Synthesis of Nitroxides for Use as Procationic Labels and Their Incorporation into Nafion Films

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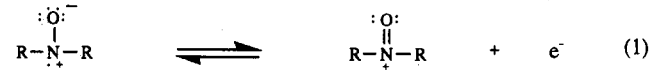
A nitroxide label was covalently attached to amphetamine and two antidepressants, nortriptyline and desipramine, by forming an amide linkage between the amino group on each of these compounds and 3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy. This nitroxide label was also attached to be biologically important compound biotin, but a spacer group, *N,N'*-dimethyl-1,6-hexanediamine, was needed to make two amide linkages between the carboxylic acids on the nitroxide and biotin. These nitroxide-labeled substances undergo a one-electron reversible oxidation between 0.5 and 0.6 V (vs. Ag/AgCl) at a naked glassy carbon (GC) elecrode and between 0.3 and 0.4 V at a GC electrode coated with a polyanionic film of Nafion. When a 0.8-V potential is applied to a GC/Nafion electrode in the presence of an aqueous buffer solution of one of these nitroxides, the nitroxide preconcentrates in the film in its oxidized form, the oxoammonium cation. Subsequent scanning of the potential in the negative direction using square wave voltammetry produces a reduction wave with a relatively large peak current making it possible to detect almost nanomolar quanitities of the procationic nitroxide. This preconcentration of procationic nitroxides makes this redox label useful in a recently developed analytical technique that combines immunoassay with Nafion-modified electrodes.

Nitroxides continue to play an important role not only as spin labels or probes in biological molecules[1-3] and polymers[4-6] but more recently as probes in inclusion complexes with cyclodextrins,[7-11] as magnetic resonance imaging (MRI) contrast enhancing agents[12,13] and as catalysts in redox processes.[14-17] As a result, considerable effort has been directed toward the synthesis of new nitroxides that can serve in these and other roles.[12,13,18-26] Undoubtedly, central to many studies involving stable nitroxides is their property of paramagnetism which allows these radicals to be observed with ESR spectroscopy.

Considerable attention has also been directed toward nitroxides as one-electron donors. Nitroxides can be reversibly oxidized at an electrode to form oxoammonium salts (1).[27,28] These salts have been used extensively for the oxidation of primary and secondary alcohols.[29,30]



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In this paper we show that nitroxides can also be useful procationic labels; that is, we take advantage of their electron-donating ability as shown in eq 1. We describe the synthesis of several new nitroxides in which the procationic label, the nitroxide, is covalently attached to two antidepressant drugs, desipramine (1) and nortriptyline (2), amphetamine (3), and the important biological molecule biotin (4). We then demonstrate that when these new nitroxides are electrochemically oxidized at an

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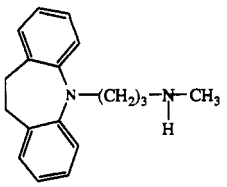

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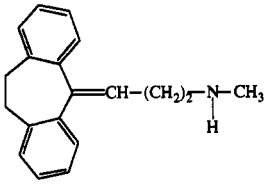

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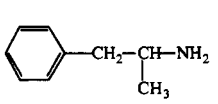

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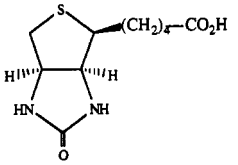

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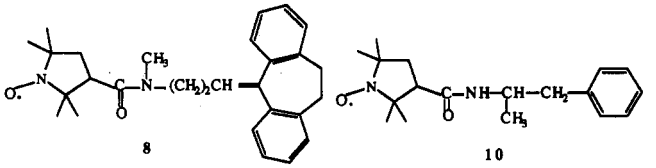
electrode surface modified with films of Nafion,[31] a perfluorinated anionic polyelectrolyte that is stable and exhibits permselectivity toward different cations,[32] the resulting cations are incorporated into these films in relatively high concentrations. This preconcentration of procationic nitroxides makes this redox label potentially useful in a new analytical technique that we have recently developed that combines immunoassay with Nafion-modified electrodes.[33a,b]

Results and Discussion

Synthesis. In Scheme I is given the sequence of reactions that were used to prepare nitroxide **5** in which desipramine is covalently attached to a nitroxide label via an amide linkage. The activated racemic ester **6** is obtained in a nearly quantitative yield from the commercially available racemic nitroxide **7**. The reaction of **6** with desipramine led to racemic **5** in a yield of 63%.

Somewhat surprisingly, the reaction of **6** with nortriptyline resulted not only in the formation of racemic nitroxide **8** but also a second nitroxide believed to be racemic **9**. The formation of the latter nitroxide can be rationalized as arising from nucleophilic attack of nortriptyline at one of the carbonyls of the succinimide. This mode of reaction has been observed previously with *N*-hydroxysuccinimide esters of organometallics.[33c] Nitroxides **8** and **9** were obtained in yields of 55 and 29%, respectively.

L-Amphetamine reacts with **6** to give a 71% yield of nitroxide **10**, which is formed presumably as a mixture of two diastereomers. No product was found resulting from nucleophilic attack of amphetamine at one of the succinimide carbonyls.



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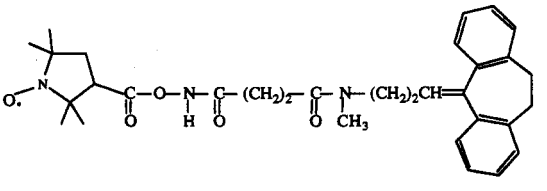

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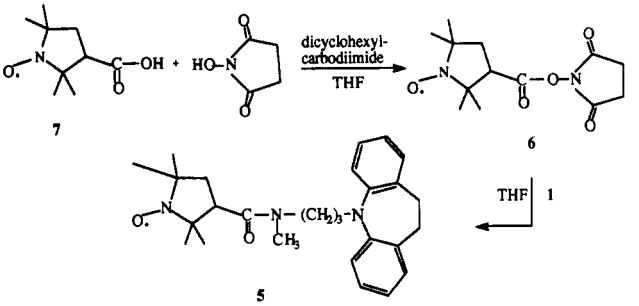
(31) Nafion is a trademark registered by E.I. Dupont de Nemours, Inc.

Scheme I



Scheme II

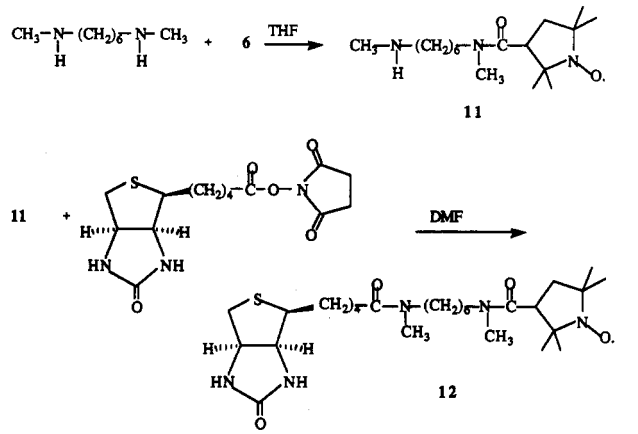


Table I. *g* Factors and Hyperfine Splitting Constants of Nitroxides 5, 8, 10, and 12

nitroxide	*g* factor	hfsc (G)
5	2.0056 ● 0.0002	14.0
8	2.0057 ± 0.0002	14.1
10	2.0058 ± 0.0002	13.8
12	2.0064 ± 0.0002	13.7

Since biotin is a carboxylic acid, a spacer group was needed to covalently attach it to nitroxide **7**. *N,N'*-Dimethyl-1,6-hexanediamine, our choice for this group, was first linked to **7** via the *N*-hydroxysuccinimide ester **6** in a yield of 33% and then, as shown in Scheme II, attached to d-biotin using a second amide group to give a 68% yield of nitroxide **12** as a mixture, presumably, of two diastereromers.

ESR Data. Electron spin resonance (ESR) spectra were obtained from nitroxides **5**, **8**, **10**, and **12**. The *g* factors and nitrogen hyperfine splitting constants (hfscs) shown in Table I are well within the range of values expected for this type of radical.[34]

Cyclic and Square Wave Voltammetry. A cyclic voltammogram (CV) of the nitroxide-labeled biotin **12** in aqueous buffer (pH 7.4) exhibits a reversible oxidation wave with a peak potential (E_p) of 0.536 V using a naked but pretreated[35] glassy carbon (GC) electrode. The

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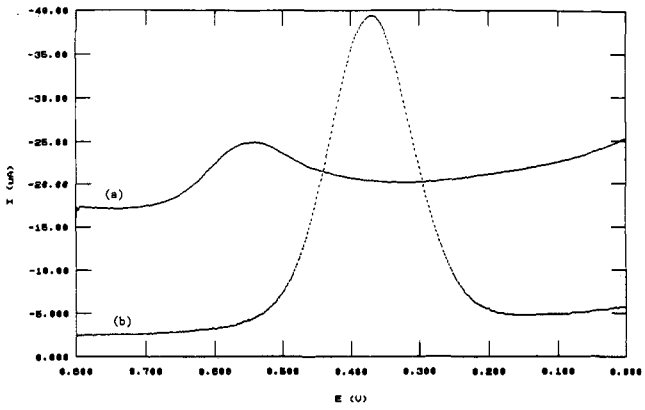

Figure 1. Square wave voltammograms of (a) 64.0 μM **10** at a naked GC electrode and (b) 1.92 μM **10** at a Nafion-coated GC electrode after a potential of +0.80 V (vs Ag/AgCl) was applied for 5 min with each electrode rotating (600 rpm). The rotation was then stopped, and a potential scan was made in the negative direction giving a wave that corresponds, therefore, to the reduction of 10^+.

difference in potential between the anodic and cathodic waves (i.e., ΔE_p) is nearly 60 mV which is consistent with the one-electron process in eq 1. Plots of i_p vs $\nu^{-1/2}$ (i_p = peak current and ν = scan rate) give a straight line with scan rates up to 500 mV/s showing that the electrode process is under diffusion, not adsorption control.[36] CVs of the nitroxide-labeled desipramine **5**, nitroxide-labeled nortriptyline **8**, and nitroxide-labeled amphetamine **10** also show one oxidation wave between 0.5 and 0.6 V. However, with **5** and **8**, $i_p/\nu^{-1/2}$ is not constant at different scan rates but increases with increasing ν suggesting that adsorption occurs at the electrode surface, a result that is not surprising given the relatively large hydrocarbon moities that are present in these nitroxides.

Square wave voltammetry (SWV) is one of the most sensitive electroanalytical techniques that can be employed to detect reversible redox systems.[37] In Figure 1 are SW voltammograms of 64.0 and 1.92 μM **10** at naked and Nafion-coated GC, respectively. The medium was aqueous phosphate buffer (pH 7.4) containing enough ethanol (3%) to dissolve **10**. In these measurements a potential of +0.8 V was applied to the GC or GC/Nafion for 5 min at 600 rpm before the potential scan was made in the negative direction. Thus, the waves are one-electron reductions corresponding to the reverse reaction in eq 1. The peak potentials for the reduction of 10^+ at naked and Nafion-coated GC are 544 and 360 mV, respectively, giving a difference of 184 mV. Rather large differences in E_p were also found for 5^+, 8^+, and 12^+ as is shown in Table II along with their peak potentials. A substantial negative shift in potential has also been observed for the bound ferrocene/ferricinium couple in Nafion films.[38]

Preconcentration in Nafion. Figure 1 shows that the Nafion coating not only decreases the potential at which 10^+ reduced but also, as expected, greatly increases i_p. This can be quantified by comparing the i_p/C values for the naked and Nafion-coated electrodes which are calculated to be 0.096 and 18.6 μA/μM. These values are fairly constant over a wide range of concentrations for **10** (Table III). It seems quite apparent then that the polyanionic Nafion film concentrates the amphetamine nitroxide in its oxidized form (i.e., 10^+) by cation exchange and that the rate of exchange is proportional to the concentration of **10** in the bulk solution provided that the anionic sites are not saturated. This preconcentration enhances the peak current considerably when 10^+ is reduced thereby providing an electrochemical method with a detection limit of 5×10^{-9} M (accumulation time = 15 min) for this nitroxide. Similar results were obtained from **5**, **8**, and **12**.

Table II. Peak Potentials for 5^+, 8^+, 10^+, and 12^+ at GC and GC/Nafion Electrodes[a]

analyte	E_p GC/naked[b] (V)	E_p GC/Nafion[b] (V)	[(GC/naked) − (GC/Nafion)][c]
5^+	0.548	0.330	218
8^+	0.530	0.336	194
10^+	0.544	0.360	184
12^+	0.536	0.350	186

[a] Aqueous phosphate buffer, pH 7.4. [b] Vs[Ag/AgCl, Cl^{-1} (0.056 M)]. [c] mV.

Summary and Conclusions

This work demonstrates then that a stable nitroxide possessing a carboxylic acid can be easily attached to an amine via an amide linkage and to another carboxylic acid via the spacer group, *N,N'*-dimethyl-1,6-hexanediamine, by employing two amide linkages. Since the nitroxide undergoes a one-electron oxidation to a relatively stable cation, it is concentrated in a polyanionic film of Nafion when a sufficiently positive potential is applied to an electrode modified with the film. This preconcentration of the cationic form of a nitroxide followed by its reduction in the film using square wave voltammetry allows this technique to be used to detect nanomolar quantities of substances bearing the procationic label and should, therefore, also be useful in homogeneous competetive immunoassays.[39] In fact, work is in progress aimed at using this label in a new analytical technique that we have recently developed that combines immunoassay with Nafion-modified electrodes.[33a,b]

Experimental Section

General. The phosphate buffer (pH 7.4) was 8.7 mM NaH_2PO_4, 30.4 mM Na_2HPO_4, and 56.0 and mM NaCl. GC rods were obtained from Carbone Lorraine. L-Amphetamine was prepared from its sulfate salt (Sigma) by treatment with aqueous NaOH followed by extraction with ether. Infrared spectra were obtained with a Nicolet 205 FT spectrometer. Elemental analyses were performed by Centre National de la Recherche Scientifique (CNRS). Mass spectral analyses were made by CNRS (Lyon) and the University of Rennes. All reagents were of analytical grade, and the water was deionized and doubly distilled.

Electrode Preparation. GC rods were sanded flat with 1200-grit silicon carbide paper and polished with 0.05 μm aqueous alumina suspension (ESCIL). Immediately after polishing, the electrodes were ultrasonically cleaned in ethanol, rinsed with doubly distilled water, and dried at 100 °C in an oven.

In the preparation of Nafion-coated GC, 0.4 mL of a Nafion solution (Aldrich, ref 27,470-4) was combined with 19.28 mL of DMF and 0.32 mL of aqueous 0.05 M LiOH to give the Li^+ salt of the Nafion. The Nafion coating was made by applying 5 μL

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Table III. Effect of Concentration on Peak Currents from the Reduction (SWV) of 10^+ at Naked and Nafion-Coated Glassy Carbon Electrodes[a]

naked				Nafion-coated			
% ethanol	C (μM)	i_p (μA)	C/M (μM/μA)	% ethanol	C (μM)	i_p (μA)	C/M (μM/μA)
0.37	8.00			*b*	0.192	3.40	17.7
0.75	16.0	1.54	0.0962	*b*	0.480	9.52	19.8
1.50	32.0	3.00	0.0931	*b*	0.950	17.8	18.7
3.00	64.0	6.13	0.0958	*b*	1.92	35.7	18.6

[a] Aqueous phosphate buffer (pH 7.4) containing the given amounts of ethanol needed to dissolve **10**. A +0.8 V potential was applied for 5 min at a naked GC or a Nafion-coated GC electrode with rotation (600 rpm) before the rotation was stopped and the potential was scanned in the negative direction. [b] Negligible.

of this diluted solution to the pretreated GC surface[35] and removing the bulk of the solvent at 140 °C for 5 min under an atmosphere saturated with DMF vapor. To assure complete removal of solvent, the electrode was placed in an oven for 10 min at 140 °C. For each measurement, a GC/Nafion rod was pressure-fitted into a narrow cylindrical hole of a Teflon tube in such a way that only the modified surface was exposed to the nitroxide solution. A film thickness of 0.4 μm was calculated by assuming a density of 1.58 g/cm^3.

Cyclic and Square Wave Voltammetry. Electrochemical measurements were made at 22 °C in a one-compartment cell (2 mL working volume) using a Princeton Applied Research 273 Potentiostat/Galvanostat interfaced to a IBM XT 286 computer system with PAR M270 software. In CV the working electrode was GC or GC modified with a Nafion film as described above; the reference electrode was Ag/AgCl (0.05 M Cl$^-$), and the counter electrode was a Pt wire. In SWV the working electrode was GC or GC/Nafion mounted on a Tacussel rotating-disk electrode; the reference electrode was Ag/AgCl (0.05 M Cl$^-$); and the counter electrode was a PT wire. The potential step increment (δE) was 2 mV; the square wave amplitude (E_{SW}) was 50 mV; and the frequency (f) was 100 Hz.

ESR Measurments. ESR measurements were made at 25 °C using a Bruker 200D spectrometer with a field modulation of 100 kHz and a microwave frequency of 9.75 GHz. A solution of the nitroxide in CH_3CN was introduced into a quartz flat cell to which was attached a capillary tube containing α,α'-diphenyl-β-picrylhydrazyl (DPPH, $g = 2.0036 \pm 0.0002$). The g factor was calculated from the field difference between the spectral centers of the nitroxide and DPPH.

Nitroxide 6. A mixture of commercially available (Aldrich) racemic 3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (200 mg, 1.07 mmol), *N*-hydroxysuccinimide (136 mg, 1.18 mmol), and *N,N'*-dicyclohexylcarbodiimide (243 mg, 1.18 mmol) in 15 mL of dry THF (freshly distilled from CaH_2) was stirred under N_2 at room temperature for 54 h. The urea byproduct was removed by gravity filtration. The solvent in the yellow filtrate was removed under reduced pressure (rotary evaporator), and the resulting viscous liquid residue was chromatographed on silica gel and eluted with CH_2Cl_2–acetone (5:1). A single yellow band was collected and evaporated to dryness giving racemic **6** as a semisolid: IR (Nujol) 1820 (s), 1790 (s), 1740 (s), 1440, 1305, 1290, 1260 (w), 1240, 1205 (s), 1095 (s), 1065 (s), 1045, 995, 965, 930, 895, 840, (w), 815, 770, 735 (w), 650 (s) cm^{-1}; MS (70eV) *m/e* (relative intensity) 283 (24), 269 (8), 253 (8), 169 (8), 154 (20), 138 (21), 126 (11), 111 (21), 97 (23), 83 (100), 74 (29), 69 (51), 58 (44), 55 (37); HRMS calcd for (M^+) $C_{13}H_{19}N_2O_5$ 283.1294, found 283.1291. Anal. Calcd for $C_{13}H_{19}N_2O_5$: C, 55.11; H, 6.76; N, 9.89. Found: C 54.75; H, 6.80; N, 9.81.

Nitroxide 5. To a solution of desipramine (188 mg, 0.706 mmol) in 10 mL of dry THF (freshly distilled from CaH_2) was added racemic nitroxide **6** (100 mg, 0.352 mmol). The yellow solution was allowed to stand under N_2 at room temperature for 4 days and was then heated in an oil bath at 50–60 °C for 3 days. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel and eluted with CH_2Cl_2 followed by CH_2Cl_2–acetone mixtures of 10:1 and 3:1. A single yellow band was collected which, following removal of solvent under reduced pressure, gave racemic nitroxide **5** as a yellow solid (97 mg, 63%). Recrystallization from heptane/toluene (twice) gave yellow plates: mp 186–9 °C; IR (Nujol) 1649 (s), 1600 (w), 1590 (w), 1489, 1413 (w), 1379, 1362, 1348 (w), 1320 (w), 1292 (w), 1252, 1235, 1223, 1199, (w), 1180 (w), 1172 (w), 1130 (w), 1115 (w), 1110 (w), 1065 (w), 988 (w), 934 (w), 917 (w), 773, 760, 750, 734, 658 cm^{-1}; MS (70 eV) *m/e* (relative intensity) 434 (28), 404 (10), 348, (7), 234, (28), 193 (38), 168 (18), 154 (15), 153 (16), 141 (18), 138 (39), 126 (20), 110 (20), 99 (41), 92 (75), 91 (100), 65 (12), 58 (98). Anal. Calcd for $C_{27}H_{36}N_3O_2$: C, 74.62; H, 8.35; N, 9.67. Found: C, 74.82; H, 8.11; N, 9.51.

Nitroxides 8 and 9. To a solution of nortripyline (186 mg, 0.706 mmol) in 10 mL of dry THF (freshly distilled from CaH_2) was added nitroxide **6** (100 mg, 0.352 mmol). The yellow solution was allowed to stand under N_2 at room temperature for 4 days and was then heated in an oil bath at 50–60 °C for 3 days. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel and eluted with CH_2Cl_2 followed by CH_2Cl_2–acetone mixtures starting with a radio of 10:1 and ending with 1:1. Two yellow fractions were collected. Removal of solvent from the first fraction under reduced pressure gave 83 mg (55%) of racemic nitroxide **8** as a yellow solid. Recrystallization (three times) from heptane gave 50 mg of yellow plates: mp 183–6 °C; IR (Nujol) 1633 (s), 1584, 1413, 1338 (w), 1321, 1295 (w), 1261, 1242, 1190 (w), 1158 (w), 1116, 1100, 1053 (w), 1042 (w), 1000 (w), 978 (w), 895 (w), 889 (w), 784, 763, 753 (w), 726 (w), 663 (w), 637 (w) cm^{-1}; MS (70 eV) *m/e* (relative intensity) 431 (35), 416 (8), 401 (28), 232 (47), 219 (12), 217 (17), 191 (12), 138 (20), 126 (13), 99 (27), 91 (44), 44 (100); HRMS calcd for (M^+) $C_{28}H_{35}N_2O_2$ 431.2698, found 431.2702. Anal. Calcd for $C_{28}H_{35}N_2O_2$: C, 77.92; H, 8.17; N, 6.49. Found: C, 78.07; H, 8.22; N, 6.42. Removal of solvent from the second yellow fraction gave a light yellow solid (55 mg, 29%) of racemic nitroxide **9**: mp 55–8 °C; IR (Nujol) 1785 (s), 1710 (s), 1625 (s), 1305, 1250 (w), 1160, 1115 (s), 1095 (s), 1068, 1038, 973, 780, 760, 741, 718 cm^{-1}. Anal. Calcd for $C_{32}H_{40}N_3O_5$: C, 70.30; H, 7.37; N, 7.68. Found: C, 70.90; H, 7.38; N, 7.56.

Nitroxide 10. To a solution of L-amphetamine (96 mg, 0.706 mmol) in 10 mL of dry THF (freshly distilled from CaH_2) as added the *N*-hydroxysuccinimide ester of nitroxide **6** (100 mg, 0.352). The yellow solution was allowed to stand under N_2 at room temperature for 4 days and was then heated in an oil bath at 50–60 °C for 3 days. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel and eluted with CH_2Cl_2–acetone (4:1). A single yellow band was collected which gave a yellow solid (93 mg) after removal of solvent under reduced pressure. Recrystallization (twice) from heptane/toluene gave yellow plates of nitroxide **10** presumably as a mixture of two diastereomers (75 mg, 71%): mp 194–7 °C; IR (Nujol) 3320, 1645 (s), 1553, 1315 (w), 1260 (w), 1232 (w), 1212 (w), 1170 (w), 1155 (w), 1137 (w), 1102 (w), 1064 (w), 1011 (w), 977 (w), 920 (w), 860 (w), 745, 710, 653 (w) 613 (w) cm^{-1}; MS (70 eV) *m/e* (relative intensity) 303 (8), 289 (7), 273 (32), 230 (5), 212 (11), 197 (6), 138 (15), 126 (22), 119 (15), 118 (20), 112 (16), 110 (16), 99 (13), 91 (51), 83 (35), 74 (11), 69 (20), 58 (19), 44 (100); HRMS calcd for $C_{18}H_{27}N_2O_2$ 303.2072, found 303.2071. Anal. Calcd for $C_{18}H_{27}N_2O_2$: C, 71.25: H, 8.97; N, 9.23. Found: C. 70.94; H, 8.84; N, 9.15.

Nitroxide 11. A solution of a large excess of *N,N'*-dimethyl-1,6-hexanediamine (2.43 g, 16.9 mmol) and nitroxide **6** (530 mg, 1.89 mmol) in 6 mL of dry THF (freshly distilled from CaH_2) was allowed to stand under N_2 at room temperature for 6 days during which time a white solid formed. The solid was filtered (180 mg), and the THF was removed from the filtrate under reduced pressure giving a yellow liquid residue. The residue was combined with 10 mL of H_2O and extracted with hexane (2 × 20 mL) and

ether (2 × 10 mL). The colorless extracts containing excess diamine were discarded. The yellow aqueous layer was extracted with CH_2Cl_2 until the extracts were no longer yellow (4 × 10 mL). The CH_2Cl_2 extracts were combined, washed with water (4 × 20 mL), and dried over anhydrous Na_2SO_4. Removal of CH_2Cl_2 gave a viscous, yellow liquid which was chromatographed on silica gel and eluted with acetone–water–NaCl (50 mL:50 mL:150 mg). A single yellow band was collected, and the bulk of the acetone was removed under reduced pressure. The aqueous solution was basified with 1.0 M NaOH and extracted with CH_2Cl_2 (4 × 10 mL). The CH_2CL_2 extracts were combined, dried over Na_2SO_4, and evaporated to dryness under reduced pressure in a rotary evaporator. Further drying at 40–50 °C (0.1 Torr) gave 197 mg (33%) of racemic nitroxide **11** as a viscous, yellow liquid: IR (film) 3475, 2975, 2940 (s), 2860, 2810 (w), 1640 (s), 1470, 1420, 1370, 1310 (w), 1245 (w), 1196 (w), 1145 (w), 1105 (w), 1070 (w) cm^{-1}; MS (70 eV) *m/e* (relative intensity) 312 (21), 297 (3), 282 (11), 239 (3), 226 (5), 199 (4), 154 (7), 138 (33), 114 (13), 112 (21), 110 (14), 100 (25), 99 (13), 83 (16), 58 (15), 55 (17), 44 (100); HRMS calcd for $C_{17}H_{34}N_3O_2$ 312.2651, found 312.2652. Anal. Calcd for $C_{17}H_{34}N_3O_2$: C, 65.34; H, 10.97; N, 13.45. Found: C, 63.83; H, 10.76; N, 13.02. Attempts to obtain an analytically pure sample were unsuccessful.

Nitroxide 12. A solution of nitroxide **11** (164 mg, 0.550 mmol) and the *N*-hydroxysuccinimide ester of biotin[40] (187 mg, 0.550 mmol) in 1.25 mL of DMF was heated at 40–5 °C for 8 days. The reaction mixture was combined with 40 mL of ether resulting in the separation of a viscous, yellow liquid. Chromatography of this liquid on silica gel followed by elution with acetone–H_2O (10:1) gave a single yellow band which was collected and evaporated to dryness at 40 °C (0.1 Torr) giving a light yellow solid of nitroxide **12** presumably as a mixture of two diastereomers: mp 45–50 °C; IR (melt) 3300, 3060 (w), 2990, 2940, 2870, 1710 (s), 1635 (s), 1470, 1425, 1370 (w), 1310 (w), 1270 (w), 1245 (w), 1225 (w), 1165 (w), 1120 (w), 1105 (w), 1080 (w), 1040 (w), 735, 705 (w) cm^{-1}; MS (FAB) $(M + H)^+$ 539. Anal. Calcd for $C_{27}H_{48}N_5O_4S$: C, 60.19; H, 8.98; N, 13.00; S, 5.95. Found: C, 59.54; H, 8.65; N, 12.67; S, 6.00.

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