Synthesis of Spin Labels for ESR Imaging of Living Rat Head

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Spin labels (7, 10, 13, 16, 22, 27) were synthesized from piperidinyloxyl (1), pyrrolidinyloxyl (2), and oxazolidinyloxyl (3). These compounds were injected into the carotid artery of anesthetized rats, and the ESR spectra of the rat brain were immediately recorded by the use of an L-band ESR spectrometer. Based on the spectra obtained, we considered whether or not these spin labels can pass the blood brain barrier and bind to brain tissue components.

Key words L-band ESR; piperidinyloxyl; pyrrolidinyloxyl; oxazolidinyloxyl; psychotropic agent

Spin labeling is a method to study the environmental status of a target into which a stable nitroxide radical (spin label) has been inserted by measuring the change of the ESR spectrum caused by the change of motion of the spin label.[1,2] Typical spin labels [piperidinyloxyl (**1**), pyrrolidinyloxyl (**2**), oxazolidinyloxyl (**3**)][1-3] are shown in Chart 1. Spin labeling is used in various fields, *e.g.*, studies of cell membrane state, spin label oximetry, spin-immunoassay, spin-clearance of a drug, and ESR imaging. In principle, if a spin label combines with some tissue component, the ESR spectrum should change.[4] Accordingly, this method should be applicable to ESR imaging of a specific tissue.

Cytidine 5′-diphosphate choline, nordiazepam, haloperidol, and phenothiazine have been used as psychotropic agents, and are considered to pass the blood brain barrier. They have been reported[5] to act at neurotransmitter receptors of acetylcholine, dopamine, norepinephrine, and serotonin.

Extensive work has been done on the synthesis of spin label reagent such as oxazolidine,[6] oxazolidinyloxyl,[7] pyrrolidinyloxyl,[8] piperidinyloxyl (**4**),[9] 2,2,6,6-tetramethyl-4-(uridine 5′-diphospho)-1-piperidinyloxyl,[10] 2-chloro-10-[3-(3-carbonyl-2,2,5,5-tetramethyl-1-pyrrolinyloxyl)aminopropyl]phenothiazine.[11]

We utilized the procedures of Weiner[9] and Berliner and Wong,[10] to condense **4** with **5** and **6**, affording the piperidinyloxyl (**7**) in 11% and 9% yields, respectively (Chart 2). When **8**[12] was added to the diphosphate (**9**), **10** was obtained in 20% yield (Chart 3). Reaction of nordiazepam (**11**)[13] with the pyrrolidinyloxyl (**12**) gave the pyrrolidinyloxyl (**13**) in 72% yield (Chart 4). Similarly, the reaction of **11** with 2-chloroethylamine monohydrochloride gave **14**, which was condensed with the oxazolidinyloxyl (**15**) to give the oxazolidinyloxyl (**16**) in 69% yield (Chart 5). **19** was prepared from the reaction of **17** with ethyl isonipecotate (**18**) by a standard method.[14] It was treated with 0.1 N NaOH to give the carboxylic acid (**20**) in good yield. Then **20** was allowed to react with **21**, giving the piperidinyloxyl (**22**) in 62% yield (Chart 6).

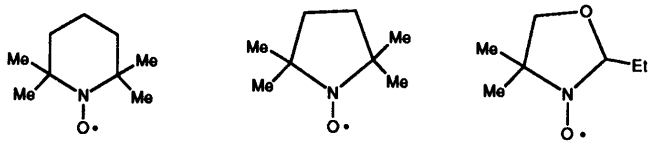

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Chart 1

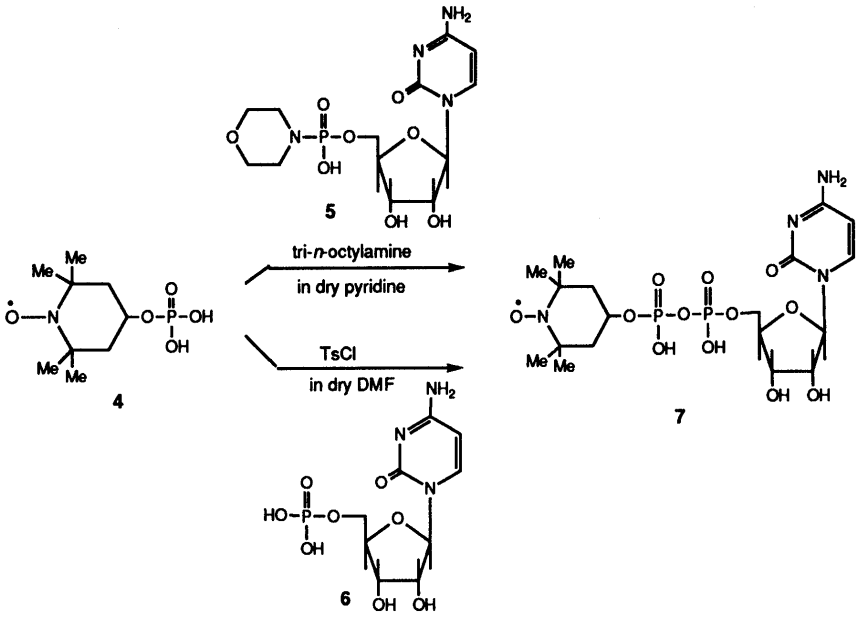

Chart 2

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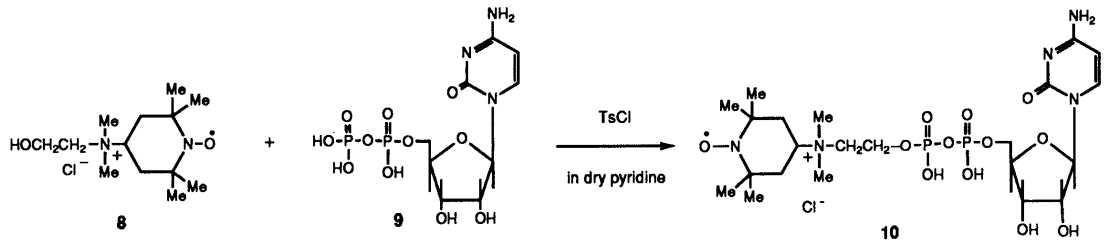

Chart 3

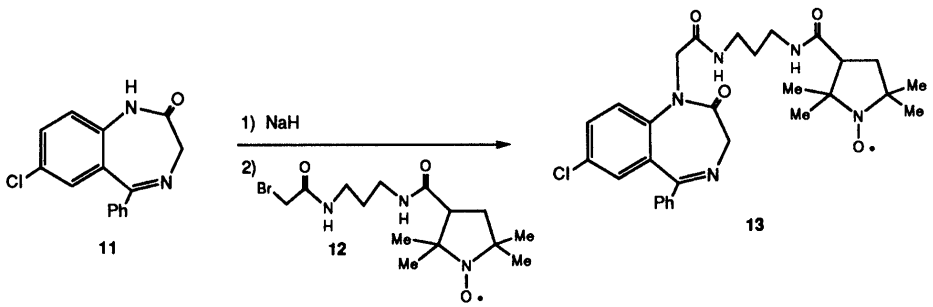

Chart 4

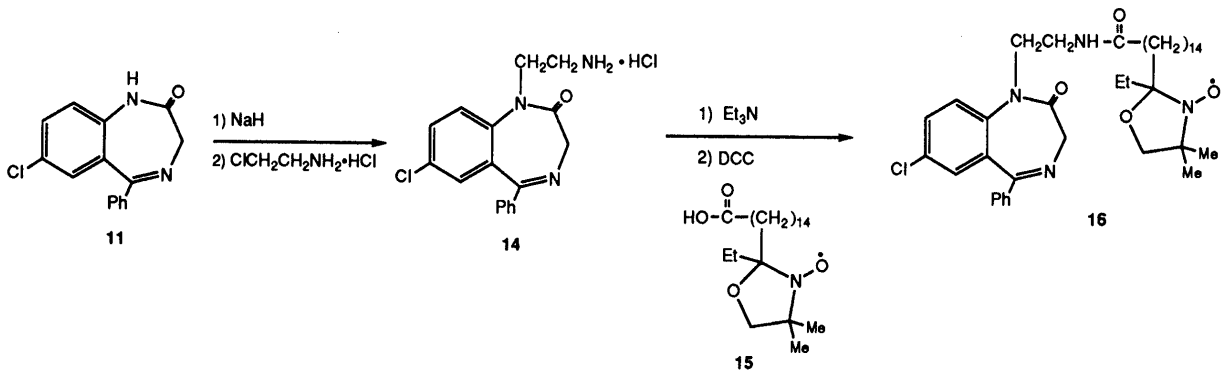

Chart 5

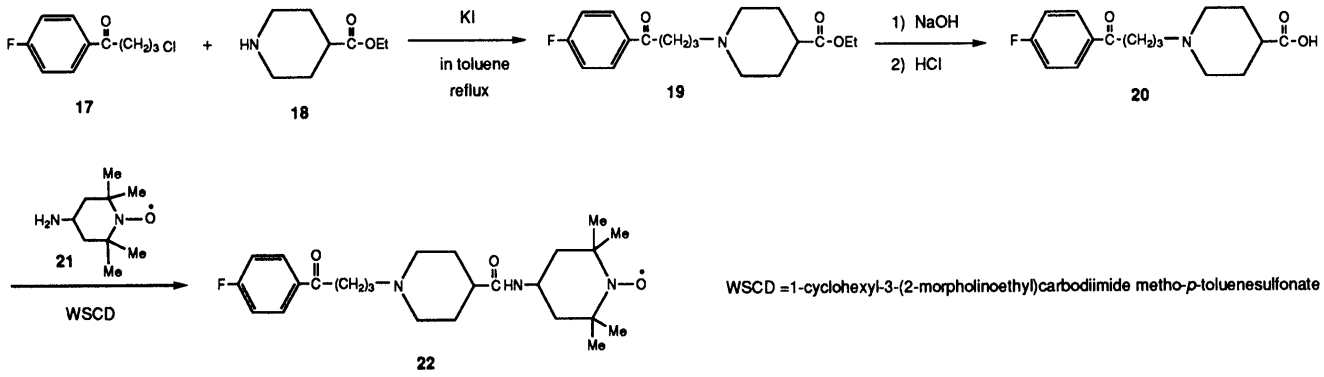

Chart 6

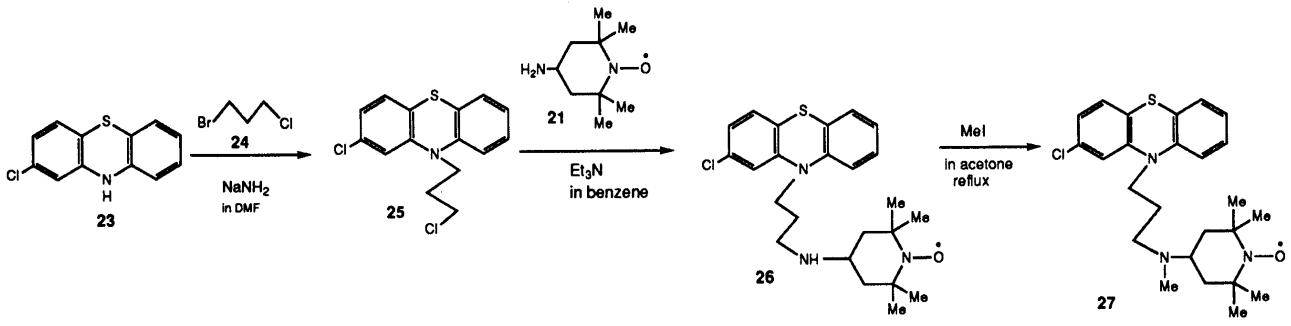

Chart 7

Table 1. Analytical and Spectral Data for **7**, **10**, **13**, **16**, **19**, **20**, **22**, **25**, **26**, and **27**

Compd.	Formula	Analysis (%) Calcd			Found			IR (KBr) cm^{-1}	EI-MS *m/z*
		C	H	N	C	H	N		
7	$C_{18}H_{31}N_4O_{12}P_2$	38.78	5.61	10.05	39.08	5.90	9.90	1710, 1610	557 (M^+), 527 (M^+-NO)
10	$C_{22}H_{41}ClN_5O_{12}P_2$	39.73	6.21	10.53	40.01	6.49	10.61	1715, 1620	629 (M^+-Cl), 599 ($M^+-Cl-NO$)
13	$C_{29}H_{35}ClN_5O_4$	62.97	6.38	12.66	63.01	6.35	12.64	1660	554 (M^++2), 552 (M^+), 522 (M^+-NO)
16	$C_{39}H_{56}ClN_4O_4$	68.85	8.30	8.24	68.71	8.33	8.11	1650	681 (M^++2), 679 (M^+), 649 (M^+-NO)
19	$C_{18}H_{24}FNO_3$	67.27	7.53	4.36	67.01	7.33	4.26	1690, 1660	321 (M^+)
20	$C_{16}H_{20}FNO_3$	65.51	6.87	4.87	65.69	6.95	4.97	1670, 1620	293 (M^+)
22	$C_{25}H_{37}FN_3O_3$	67.23	8.35	9.41	67.40	8.50	9.30	1620	446 (M^+), 416 (M^+-NO)
25	$C_{15}H_{13}Cl_2NS$	58.07	4.22	4.52	58.31	4.40	4.47	—	311 (M^++2), 309 (M^+)
26	$C_{24}H_{31}ClN_3OS$	64.77	7.02	9.44	65.01	7.21	9.37	—	446 (M^++2), 444 (M^+), 414 (M^+-NO)
27	$C_{25}H_{33}ClN_3OS$	65.41	7.25	9.15	65.60	7.36	9.01	—	460 (M^++2), 458 (M^+), 428 (M^+-NO)

Lastly, the phenothiazine (**25**), prepared from **23**, was allowed to react with **21** to give the piperidinyloxyl (**26**). The reaction of **26** with methyl iodide gave **27** in 87% yield (Chart 7). Elemental analyses and spectral data for the spin labels (**7**, **10**, **13**, **16**, **19**, **20**, **22**, **25**, **26**, **27**) are summarized in Table 1.

The X-band ESR spectra of nitroxide in the spin labels (**7**, **10**, **13**, **16**, **22**, **27**) in dimethyl sulfoxide (DMSO) showed the characteristic three lines in a symmetrical pattern with $g = ca.$ 2.006 —and the nitrogen hyperfine splitting constant (*hfsc*) $a_N = ca.$ 1.6 mT—. For example, Fig. 1 shows the ESR spectrum of **16** in DMSO ($g = 2.0061$, $a_N = ca.$ 1.68 mT).

The spin labels (**7**, **10**, **13**, **16**, **22**, **27**) in DMSO were injected into the left carotid artery of anesthetized living rats. Immediately, the ESR spectra of the rat head were measured using L-band ESR with a loop gap resonator. The ESR spectra of **7**, **10**, **13**, **22**, and **27**, but not **16**, showed a three line signal, similar to that in Fig. 1, at the expected magnetic field. For example, Fig. 2 shows the ESR spectrum of **13**. Thus, these compounds do not combine with any brain tissue component.

Compound **16** showed a change from the symmetrical pattern under the same ESR conditions as used for the other spin labels (Fig. 3). Usually, when a spin label combines with some high-molecular substance, the ESR spectrum shows an unsymmetrical pattern.[15] Therefore, **16** seems to have passed the blood brain barrier and combined with some brain tissue component. This result suggests the feasibility of using ESR-CT (computed tomography) to image pathologic foci in the brain.

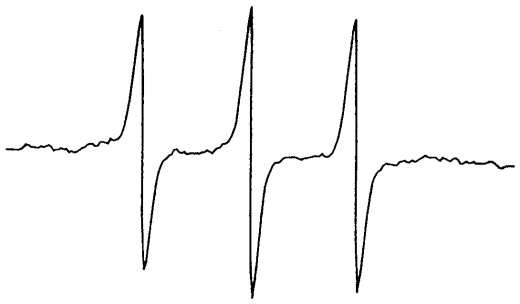

Fig. 1. ESR Spectrum of **16** in DMSO

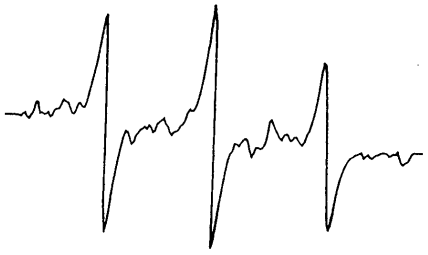

Fig. 2. ESR Spectrum in Living Rat Head after Injection of **13** into the Carotid Artery

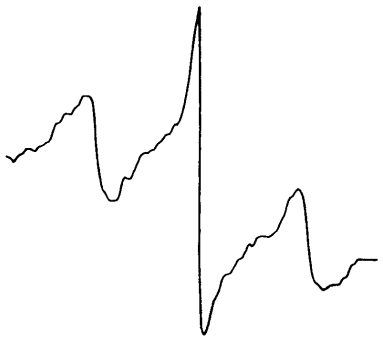

Fig. 3. ESR Spectrum in Living Rat Head after Injection of **16** into the Carotid Artery

Experimental

Melting points were determined on a Yanaco model MP apparatus and are uncorrected. IR spectra were taken with a JASCO IR-810 spectrophotometer. ^{1}H-NMR spectra were recorded by using tetramethylsilane as an internal standard on JEOL JNM PMX-60 spectrometers at 60 MHz. Mass spectra were recorded with a JEOL JMS-OISG-2 mass spectrometer. Wako gel (C-200) was employed for silica gel column chromatography.

4-(Cytidine 5′-Diphosphoxy)-2,2,6,6-tetramethyl-1-piperidinyloxyl (7). a) Reaction of Cytidine 5′-Monophosphonomorpholidate (5) with 2,2,6,6-Tetramethyl-4-phosphoxy-1-piperidinyloxyl (4) A solution of tri-*n*-octylamine (6.7 ml) in dry pyridine (10 ml) was added dropwise to a solution of **4** (6.31 g, 25.0 mmol) in dry pyridine (10 ml) at 5—10 °C with stirring. This mixture was stirred at 5—10 °C for 2 h, then a solution of **5** (1.96 g, 5.00 mmol) in dry pyridine (10 ml) was added dropwise at room temperature with stirring. The reaction mixture was concentrated *in vacuo*, and the residue was coevaporated with dry pyridine *in vacuo*. The reaction was allowed to proceed at room temperature in dry pyridine (15 ml) for 5 d and was terminated by the addition of water followed by lithium acetate (1.0 g). The tri-*n*-octylamine was extracted with ether. The aqueous fraction was applied to a Dowex 1 × 2 (200—400 mesh) column in the chloride form. The product was eluted as the second-to-last band using a 3-l gradient of 0.01—0.5 M LiCl in 0.1 M NH_4Cl. The fractions were pooled and concentrated to a small volume for desalting on a Sephadex G-10 (3.0 × 50 cm) column. Further purification was

achieved by repeated elution through another Dowex 1 × 2 (1.0 × 20 cm) column with a 1-l gradient of 0.005—0.6 M LiCl in 0.003 N HCl (25 ml/h flow rate). The product fractions were pooled, adjusted to pH 10 with LiOH, and desalted on Sephadex G-10. The solution was evaporated and reconstituted with MeOH. Precipitation with ether gave **7**, colorless powder, mp 216—219 °C, 0.31 g (11%).

b) Reaction of Cytidine 5′-Monophosphate (6) with 4 Compound **6** (1.62 g, 5.02 mmol) was dissolved in dry *N*,*N*-dimethylformamide (DMF) (30 ml), then **4** (6.31 g, 25.0 mmol) and *p*-TsCl (0.5 g) at 5—10 °C were added with stirring. This mixture was stirred at room temperature for 1 d, then water (20 ml) was added with stirring and the whole was applied to a Dowex 1 × 2 (200—400 mesh) column in the chloride form. The product was eluted using a 10-l gradient of 0.01—0.5 M LiCl in 0.1 M NH_4Cl, concentrated on a Sephadex G-10 (3.0 × 50 cm) column and precipitated with ether to give **7**, 0.25 g (9%).

4-{*N*-(Cytidine 5′-Phosphophosphonoxyethyl)-*N*,*N*-dimethylamino}-2,2,6,6-tetramethyl-1-piperidinyloxyl (10) Following the same procedure described above, **8**[9] (7.00 g, 25.0 mmol), *p*-TsCl (0.5 g) was added to a solution of **9** (1.40 g, 5.00 mmol) in dry pyridine (30 ml) at 5—10 °C with stirring. Work-up as above gave **10**, colorless powder, mp 234—237 °C, 0.67 g (20%).

3-[3-{2-(7-Chloro-1,3-dihydro-2-oxo-5-phenyl-2*H*-1,4-benzodiazepinyl)-acetamido}propylcarbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (13) A solution of **12** (2.18 g, 6.01 mmol) in DMF (10 ml) was added dropwise to a suspension of **11** (1.35 g, 4.98 mmol), sodium hydride (63% dispersion in mineral oil) (132 mg, 5.50 mmol) in DMF (10 ml) with stirring and ice-cooling. The addition required about 10 min to complete. The mixture was stirred at room temperature for 4 h, then allowed to stand at room temperature overnight. It was poured into ice water, and was extracted with ethyl acetate–ether. The organic layer was washed with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was subjected to silica gel (100 g) column chromatography. Elution with hexane–ethyl acetate (1 : 1) gave **13** as colorless needles, mp 241—244 °C (from ethyl acetate). Yield, 1.99 g (72%).

2-{14-[(7-Chloro-1,3-dihydro-2-oxo-5-phenyl-2*H*-1,4-benzodiazepinyl)-*N*-ethylcarbamoyl]tetradecyl}-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (16) A solution of 2-chloroethylamine monohydrochloride (0.70 g, 6.03 mmol) in DMF (10 ml) was added dropwise to a suspension of **11**[13] (1.35 g, 4.98 mmol) and sodium hydride (63% dispersion in mineral oil) (132 mg, 5.50 mmol) in DMF (10 ml) with stirring and ice-cooling. The mixture was stirred at room temperature overnight. A solution of triethylamine (0.61 g, 6.04 mmol) in DMF (10 ml) was added dropwise with stirring and ice-cooling. The whole was stirred for 2 h at ice-cooling. Next, a solution of **15** (2.69 g, 6.99 mmol) in ether (20 ml) was added with stirring and ice-cooling, and then a solution of 1,3-dicyclohexylcarbodiimide (DCC) (1.65 g, 8.01 mmol) in dry ether (20 ml) was added with stirring and ice-cooling. The reaction mixture was stirred at room temperature for 4 h. The precipitate was filtered off, and the filtrate was concentrated *in vacuo*. The residue was subjected to silica gel (200 g) column chromatography. Elution with hexane–ethyl acetate (3 : 1) gave **16**, colorless needles (from ethyl acetate), mp 238—240 °C. Yield, 2.34 g (69%).

4-[*N*-{1-(4-Oxo-4-*p*-fluorophenylbutyl)piperidine-4-carboxyamido}]-2,2,6,6-tetramethyl-1-piperidinyloxyl (22) A mixture of **17** (2.00 g, 9.95 mmol), ethyl isonipecotate (**18**) (2.36 g, 15.0 mmol) and potassium iodide (2.49 g, 15.0 mmol) in toluene (50 ml) was heated under reflux for 4 h. The solid residue, obtained by filtration of the cooled reaction mixture, was washed with water and ether, and the ether layer was added to the filtrate of the original reaction mixture. The dried (K_2CO_3), combined solutions were then filtered and concentrated to a quarter of their initial volume, and cooled. The precipitate was filtered off and recrystallized from hexane–ether to give **19** (2.50 g, 78%), mp 69—71 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7 Hz, OEt), 1.55—3.69 (15H, m, piperidine ring-H), 4.13 (3H, m, OEt), 7.10—8.02 (4H, m, aromatic-H). The ester (**19**) (2.40 g, 7.48 mmol) was added to a solution of 0.1 N NaOH (50 ml) with stirring and ice-cooling. After having been stirred at room temperature for 10 min, the reaction mixture was acidified with 0.1 N HCl.

The resulting mixture was concentrated *in vacuo*. The residue was dissolved in chloroform. The chloroform solution was washed with water, and dried over anhydrous sodium sulfate. Evaporation of the solvent gave a residue, which was purified by column chromatography on silica gel (200 g) with chloroform to give a crystalline substance, which was recrystallized from ether to give 1.99 g (91%) of **20**, mp 180—183 °C. ^{1}H-NMR (CD_3OD) δ: 1.58—3.75 (15H, m, piperidine ring-H), 7.11—8.12 (4H, m, aromatic-H). A mixture of **20** (1.94 g, 6.62 mmol) and **21** (1.25 g, 7.31 mmol) was suspended in water (20 ml), and a solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (3.39 g, 8.00 mmol) in water (10 ml) was added with stirring and ice-cooling. The mixture was stirred for 12 h at 0—5 °C, then extracted with ether. The ether solution was washed with water, dried over anhydrous sodium sulfate, and evaporated. The residue was purified by silica gel (200 g) column chromatography with hexane–ethyl acetate (1 : 1) to give a crystalline substance, which was recrystallized from ether to give 1.83 g (62%) of **22**, mp 224—227 °C.

4-[*N*-{3-(2-Chloro-10-phenothiazinyl)propyl}-*N*-methylamino]-2,2,6,6-tetramethyl-1-piperidinyloxyl (27) Sodium amide (0.39 g, 10.0 mmol) was suspended in DMF (50 ml), and a solution of **23** (2.34 g, 10.0 mmol) in DMF (30 ml) was added to it with stirring and ice-cooling. The mixture was stirred at room temperature for 10 min. A solution of 1-bromo-3-chloropropane (**24**) (1.57 g, 10.0 mol) in DMF (20 ml) was then added dropwise at room temperature with stirring. The reaction mixture was stirred at room temperature for 15 h, poured into ice water, and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was subjected to silica gel (200 g) column chromatography. Elution with hexane–ethyl acetate (10 : 1) gave **25** as colorless needles, mp 130—133 °C (from hexane–ethyl acetate). Yield, 1.90 g (61%). ^{1}H-NMR (CD_3OD) δ: 2.10—2.60 (2H, m, propyl-H), 3.60—3.90 (4H, m, propyl-H), 6.50—7.00 (7H, m, aromatic-H). A solution of **25** (1.80 g, 5.80 mmol) in benzene (70 ml) was added dropwise to a solution of **21** (1.10 g, 6.43 mmol) and triethylamine (0.65 g, 6.44 mol) in benzene (30 ml) with stirring and ice-cooling. The reaction mixture was stirred at room temperature for 2 h, then washed with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was subjected to silica gel (100 g) column chromatography. Elution with hexane–ethyl acetate (5 : 1) gave **26** as colorless needles, mp 166—169 °C (from hexane–ethyl acetate). Yield, 1.10 g (43%). A solution of **26** (0.90 g, 2.02 mmol) and iodomethane (0.57 g, 4.01 mmol) in acetone (20 ml) was heated under reflux for 12 h. The solvent was evaporated off under reduced pressure to give a residue, which was subjected to silica gel (100 g) column chromatography. Elution with hexane–ethyl acetate (6 : 1) gave **27** as colorless needles, mp 179—182 °C (from hexane–ethyl acetate). Yield, 0.81 g (87%).

ESR Measurement Male Wistar rats, 200 g, were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital. A polyethylene tube (0.25 × 0.75 mm), filled with saline was inserted into the cervical portion of the left carotid artery. Following arterial cannulation, the head was inserted into a loop gap resonator centered between the ESR magnets. Then a spin label agent (0.1 ml of 0.4 M solution) was infused over 30 s through the intra-carotid canula. The L-band ESR spectrometer (JEOL, Tokyo) consists of a pair of field gradient coils (Yonezawa Electric Wire Co., Ltd., Yonezawa, Yamagata) and a computer (5450, Concurrent Computer Corporation, Massachusetts, U.S.A.). ESR spectra were recorded at 700 mHz by the L-band ESR spectrometer with an electrical shield in the loop of the gap resonator. The loop gap resonator was 41 mm in diameter and 10 mm in axial length.

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