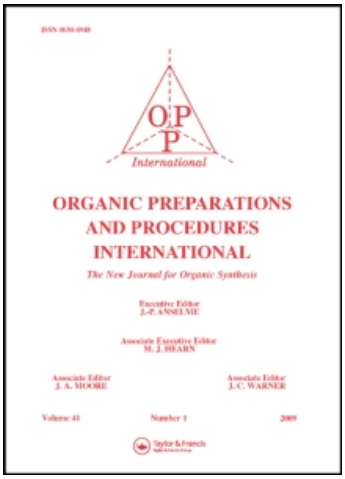

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APPLICATION OF SPIN LABELING TO DRUG ASSAYS. IV. SPIN- AND RADIOLABELED LIDOCAINE

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APPLICATION OF SPIN LABELING TO DRUG ASSAYS.

IV. SPIN- AND RADIOLABELED LIDOCAINE

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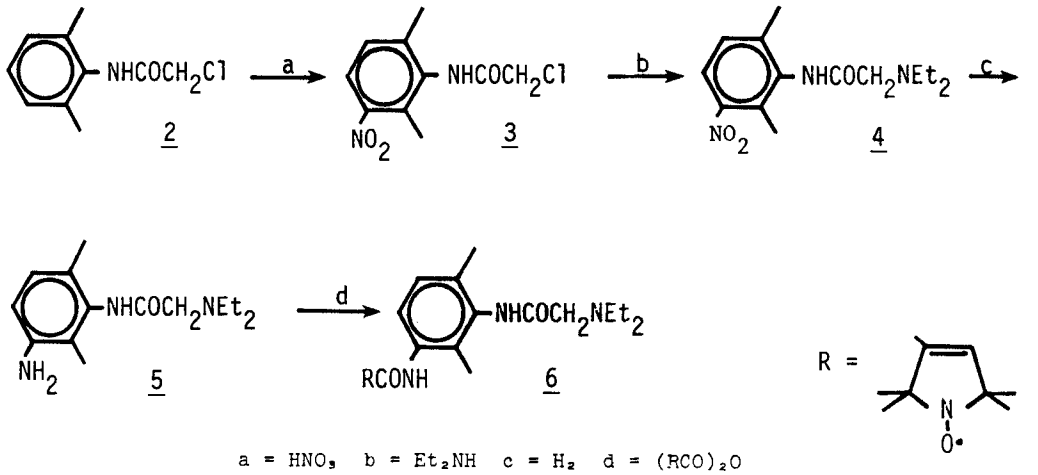
Drugs in the blood are either free in solution or bound to serum proteins. Since only the free drug is therapeutically active, it is important, during the monitoring of drug levels, to determine the free fraction. The current methods for the estimation of the free fraction are primarily based on either ultrafiltration or on equilibrium dialysis. These techniques are time consuming, expensive and fraught with technological problems. In an effort to overcome these problems, we have in recent years been investigating the application of the electron spin resonance (ESR) spectrometry for monitoring drug levels. Since the ESR spectra of the free and the bound spin label differ in shape and intensity, the free and the bound fractions of the drug can be readily determined without the physical separation of the two fractions. We have recently reported studies on the binding of spin-labeled morphine[1] and phenytoin[2] to serum proteins. We now report the synthesis of several spin-labeled derivatives of the antiarrhythmic drug lidocaine (1, ω-diethylamino-2,6-dimethylacetanilide) where the labels were linked to the xylidine or the glycine portion of 1. Spin labels are conveniently attached *via* a carbonyl or an amino group.

Our attempt to introduce an amino on 1 *via* nitration of 1 was unsuccessful. However, 2, (2-chloro-2',6'-acetoxylidide, Aldrich), a

precursor of 1, could be readily nitrated to give 2-chloro-N-(2,6-dimethyl-3-nitrophenyl)acetamide (3, Scheme 1). Substitution of the halide with an



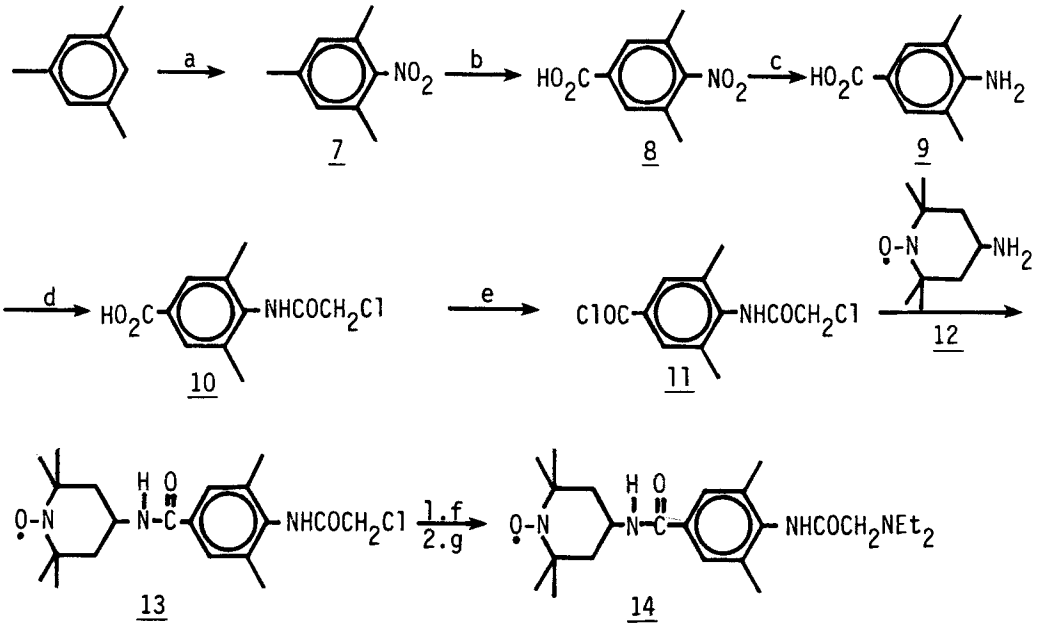
2 3 4 5 6

a = HNO_3 b = Et_2NH c = H_2 d = $(RCO)_2O$

Scheme 1

N,N-diethylamino group gave 4 and reduction of the nitro group gave 5. The carbonyl of the spin label was linked to the amino group of 5 to give 6.

The synthesis of 14, a 4'-carboxy substituted 1, was carried out as shown in Scheme 2.

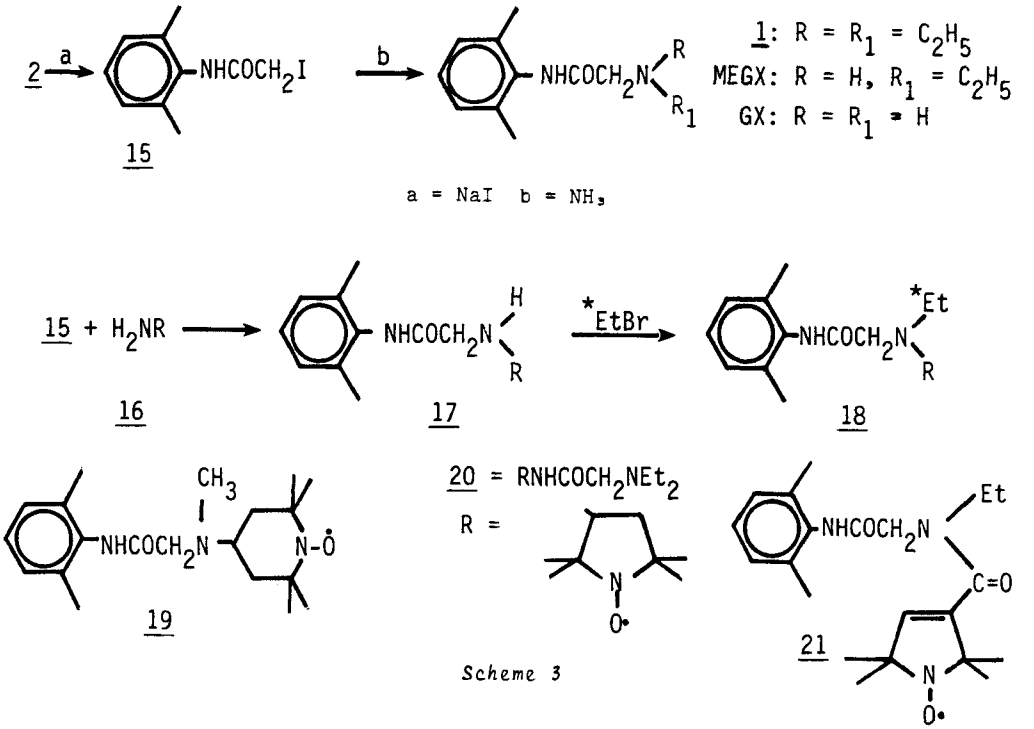


7 8 9 10 11 12 13 14

a = HNO_3 b = CrO_3 c = H_2/Pt d = $(ClCH_2CO)_2O$ e = $(COCl)_2$ f = NaI g = Et_2NH

Scheme 2

Various other analogs of 1 were prepared by coupling spin labels to the amino N-atom, as shown on Scheme 3. These derivatives are more readily prepared from 2-iodo-N-(2,6-dimethylphenyl)acetamide (15), because the iodide ion is more readily displaced than the chloride. The chlorine atom on the acetamido moiety in Scheme 2 structure 11 was retained thus inhibiting 12 from reacting at the ω-carbon.



Scheme 3

To conduct competitive binding studies of spin-labeled 1 with proteins, radiolabeled 1 was required. Reaction of 2 with ethylamine gave monoethylglycylxylidide (MEGX) which upon ethylation with [^{14}C]-bromoethane gave radiolabeled 1. Similarly, the major metabolite of 1, MEGX, was radiolabeled by ethylating glycinexylidide (GX) with [^{14}C]-bromoethane. The products were co-chromatographed with authentic material on TLC and HPLC. Moreover, a spin-labeled analog of 1 was also radiolabeled by N-alkylation of 17 with [^{14}C]-EtBr to give 18 in about 5% yield. The low yield is

apparently due to the resistance to N-alkylation of the secondary amines on nitroxides, previously noted.[5] The identity of the products was confirmed by spectral data and by the use of special test reactions. For example, 2 could not be N-ethylated because of hindrance at the nitrogen, therefore with ethyl bromide, 17 gave 18 and not a tertiary amide. The reaction of MEGX with 3,5-dibromo-2,2,6,6-tetramethylpiperidin-4-one-1-oxyl gave N-(2,6-dimethylanilino-carbonylmethylene)-N-ethyl-2,2,5,5-tetramethylpyrroline-3-carboxamide-1-oxyl (21).[9] All spin-labeled probes gave ESR spectra typical of nitroxides (three peaks). Binding studies of these probes to serum proteins will be reported elsewhere.

EXPERIMENTAL SECTION

All mps. are uncorrected. Infrared spectra were obtained as KBr pellets on a Beckman IR-10 spectrometer; NMR spectra were determined on a Varian T-60 spectrometer; mass spectra were obtained on an AEI Model MS-30 spectrometer; ultraviolet spectra were obtained on a Beckman Model DK-2.

2-Chloro-N-(2,6-dimethyl-3-nitrophenyl)acetamide (3). — 2-Chloro-N-(2,6-dimethylphenyl)acetamide (2, 590 mg, 3.0 mmol, Aldrich) in acetic acid (4 ml) and conc. nitric acid (0.5 ml) was cooled in an ice bath and conc. sulfuric acid (0.30 ml) was then added. The mixture was then kept at 85° for 4 hrs. It was cooled, concentrated under vacuum and diluted with cold water (5 ml). The precipitate was recrystallized from 95% ethanol-water, giving 490 mg (67%) of white crystals, mp. 155-158°; IR (KBr): 3250(NH), 1660(C=O), 1530, 1340(NO) cm^{-1}; NMR (DMSO-d_6): δ 2.25(CH_3), 4.10 (CH_2) and doublets at 7.25 and 7.70, J = 8 Hz, ortho aromatic H; MS: m/e 242 (M^+).

2-Diethylamino-N-(2,6-dimethyl-3-nitrophenyl)acetamide (4). — Diethylamine (2 ml) and 3 (175 mg) in toluene (10 ml) were heated at 65° in a tightly stoppered flask for 2 hrs. The mixture was cooled to 20° and a white precipitate was filtered off. The filtrate was concentrated to 4 ml. Upon cooling it gave the product which was collected by filtration (110 mg,

55%), mp. 88°. IR (KBr): 3280(NH), 1690(C=O), 1520 and 1340(NO) cm^{-1}; MS: m/e Calcd. for $C_{14}H_{21}N_3O_3$: 279.1575; found: 279.1561.

N-(3-Amino-2,6-dimethylphenyl)-2-diethylaminoacetamide (5). – Compound 4 (100 mg) in ethanol was hydrogenated in the presence of 10% Pd-C for 5 hrs. The catalyst and the solvent were removed. 5 was purified by TLC (ethyl acetate), Rf - 0.27, (52 mg, 58%), mp. 95-98°. IR (KBr): 3450, 3360, 3320, 3250(NH) and 1670(C=O) cm^{-1}; UV (EtOH): 244 (11,000) and 290 (2,200) nm; MS: m/e 249 (M^+).

N-[2,4-Dimethyl-3-(2-diethylaminoacetamido)phenyl]-2,2,5,5-tetramethyl pyrroline-1-oxyl-3-carboxamide (6). – Equimolar amounts of 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid anhydride (Molecular Probes, Junction City, OR 97448) and 5 were heated in benzene for 4 hrs at 50°. The reaction mixture was purified on silica gel TLC plates with methanol:ethyl acetate (1:7). The band (Rf 0.50) gave 5 and the yellow band (Rf 0.37) gave the product 6, mp. 192-194° in 62% yield. IR (KBr): 3300(NH), 1670(C=O); MS: m/e Calcd. for $C_{23}H_{35}N_4O_3$: 415.2708; found: 415.2712 (M^+).[6]

Nitromesitylene (7). – Nitromesitylene was prepared by a modification of the method of Powell and Johnson.[3] In our procedure we have omitted the steam and the fractional distillation steps. Instead the precipitated product from the aqueous phase was collected by filtration and air-dried (54 g) mp. 40-42°. It was then dissolved in ether (300 ml). Sodium bicarbonate (3 g) and magnesium sulfate (5 g) were then added with stirring. The sediment was removed by filtration and the filtrate was concentrated by heating to 60°. Hot 95% ethanol (25 ml) was added and the solution was cooled. The product was collected by filtration and washed

with cold petroleum ether (43 g, 78%), mp. 42°, lit.[3] mp. 43°. Additional product was obtained by concentration of the filtrate; total yield 87%.

3,5-Dimethyl-4-aminobenzoic Acid (9). — 3,5-Dimethyl-4-nitrobenzoic acid,[4] 8, (5 g, 25 mmol) in absolute ethanol (100 ml) was hydrogenated for 4 hrs at 40 psi in the presence of 10% Pd-C (0.2 g). The mixture was then heated to 65°, the catalyst was filtered off and the filtrate was cooled. The product was collected by filtration (1.66 g) mp. 250° (dec.) The filtrate was concentrated to give more product (total yield 84%). IR (KBr): 3520, 3420(NH), 3000-2400(OH), 1650(C=O) cm^{-1}; MS: m/e 165 (M^+), 148 (M^+- OH).

4-(2-Chloroacetamido)-3,5-dimethylbenzoic Acid (10). — A 20 ml scintillation vial containing 9 (365 mg, 2.2 mmol) and chloroacetic anhydride (374 mg, 2.2 mmol) in DMF (0.8 ml) was flushed with nitrogen, capped and heated at 80° for 2 hrs. The solvent was removed by passing a stream of nitrogen and the solid residue was dissolved in hot ethanol (2 ml). Upon cooling, the mother liquor was pipetted off. The residue was the product (360 mg, 68%), mp. > 250° (dec.) IR (KBr): 3220(NH), 3200-2500(OH), 1690 and 1670(C=O), 1530 amide II; and bands at 1440, 1320, 1230 cm^{-1}; MS: m/e Calcd. for $C_{11}H_{12}NO_3Cl$ 241.0505; found: 241.0500.

N-(2,2,6,6-Tetramethylpiperidinoxy-4-yl) 4-(2-diethylaminoacetamido)-3,5-dimethylbenzamide (14). — A suspension of 10 (198 mg, 0.80 mmol) and oxalyl chloride (0.5 ml) in dry 1,2-dichloroethane (8 ml) and dry tetrahydrofuran (4 ml) was stirred at 25° until it dissolved (1 hr). Stirring was continued for two additional hours. The solution was concentrated with a stream of nitrogen followed by application of vacuum. The oily residue was dissolved in 1,2-dichloroethane (5 ml) and the solvent was removed. The residue 11 was redissolved in 1,2-dichloroethane (10 ml). 4-Amino-2,2,6,6-

tetramethylpiperidine-1-oxyl, 12 (140 mg, 0.8 mmol) and triethylamine (0.8 mmol) were added. The mixture was stirred at 25° for 2 hrs and then warmed to 50°. The solution was first washed with 1 N hydrochloric acid (2 x 2 ml), then with 1 N sodium hydroxide and finally with brine. The organic phase was dried over sodium sulfate and the solvent was removed in vacuo. The residue dissolved in dry acetone (5 ml) containing sodium iodide (0.5 g) was stirred for 3 hrs. The solvent was removed under a stream of nitrogen. The residue was triturated with dichloromethane and the sediment was removed by filtration. The filtrate was concentrated and the resulting residue was dissolved in methanol (2 ml). Diethylamine (1 ml) was added and allowed to react for 16 hrs at room temperture. The solution was concentrated and purified by TLC silica gel (ethyl acetate:methanol, 9:1). The material from the slowest major band (Rf 0.5) was eluted with ether and rechromatographed with acetone. The major band (Rf 0.7) gave 14 (50 mg, 15%). MS: m/e Calcd. for $C_{24}H_{39}N_4O_3$: 431.3022; found: 431.3016 (M^+)[6]; with low resolution m/e: 432 ($M^+ + 1$) and 431 (M^+) in a ratio of 2:1.[6]

2-Iodo-N-(2,6-dimethylphenyl)acetamide (15). — Compound 2 (1.20 g, 6.0 mmol) was added to a solution of sodium iodide (2.7g, 18 mmol) in acetone (25 ml). The solution was refluxed for 15 min and then cooled to 20°, the precipitated, sodium chloride (360 mg) was removed by filtration. An aliquot of the filtrate showed by TLC on silica gel with chloroform the product (Rf 0.42) and a trace of the starting material (Rf 0.60). The filtrate was brought to dryness under a stream of nitrogen at 40°. The solid residue was boiled in chloroform (40 ml) and the mixture was filtered to remove the excess sodium iodide (1.86 g).

The filtrate was concentrated to 25 ml by boiling. Hot benzene (10 ml) was added, the mixture was cooled and white needles precipitated. The mother liquor was decanted and the crystals were washed with n-hexane to

give chromatographically pure 15 (1.2 g, 70%), mp. 172-174°. IR (KBr): 3260(NH) and 1645(C=O); NMR (DMSO-d_6): 3.1 (CH_3, 6H), 3.8 (CH_2, 2H) and 7.0 (aromatic, 3H) ppm. MS: m/e Calcd. for $C_{10}H_{12}NOI$: 288.9968; found: 288.9957. When water (2 drops) was added to the NMR sample, a new peak appeared at 4.0 ppm which after 6 days had an intensity equal to that at 3.8 ppm. The peak at 4.0 ppm was apparently due to methylene protons of the 2-hydroxy-N-(2,6-dimethylphenyl)acetamide, as inferred from the MS; m/e: 289 (M^+ title compd) and 179 (M^+ of putative 2-hydroxy-N-(2,6-dimethylphenyl)acetamide) in a ratio of 2:3. This hydroxy acetamide was not observed using 2, thus showing that the ω-carbon of 15 is a better electrophile than that of 2.

N-(2,6-Dimethylanilinocarbonylmethylene)-2,2,5,5-tetramethylpyrrolidin-3-amin-1-oxyl (17). – Compound 15 (50 mg) and 3-amino-2,2,5,5-tetramethylpyrrolidin-1-oxyl (16, 50 mg, Molecular Probes) and sodium carbonate (10 mg) in tetrahydrofuran (4 ml) were stirred overnight at 38° in a closed vial. The mixture was separated by chromatography (prep. TLC) with chloroform/acetone (3:1). The major band (Rf 0.50) gave yellow crystals (30 mg; 55%), mp. 121-123°. IR (KBr): 3320(NH) and 1685(C=O) cm^{-1}; MS: m/e 318(M^+), 319(M^++1). Calcd. for $C_{18}H_{29}N_3O_2$: 319.2259; found: 319.2280.

N-(2,6-Dimethylanilinocarbonylmethylene)-N-ethyl-2,2,5,5-tetramethylpyrrolidin-3-amine-1-oxyl (18). – Compound 17 and ethyl iodide (50 µl) in tetrahydrofuran (3 ml) were stirred overnight at 50° in a capped vial. The mixture was chromatographed with chloroform/acetone (3:1). The major band (Rf 0.66) gave 18 (2 mg, 5%); MS: m/e 346(M^+) and 347(M^++1).[6] Calcd. for $C_{20}H_{33}N_3O_2$ 347.2572; found:347.2599. The second major band (Rf 0.50) gave 17 (18 mg).

In a second experiment a solution of 17 (10 mg) in tetrahydrofuran

(1 ml) was added to an ampule which contained [^{14}C]-ethyl bromide. The radiochromatogram showed radioactivity at the origin and at Rfs of 0.25, 0.33, 0.66 and 0.80. The band at Rf 0.66 gave 18 (0.4% yield based on radioactivity).

2-[N-Methyl-4-(2,2,6,6-tetramethylpiperidinoxy)]amino-N-(2,6-dimethylphenyl)acetamide(19). – Compound 15 (97 mg, 0.33 mmol), 4-methylamino-2,2,6,6-tetramethylpiperidine-1-oxyl (Molecular Probes, 92 mg, 0.50 mmol) and sodium carbonate (20 mg) in dry tetrahydrofuran (5 ml) were stirred in a capped vial for 2 days. The mixture was chromatographed by TLC on silica gel with chloroform:acetone (3:1). The pink band (Rf 0.77) was eluted with acetone. The eluate was concentrated and diluted with hot n-heptane. Upon cooling, yellow needles settled, (35 mg, 30%), mp. 167-168°. IR (KBr): 3300(NH) and 1670(C=O) cm^{-1}; UV (EtOH): 262 nm infl. (e= 1700 $M^{-1}cm^{-1}$); MS: m/e Calcd. for $C_{20}H_{32}N_3O_2$: 346.2494; found: 346.2486.

2-Diethylamino-N-(2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-yl)acetamide (20). 2-Iodo-N-(2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-yl)acetamide (Aldrich, 7 mg) in diethylamine (0.4 ml) in a closed vial was stirred overnight at 25°. The mixture was purified by TLC on silica gel with ethyl acetate. The red band (Rf 0.33) gave 20 (46%), mp. 58-61°. MS: m/e Calcd. for $C_{14}H_{28}N_3O_2$: 270.2181; found: 270.2188.

In another experiment, 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy and diethylamine gave, by the procedure as for 20 the 2-diethylamino-N-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-yl)acetamide (Rf 0.37) MS: m/e Calcd. for $C_{15}H_{30}N_3O_2$: 284.2337; found: 284.2332.

2-Amino-N-(2,6-dimethylphenyl)acetamide (GX). – Compound 15 (1.25 g, 0.043 mol) in ethanol (20 ml) and conc. ammonium hydroxide (2 ml) in a tightly closed vial was warmed to 50° for 1 hr. The mixture was then concentrated,

diluted with 0.1 N sodium hydroxide and washed with ethyl acetate. The organic phase was dried with magnesium sulfate and the solvent was evaporated. The solid residue was recrystallized from 95% ethanol-benzene (0.45 g, 58%) mp. 216-220°. IR (KBr): 3380, 3220(NH) and 1650(C=O) cm^{-1}; Ret. time on HPLC identical to that of authentic GX.[7] MS: m/e Calcd. for $C_{10}H_{14}N_2O$: 178.1106; found: 178.1091.

2-[^{14}C]-Ethylamino-N-(2,6-dimethylphenyl)acetamide (MEGX). — GX (12 mg), in 95% ethanol (0.7 ml) was placed into an ampule which contained [^{14}C]-ethyl bromide (1 mCi, sp. act. 48 mCi/mmol). The sealed ampule was kept at 70° for 2 hrs. The title compound was isolated by HPLC using Spherosorb C_{18} RP column (Regis). In this procedure about 10 µl of the reaction mixture was injected and eluted with 35% acetonitrile in water as reported elsewhere.[7] Alternatively, the mixture was purifed by TLC on silica gel with chloroform. The band (Rf 0.2) gave the title compound.

[^{14}C]-Lidocaine (^{14}C-1). — MEGX (13 mg) in abs. ethanol (1 ml) was placed in an ampule with [^{14}C]-ethyl bromide (1 mCi, sp. act. 10 mCi/mmol). The ampule was sealed and kept at 80° for 2 hrs. The product was then isolated by TLC on silica gel with carbon tetrachloride:ethyl acetate (2:3). The radiochromatogram showed a single radiolabeled band, the product (Rf 0.33) with a yield of 31% based on radioactivity.[8]

Compound 21. — MEGX (85 mg, 0.4 mmol) and 3,5-dibromo-2,2,6,6-tetramethyl-piperidin-4-one-1-oxyl[9] (45 mg, 0.13 mmol) in methylene chloride (3 ml) were stirred for 2 hrs at 25°. The mixture was separated by chromatography (prep. TLC) with ethyl acetate/methylene chloride (3:1). The second fastest, major band (Rf 0.66) was eluted with chloroform giving the product (15%), mp. 110-114°; MS: m/e 372(M^+). Calcd. for $C_{21}H_{30}N_3O_3$: 372.2303; found: 372.2286.

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6. Numerous nitroxides showed ions at M^+ and at $M^+ + 1$. The relative intensity of M + 1 increased with higher temperature of the solid insertion probe on mass spectrometer. Thus compound 18 gave mainly M+1 ion, the explanation being that nitroxides are efficient H atom scavengers at temperatures over 100°C (see A. Morrison and A. P. Davis, Org. Mass. Spectrom. 3, 353 (1970)).

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8. Many basic compounds, as amines like GX and 1, when chromatographed on acidic TLC media (e.g. silica gel) have Rfs which are a function of the amount of amine applied. These differences are probably due to overloading of the binding sites on the gel. Hence, the Rf of [^{14}C]-1, as determined from the radiochromatogram, did not always agree with the Rf of the standard 1 as visualized by UV light if the two were run in separate channels on the TLC plate. In these studies when the radiolabeled and unlabeled amines were applied to the same spot, then detection by radioscanning and UV light showed identical Rfs.

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