (30 mL) 2% Pd/SiO_2 (1.0 g) was added and the mixture hydrogenated at normal pressure and room temperature, under

Table 1. Syntheses of labeled amino esters

Compd	Molar ratios (based on starting ester)		Color	Mp (°C)	Purification	Yield (%)	
	alcohol **8**	cat.				Crude product	Purified product
4	1.1	0.3	Light-pink	104–106	Recryst.[a] (MeOH–H_2O)	87.0	54.1
5	1.2	0.5	Pink–yellow	134–136	Recryst (MeOH)	84.5	59.2
9	1.2	0.3	Red	viscous oil	Column[b] chromatography	76.0	40.2

[a]Purification by two subsequent recrystallizations or column chromatography on alumina (eluent hexane), followed by recrystallization.
[b]Column chromatography on silica gel (eluent ethyl ether).

Table 2. Elemental analyses of compounds **4**, **5**, and **9**

Compd	Elemental analysis			
		%C	%H	%N
4	Calcd for $C_{16}H_{23}N_2O_3$	65.96	7.96	9.61
	Found	65.72	8.01	9.47
5	Calcd for $C_{16}H_{23}N_2O_3$	65.96	7.96	9.61
	Found	65.68	7.71	9.70
9	Calcd for $C_{25}H_{33}N_2O_4$	70.56	7.82	6.58
	Found	70.33	7.94	6.65

vigorous stirring. After the absorption of hydrogen had ceased (4 h), the catalyst was filtered off and the filtrate evaporated under reduced pressure yielding **10** (0.35 g, 74.0%), white hygroscopic solid, mp 66–68 °C (after drying in vacuo). The product gave no ESR signal and exibited an intense IR absorption at 3570 cm^{-1} (phenolic OH). Hydroxylamine **10** is air sensitive; on standing in air for a few hours spontaneous partial oxidation of its piperidinic N—OH group to the corresponding nitroxide (**6**) occurs.

(*S*)-Tyrosine, (1-oxyl-2,2,6,6-tetramethylpiperidinyl) ester (6). A mixture of lead dioxide (2 g) and crude **10** (0.3 g) in ethyl ether (50 mL) was stirred in air for 15 h, then the catalyst filtered and the filtrate evaporated under reduced pressure to give 0.2 (66.6%) of a viscous, red oil that crystallized on standing; mp 112–115 °C (ethyl ether:hexane). Anal. calcd for $C_{18}H_{27}N_2O_4$: C, 64.46; H, 8.11; N, 8.35. Found: C, 64.25; H, 8.34; N, 8.50%. $[\alpha]_D^{22} -5.8+0.6°$ (*c* 1.2, $CHCl_3$). IR spectrum (CH_2Cl_2) ν_{max}: 1720 (C=O), 3320 (assoc. NH_2) and 3570 cm^{-1}(OH). ESR spectrum (benzene): triplet with $a_N = 15.4$ G; $g = 2.0048$.

Optical purity of compounds 6, 7, and 9. The *S* configuration was assigned to ester **7** prepared from *S*-tyrosine.[13] For reasons stated within the Discussion the following experiments were carried out to unambigously determine the optical purity of compounds **6**, **7**, and **9** (Scheme 1): (a) Ester **7** (obtained from its purified chlorohydrate)[13] has the specific rotation $[\alpha]_D^{20} = +5.1 \pm 0.5°$ (*c* 1.1, $CHCl_3$); (b) **7** was converted into (possibly diastereomeric) salt(s) by treatment, in the NMR tube, with equimolar (1*S*)-(+)-10-camphorsulfonic acid; the NMR spectrum evidenced only one signal for the proton at the asymmetric carbon (shifted by 0.3 ppm relative to the signal of the same proton in **7**, as expected for conversion of amino group to $N\dot{H}_3^+$). This means that either only the (*S*) enantiomer is present or the NMR signals of corresponding protons in the diastereomers are too close together for resolution; (c) GC analysis of nonparamagnetic ester **7** on a chiral column (permethylated β-cyclodextrin) revealed a single peak (vaporizer: 150 °C; column: temperature programed from 100 to 150 °C with an increase of 1 °C/min and from 160 to 230 °C with 3 °C/min). The same conditions enabled distinct separation in enantiomers of racemic methyl phenylalaninates, prepared from authentic DL-phenylalanine by the procedure used for **7**; and (d) ester **7**, refluxed for 3 h in toluene in the presence of MeONa showed practically no change of specific rotation; its GC analysis under the above conditions revealed a single peak with exactly the same retention time as before methoxide treatment. Consequently, the strong base did not disturb the chiral center.

Radicals **9** and **6** and their reduced counterparts (e.g., **10**) are too unstable to withstand the drastic thermic conditions of VPC analysis. However, since **7** did not racemize on exposure to MeONa we may safely conclude that **9** and **6** are optically pure and have absolute configuration *S*.

***N*-(1-Oxyl-3-carbonyl-2,2,5,5-tetramethylpyrroline)tyramine (16) and *N,O*-bis(1-oxyl-3-carbonyl-2,2,5,5-tetramethylpyrroline)tyramine (17).** 1.65 g (8.97 mmol) of 2,2,5,5-tetramethyl-1-pyrrolinyloxy-3-carboxylic acid (**20**) and 0.65 mL (8.97 mmol) thionyl chloride were reacted in 20 mL of benzene and 1.3 mL (16.16 mmol) pyridine, as described previously to give acid chloride **15**.[10a] After dilution of the reaction mixture with 40 mL of benzene and filtration of pyridine hydrochloride, the orange, clear solution of **15** was added to a solution of 0.75 g (5.47 mmol) tyramine (Sigma) in 20 mL of aqueous 10% sodium hydroxide solution. The flask was stoppered and shaken vigorously for 40 min, then layers were separated.

The red *aq layer* containing **16** (as its sodium phenolate) was extracted several times with benzene to remove traces of unreacted **15**, then acidified with hydrogen chloride and repeatedly extracted with methylene chloride. The yellow extracts were washed with 3 × 20 mL of a 10% sodium bicarbonate soln [to recover acid **20** (0.35 g) resulted from accompanying hydrolysis of **15**] and water, dried on magnesium sulfate, and evaporated to give 0.35 g (21.1%, based on tyramine) of **16**, yellow crystals, mp 173–174.5 °C. Anal. calcd for $C_{17}H_{23}N_2O_3$: C, 67.29; H, 7.65; N, 9.24. Found:

Table 3. Spectra of compounds **4**, **5**, and **9**

Compd	IR (CCl_4; ν, cm^{-1})	ESR[a]	
		a_N (G)	*g*
4	1255, 1290 (C—O—C); 1610 (C=C); 1680 (C=O); 3375, 3500 (NH_2)	15.4	2.0061
5	1215, 1275 (C—O—C); 1612 (C=C); 1700 (C=O); 3405, 3495 (NH_2)	15.5	2.0061
9	1600 (C=C); 1720 (C=O); 3390 (NH_2)	15.4	2.0062

[a]Triplets; in benzene, at 20 °C.

C, 67.01; H, 7.55; N, 9.35%. IR spectrum (CH_2Cl_2) ν_{max}: 1510, 1660 and 3435 (amide), 1610 (double bond) and 3570 cm^{-1} (assoc. OH). ^1H NMR spectrum (CD_3OD, δ (ppm); after reduction of NO· with phenylhydrazine): 1.28 (s) and 1.40 (s) (12H, CH_3); 2.78 (t) (2H, —$C\underline{H}_2C_6H_4OH$), 3.47 (t) (2H, $C\underline{H}_2NH$), 7.19 (d) ($2H_{arom}$ adjacent to OH; $J=8.5$ cps) and 7.51 (d) ($2H_{arom}$; $J=8.5$ cps). ESR spectrum (benzene): triplet, $a_N=15.03$ G, $g=2.0059$.

The *benzene layer* separated after reaction yielded on evaporation a solid that was twice recrystallized from ethyl ether:hexane to give 0.3 g (11.7%, based on tyramine) of biradical **17**, light-yellow crystals, mp 183–185 °C. Anal. calcd for $C_{26}H_{35}N_3O_5$: C, 66.50; H,7.51; N, 8.95. Found: C, 66.31; H, 7.45; N, 9.04%. IR spectrum (CCl_4) ν_{max}: 1505 and 1670 (amide), 1620 (double bond) and 1730 cm^{-1} (ester CO). ESR spectrum (benzene) showed a quintet with $a_N=7.02$ G, $g=2.0059$.

1-Oxyl-3-(2-bromoethoxycarbonyl)-2,2,5,5-tetramethylpyrroline (18). 1,2-Dibromoethane (6 g, 32 mmol) was added to a hot solution of salt **19** (2.2 g; 10 mmol) in dimethylformamide (25 mL) and the resulted solution heated with stirring, at 80 °C, for 8 h. The solvent was removed under reduced pressure and the solid residue treated with water and dichloromethane. The organic layer was separated, washed with dilute aqueous potassium hydroxide solution and water, dried over magnesium sulphate, and concentrated in vacuo with heating (to remove unreacted excess of dibromoethane). A yellow oil (1.7 g) crystallizing on standing was obtained. Recrystallization from chloroform–hexane gave yellow needles, mp 59–61 °C. Anal. calcd for $C_{11}H_{17}NO_3Br$: C, 45.38; H, 5.88; N, 4.81; Br, 27.44. Found: C, 45.52; H, 6.01; N, 4.73; Br, 27.62%. ^1H NMR [$CDCl_3$, δ (ppm); after phenylhydrazine reduction] 1.33 (s) and 1.43 (s) (12H, CH_3), 3.63 (m, 2H, CH_2Br), 4.46 (m, 2H, CH_2O). IR spectrum (CCl_4) ν_{max}: 1250, 1290 (C—O—C); 1621 (C=C); 1715 (C=O); 2865, 2892 (sh.), 2930 and 2979 cm^{-1} (CH_2, CH_3). ESR spectrum (benzene): triplet, $a_N=14.0$ G; $g=2.0058$.

1,2-Bis(1-oxyl-3-oxycarbonyl-2,2,5,5-tetramethylpyrroline)-ethane (21). The same procedure as above was followed, but the reaction was carried out with an excess of salt **19** (2.2 g, 10 mmol) relative to dibromoethane (0.6 g, 3.2 mmol). Work up afforded a yellow solid (1.4 g) that on recrystallization from chloroform:hexane (1:30, vol) gave light-yellow crystals (1.0 g, 79% yield), mp 176–178 °C. Anal. calcd for $C_{20}H_{30}N_2O_6$: C, 60.90; H, 7.67; N, 7.10. Found: C, 61.18; H, 7.95; N, 6.88%. ^1H NMR [$CDCl_3$, δ (ppm); after phenylhydrazine reduction]: 1.32 (s) and 1.43 (s) (24H, CH_3), 4.48 (m, 4H, CH_2). IR spectrum (CCl_4) ν_{max}: 1250, 1290 (C—O—C); 1620 (C=C); 1715 (C=O); 2867, 2890, 2930, and 2980 cm^{-1} (CH_2, CH_3). ESR spectrum (benzene): quintet, $a_N=14.1$ G and $g=2.0059$.

***N*-[1-Oxyl-3-(ethylenoxycarbonyl)-2,2,5,5-tetramethylpyrroline]ethylenediamine (22).** A solution of bromoderivative **18** (4.5 g, 15.46 mmol) in ethylenediamine (19 g; 316.6 mmol) was heated at 55–60 °C for 4 h, then at 65–70 °C for 6 h in an inert atmosphere. Unreacted ethylenediamine was removed from the reaction mixture by distillation under reduced pressure and the residue purified by column chromatography on neutral alumina (100 g, eluent: chloroform:dichloromethane, 1:1) yielding a pink–yellow oil (2.55 g, 61.0%). Anal. calcd for $C_{13}H_{24}N_3O_3$: C, 57.76; H, 8.95; N, 15.54. Found: C, 57.61; H, 9.07; N, 15.80%. ^1H NMR [$CDCl_3$, δ (ppm); after phenylhydrazine reduction]: 1.30 (s) and 1.43 (s) (12 H, CH_3), 2.90 (m, 6 H, $C\underline{H}_2$—NH_2 and $C\underline{H}_2$—NH), 3.78 (t) (2 H, COO—$C\underline{H}_2$). IR spectrum ($CHCl_3$) ν_{max}: 1620 (C=C), 1660 (C=O), 2800–3000 (CH_2, CH_3), 3000–3100 (assoc. NH) and 3100–3600 cm^{-1} (assoc. NH_2). ESR spectrum (water): triplet, $a_N=15.78$ G and $g=2.0054$.

2-(1-Oxyl-3-oxycarbonyl-2,2,5,5-tetramethylpyrroline)-*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)ethaneamine (23). A solution of bromoderivative **18** (2.2 g; 7.5 mmol) and Tempamine (2.6 g; 15.2 mmol) in dimethylformamide (15 mL) was heated at 80 °C for 6 h, then concentrated in vacuo. Water was added to the residue and the mixture extracted with ethyl ether. The ethereal extracts were repeatedly washed with water to remove traces of Tempamine and then with 5 × 20 mL of buffer solution (citric acid–Na_2HPO_4) having pH 5.5. Amine **23**, which separated on saturation of the red acid solution with potassium carbonate, was extracted in ethyl ether, the extracts dried over potassium carbonate and evaporated to give a red viscous oil (1.5 g; 52% yield). Anal. calcd for $C_{20}H_{35}N_3O_4$: C, 62.96; H, 9.25; N, 11.01. Found: C, 62.73; H, 9.35; N, 11.20%. IR spectrum (CCl_4) ν_{max}: 1250, 1290 (C—O—C); 1620 (C=C); 1712 (C=O); 2775, 2855, 2928, and 2977 cm^{-1} (CH_2, CH_3). ESR spectrum (benzene): quintet, $a_N=14.5$ G, $g=2.0060$. ^1H NMR [$CDCl_3$, δ (ppm); after phenylhydrazine reduction]: 1.18 (s) and 1.23 (s) (12H, piperidine methyls), 1.28 (s) and 1.40 (s) (12H, pyrroline methyls), 2.93 (m, 2H, $C\underline{H}_2$—NH), 4.28 (m, 2H, CH_2O).

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