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A novel steroidal spin label for membrane structure studies: synthesis and applications

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Abstract

2,2,6,6-Tetramethyl piperidine-*N*-oxyl nitroxyls are known to partition between aqueous and lipid phases, thus serving as probes to study membrane dynamics. The synthesis of a novel steroidal spin label, 3α-hydroxycholan-24-yl-(2″,2″,6″,6″-tetramethyl-*N*-oxyl)piperidyl butan-1′,4′-dioate, containing 2,2,6,6-tetramethylpiperidine-*N*-oxyl moiety covalently bonded to the side chain in 3,24-caprostan-diol has been described. The localization of this spin label in model biomembranes has been studied by using electron spin resonance, differential scanning calorimetry, and ^{1}H and ^{31}P NMR spectroscopic techniques. Its applicability in studying the phase transition properties of model membrane L-α-dipalmitoyl phosphatidyl choline in the presence and absence of drugs has been described by using electron spin resonance. The label has also been used to study the permeability of epinephrine into membrane. The results have shown the applicability of the spin label as a potential spin probe in the study of biomembranes.

Keywords: 3α-hydroxycholan-24-yl-(2″,2″,6″,6″-tetramethyl-*N*-oxyl)piperidyl butan-1′,4′-dioate; TEMPO nitroxyl; Spin label; Model membrane; Phase transition

1. Introduction

The spin-labeling technique is a well-established tool for probing the structure and dynamic processes of model as well as biomembranes. Various classes of molecules have been labeled with the 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) moiety to study membrane properties. These include small organic molecules like tempocholine, tempophosphate, TEMPO labeled phosphatidylcholine, long-chain acids and amines, etc. [1–4]. However, such labels are not suitable for studying hydrophobic regions of membrane and, hence, there was a need to synthesize spin labels (SLs) that would bind more strongly to the hydrophobic regions of membranes. Steroids are known for their affinity towards membranes and can serve as good parent molecules for the nitroxyls. Several derivatives of 4,4-dimethyloxazolidine-*N*-oxyl (DOXYL) and 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (PROXYL) steroids have been reported in the literature but the reports on the synthesis and applications of steroidal TEMPO derivatives are relatively few [5–7]. Moreover, the steroids containing *cis*-fused A/B ring systems have also not been explored. SLs containing a steroid molecule attached to TEMPO moiety are expected to have higher mobility due to free rotation and are expected to be a more versatile as TEMPO is known to partition between aqueous and lipid phases [8].

The synthesis and applications of the first steroidal SL having TEMPO moiety on the side chain was reported by our group [9]. In continuation with our efforts to synthesize novel steroidal SLs for membranes and spin-immunoassays [10–13], it was thought worthwhile to synthesize and study the applications of some *cis*-fused steroidal SLs containing TEMPO moiety at the side chain. Our approach utilizes the introduction of the nitroxyl moiety in the substrate molecule by a covalent bond formation between the substrate molecule and the nitroxyl. For this purpose, a bile acid steroid, lithocholic acid was chosen. Because the functional groups present on the parent steroid molecule are, in most cases, responsible for its biological activity, the site of attachment of

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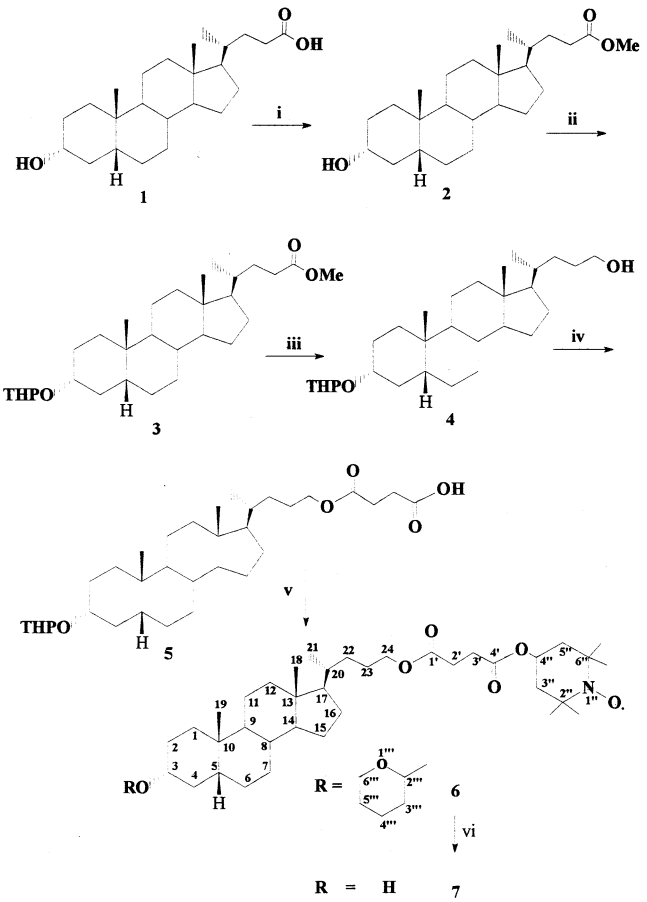

Scheme 1. Synthesis of 3α-hydroxycholan-24-yl-(2″,2″,6″,6″-tetramethyl-*N*-oxyl)piperidyl butan-1′,4′-dioate (**7**). Conditions: i) H_2SO_4, methanol, 8 h; ii) DHP, *p*-TSA, CH_2Cl_2, 12 h; iii) Lithium aluminum hydride, THF, 5 h; iv) Pyridine, succinic anhydride, 4 h; v) 4-Hydroxy TEMPO, DCC, DMAP, THF, 16 h; vi) Methanol, *p*-TSA, 4 h.

the TEMPO moiety was chosen to be as far as possible from its 3α-hydroxy group. A hemisuccinate linker would protrude the nitroxyl moiety away from the parent steroid, thus increasing the flexibility of the SL [14].

The synthesized SL would be principally utilized to study biomembrane properties. However, the lipid diversity of biological membranes makes it experimentally, as well as theoretically, difficult to characterize the thermodynamic state of the lipid membrane and its associated phase equilibria. Within a reductionisitic approach, therefore, the studies of these properties of the model membranes constitutes the first important step towards a description of complicated multi-component membranes [15,16].

In the envisaged scheme, 4-hydroxy TEMPO has been attached to a suitably modified C-24 atom of 3,24-caprostandiol via a succinyl group. The potential of the novel SL, 3α-hydroxycholan-24-yl-(TEMPO) butan-1′,4′-dioate, thus synthesized, has been exploited by incorporating it into a model membrane L-α-dipalmitoyl phosphatidyl choline (DPPC), studying certain properties of DPPC and monitoring the effect of various drugs on it with the help of electron spin resonance (ESR) spectroscopy.

2. Experimental

Melting points are reported uncorrected. All solvents were predried according to standard procedures. Petroleum ether refers to the fraction having b.p. 60–80°C. DPPC and epinephrine were purchased from Sigma Chemical Company (St. Louis, MO, USA) whereas dilitazem was received as a gift from Iswtituto di Science Fisiche, Unversity di Ancona, Italy. 4-Hydroxy TEMPO was prepared from 2,2,6,6-tetramethylpiperidine-4-one monohydrochloride that was purchased from Fluka (Switzerland). 4-Oxo TEMPO was obtained by treating 2,2,6,6-tetramethylpiperidine-4-one monohydrochloride with H_2O_2/Na_2WO_4 and was converted to the corresponding alcohol by reduction with lithium aluminum hydride. Column chromatography was carried out by using 60–120 mesh silica gel. Petroleum ether–ethyl acetate as the solvent system for column chromatography as well as TLC. Elemental analysis was carried out on CEST 1106. IR spectra were recorded on a Nicolet Impact 400 Fourier transform IR spectrophotometer. A Hewlett Packard MS Engine 5989-A spectrometer was used to record the mass spectra. ^{1}H NMR spectra were recorded on a Varian VXR 300S spectrometer. About 5 mg of the sample was dissolved in 0.6 ml of the solvent. The ^{1}H NMR spectra of nitroxyls were recorded after in situ reduction of their $CDCl_3$ solutions with 1.5 equivalents of freshly distilled phenyl hydrazine. The ^{1}H NMR and ^{31}P NMR for determination of the localization of the SL in DPPC (1:5 molar ratio) was taken in 0.6 ml of $CDCl_3$. The differential scanning calorimetry experiments were carried out on France Setaram instrument and the volume of the sample taken was 0.85 ml. ESR spectra were recorded at ambient temperature or at 50°C on Varian E-112 spectrometer operating in the X-band with tetracyanoethylene as internal standard ($g_0 = 2.00277$). Deoxygenated chloroform was used as the solvent for ESR measurements, the concentrations of the nitroxyls being ca. 10^{-5} M.

2.1. Preparation of multilamellar dispersions of DPPC

Chloroform solutions of the required SL and the lipid were taken up in a small tube. The solvent was evaporated slowly with a stream of nitrogen gas to obtain a thin film on the walls of the tube. Traces of solvent were removed by drying under vacuum for 3 h. The dried film was then hydrated with required amount of 10 mM phosphate buffer (pH = 7.2) for 20 min and then vortexed at 50°C for 10 min to get multilamellar vesicles. The concentration of the lipid in the buffer solution was 80 mM and that of the SL was 0.8 mM. The samples were taken in 50 μl glass capillaries sealed at both ends and mounted in the variable temperature accessory of the spectrometer.

2.2. Preparation of unilamellar dispersions of DPPC

A lipid dispersion of DPPC in 10 mM phosphate buffer with SL 7 was prepared according to the above mentioned method. After a vortex of 10 min at 50°C, the dispersion

was subjected to sonification by using sonifier 1210 BRANSON (Model 1210E-DTH, working frequency 47 KHz ± 6%, HF-output power nom. 35 W) at 40°C. Sonification for 30 min produced clear homogeneous solution, that was used for the ESR experiments.

For phase transition experiments with diltiazem, the multilamellar dispersion was hydrated as usual by using the appropriate amount of 10 mM phosphate buffer (pH 7.2) containing the drug. The system was allowed to equilibrate for 20 min before vortexing. The molar ratios of the drug: lipid:SL were maintained at 40:100:1.

For permeation studies with epinephrine, the temperature was kept at 50°C, that is above the phase transition temperature (41°C) of DPPC, to ensure that the lipid remained in the liquid crystalline phase. The microwave power of the instrument was set at a low value of 0.5 mW with a microwave frequency of 9.1 GHz. The modulation amplitude was set at 2.0 mW × 1.0 G and the time constant of the detector unit at 0.128 s. The scan time for each spectrum was 4 min. The receiver gain at the start of the experiment was kept at a fairly large value to give a strong signal whose decay could be monitored with time. It was not altered throughout the experiment. Only the first line in the ESR spectrum and its decay with time were recorded.

In a typical experiment, to a 50 μl of the sonicated preparation, required amount of drug solution in buffer was added. This time, at which the drug was added, corresponded to time $t = 0$. Subsequently, the decay of the ESR signal with time was monitored. The samples were taken in 50 μl glass capillaries sealed at both ends and mounted in the variable temperature accessory of the spectrometer. In all the permeation experiments, the concentration of the lipid in the buffer solution was 100 mM and that of the SL was 1.00 mM. The molar ratios of the drug:lipid:SL were maintained at 20:100:1. The quencher solutions were prepared in 10 mM phosphate buffer (pH 7.2).

2.3. Tetrahydropyranyl ether *3* of methyl lithocholate

Methyl lithocholate (**2**) (1 g, 2.56 mmol) was dissolved in dichloromethane (20 ml). Dihydropyran (0.43 ml, 5.12 mmol) and *p*-toluene sulphonic acid (0.015 g, 0.007 mmol) were added to it. The reaction mixture was stirred for 14 h, washed with 5% NaOH, water, brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified over silica gel column to obtain the required product **3** as a semisolid in 90% yield. IR (KBr): *v* 3015, 2939, 2866, 1732, 1448, 1366, 1025 cm^{-1}. MS: m/z 474 (M^+). Elemental analysis: Calculated for $C_{30}H_{50}O_4$: C, 75.90; H, 10.62%; found: C, 75.50; H, 10.40%. ^{1}H NMR ($CDCl_3$): δ 4.72 (bs, 1H, 2′-H), 3.91 (m, 1H, 6′-H), 3.66 (s, 3H, COOMe), 3.63 (m, 1H, 6′-H), 3.50 (m, 1H, 3β-H), 2.36 (ddd, J = 5.4, 10.2, 15.3, 1H, 23-H), 2.21 (ddd, J = 6.6, 9.6, 15.3, 1H, 23-H), 0.91 (s, 3H, 19-H_3), 0.40 (d, J = 6.3, 3H, 21-H_3), 0.63 (s, 3H, 18-H_3).

2.4. 3α-Tetrahydropyranyloxycholan24-ol (*4*)

Compound **3** (1 g, 2.1 mmol) was dissolved in dry tetrahydrofuran (25 ml) under nitrogen atmosphere. Lithium aluminum hydride (0.16 g, 4.2 mmol) was added to it slowly at 0°C. The reaction mixture was then stirred at room temperature for 5 h (monitored by TLC). After completion of the reaction, the solvent was evaporated under reduced pressure and the inorganic salts were filtered off followed by extraction of the compound with ethyl acetate. After evaporation of the solvent and purification over column, the required compound **4** was obtained in 76% yield. m.p. = 137–138°C. IR (KBr): *v* 3342, 2938, 2864, 1440, 1373, 1119, 1026 cm^{-1}. MS: *m/z* 428 (M-18). Elemental analysis: Calculated for $C_{29}H_{50}O_3$: C, 77.97; H, 11.28%; found: C, 77.85; H, 11.24%. ^{1}H NMR ($CDCl_3$): δ 4.73 (bs, 1H, 2‴-H), 3.91 (m, 1H, 6‴-H), 3.66 (m, 1H, 6‴-H), 3.61 (m, 2H, 24-H_2), 3.51 (m, 1H, 3β-H), 0.92 (d, J = 6.3, 21-H_3), 0.91 (s, 3H, 19-H_3), 0.64 (s, 3H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 98.8 (C-2‴), 71.8 (C-24), 68.0 (C-3), 62.7 (C-6‴), 23.3 (C-21), 18.6 (C-19), 12.0 (C-18).

2.5. Hemisuccinate *5* of 3α-tetrahydropyranyloxycholan-24-ol

Compound **4** (0.2 g, 0.45 mmol) was dissolved in pyridine (2 ml) in a round bottom flask to which succinic anhydride (0.045 g, 0.45 mmol) was added and stirred for 4 h. Ethyl acetate was then added and the reaction mixture was filtered through celite. The product was extracted with ethyl acetate, washed with dilute HCl, water and brine. The organic extract was dried over anhydrous Na_2SO_4 and concentrated in vacuo. Silica gel column chromatography afforded the pure hemisuccinate **5** in 71% yield. m.p. = 102–103°C. MS: *m/z* 546 (M^+). IR (KBr): *v* 3444, 2933, 2862, 1738, 1692, 1451, 1361, 1167, 1023 cm^{-1}. Elemental analysis: Calculated for $C_{33}H_{54}O_6$: C, 72.49; H, 9.95%; found: C, 72.44; H, 9.79%. ^{1}H NMR ($CDCl_3$): δ 4.72 (bs, 1H, 2‴-H), 4.08 (m, 2H, 24-H_2), 3.92 (m, 1H, 6‴-H), 3.64 (m, 1H, 6‴-H), 3.50 (m, 1H, 3β-H), 2.64 (m, 4H, 2′-H_2, 3′-H_2), 0.91 (s, 3H, 19-H_3), 0.64 (s, 3H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 177.5 (C-1′), 172.2 (C-4′), 96.7 and 96.4 (C-2‴, diastereomeric), 75.8 (C-24), 65.4 (C-3), 62.6 (C-6‴), 28.8 (C-2′, C-3′), 23.2 (C-21), 18.4 (C-19), 11.8 (C-18).

2.6. TEMPO derivative *6* of 3α-tetrahydropyranyloxy cholan-24-hemisuccinate

Compound **5** (0.1 g, 0.18 mmol) was dissolved in dry THF (20 ml) and to it 4-hydroxy-TEMPO (0.03 g, 0.18 mmol) in THF (5 ml), 1,3-dicyclohexylcarbodiimide (0.03 g, 0.18 mmol) and 4-dimethylaminopyridine (0.02 g, 0.18 mmol) were added under nitrogen atmosphere. The reaction mixture was stirred for 16 h followed by filtration of insoluble urea and evaporation of the solvent under reduced pressure. The crude product obtained was purified by silica

gel column chromatography to accrue compound **6** in 25% yield. m.p. = 44–45°C. MS: *m/z* 701 (M + 1). IR ($CHCl_3$): *v* 2927, 2858, 1735, 1628, 1460, 1361, 1157, 1027 cm^{-1}. Elemental analysis: Calculated for $C_{42}H_{70}O_7N$: C, 71.96; H, 10.06; N, 2.00%; found: C, 71.86; H, 10.00; N, 2.00%. ^1H NMR ($CDCl_3$ with 1.5 equivalents of $PhNHNH_2$): δ 4.72 (bs, 1H, 2‴-H), 4.05–4.10 (m, 3H, 4″-H, 24-H_2), 3.92 (m, 1H, 6‴-H), 3.64 (m, 1H, 6‴-H), 3.50 (m, 1H, 3β-H), 2.62 (m, 4H succinyl $(H_2)_2$), 1.38 (s, 6H, gem-dimethyls), 1.25 (s, 6H, gemdimethyls), 0.91 (s, 3H, 19-H_3), 0.64 (s, 3H, 18-H_3). ESR spectrum (10^{-5} M in $CHCl_3$): symmetrical triplet with $g_0 = 2.0058$ and $A_0 = 15.81$ G.

*2.7. 3α-Hydroxycholan-24-yl-(TEMPO)piperidyl butan-1′,4′-dioate (***7***)*

The tetrahydropyranyl ether derivative (**6**; 50 mg, 0.071 mmol) was dissolved in aqueous methanol (20 ml) and catalytic amount of *p*-toluene sulphonic acid was added to it. The reaction mixture was stirred at room temperature for 4 h (monitored by TLC). After completion, the solvent was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic layer was washed with sodium bicarbonate and dried over Na_2SO_4 and concentrated in vacuo. The crude product was column chromatographed to obtain the pure SL **7** as a pale yellow oil (31 mg, 70%). MS: *m/z* 617 (M + 1). IR ($CHCl_3$): *v* 3320, 2927, 2858, 1735, 1629, 1460, 1360, 1155, 1027 cm^{-1}. Elemental analysis: Calculated for $C_{37}H_{62}O_6N$: C, 72.04; H, 10.13; N, 2.27%; found: C, 72.00; H, 10.10; N, 2.05%. ^1H NMR ($CDCl_3$ with 1.5 equivalents of $PhNHNH_2$): δ 4.08 (m, 3H, 4″-H, 24-H_2), 3.62 (m, 1H, 3β-H), 2.62 (m, 4H, 2′-H_2, 3′-H_2), 1.36 (s, 6H, gemdimethyls), 1.28 (s, 6H, gemdimethyls), 0.91 (s, 3H, 19-H_3), 0.68 (s, 3H, 18-H_3). ESR spectrum (10^{-5} M in $CHCl_3$): symmetrical triplet with $g_0 = 2.0060$ and $A_0 = 15.375$ G.

3. Results and discussion

The C-24 carboxy group of lithocholic acid (**1**) was converted to its corresponding hydroxy group. For this, methyl lithocholate (**2**), characterized by the presence of 1730 cm^{-1} peak for the ester carbonyl in the IR spectrum and a three-proton singlet at δ 3.66 ppm for the ester OMe in the ^1H NMR spectrum, was prepared from compound **1**. Compound **2** was then converted to the corresponding 3α-tetrahydropyranyl (THP) ether **3**. The product formed showed the absence of -OH peak in its IR spectrum. The ^1H NMR spectrum showed resonances at 4.72, 3.91, and 3.63 ppm corresponding to the THP group. The multiplet for the 3β-H was deshielded and appeared at 3.50 ppm. The mass spectrum of compound **3** exhibited the molecular ion peak at *m/z* 474 ($C_{30}H_{50}O_4$) thus confirming it to be tetrahydropyranyl methyl lithocholate. Lithium aluminum hydride reduction of the methyl ester **3** yielded the C-24 primary alcohol **4** that showed the -OH absorption band at 3342 cm^{-1} in the IR spectrum. The mass spectrum of compound **4** ($C_{29}H_{50}O_3$) exhibited the molecular ion peak at *m/z* 428 (M-18). The absence of a three-proton singlet for the -OCH_3 group was eminent in the ^1H NMR spectrum of alcohol **4**. Instead, a two-proton multiplet, characteristic of -OCH_2- group was observed at δ 3.61 ppm, that was assigned to the C-24 protons. The ^{13}C NMR spectrum of compound **4** also lacked in carbonyl resonances but showed a shielded C-24 peak at 71.8 ppm. The peaks at 98.8 (C-2‴) and 62.7 ppm (C-6‴) were characteristic of the THP moiety. Alcohol **4** was then converted to its hemisuccinate derivative **5**, that was characterized by the presence of an ester and acid carbonyl signal at 1738 and 1692 cm^{-1}, respectively, in its IR spectrum. Mass spectrum of compound **5** exhibited the M^+ peak at *m/z* 546 ($C_{33}H_{54}O_6$). The ^1H NMR spectrum of compound **5** showed a multiplet at δ 2.64 ppm (4H) characteristic of the hemisuccinate methylene protons in addition to the downfield shift of the 24-H_2 resonance by ca. 0.5 ppm. The presence of an ester carbonyl peak at 177.5 ppm along with a peak at 172.28 ppm for -COOH in its ^{13}C NMR spectrum confirmed its formation. The hemisuccinate **5** hence obtained was then condensed with 4-hydroxy-TEMPO in the presence of dicyclohexyl carbodiimide and *N*,*N*-dimethylamino pyridine to yield the nitroxyl **6**. Product formation was indicated by the absence of the acid carbonyl peak and the appearance of ester carbonyl peaks at 1735 cm^{-1} in the IR spectrum. The mass spectrum of compound **6** showed M + 1 peak at *m/z* 701 ($C_{42}H_{70}O_7N$). The ^1H NMR spectrum after reduction of the nitroxyl with 1.5 equivalents of phenyl hydrazine showed additional singlets at 1.38 and 1.25 ppm assigned to the gem-dimethyls of the TEMPO moiety. The ESR spectrum of the nitroxyl showed a symmetrical triplet with $g_0 = 2.0058$ and $A_0 = 15.81$ G.

The need for the deprotection of the 3α-hydroxy group of the TEMPO ester **6**, stemmed from the fact that the SL would thus possess a free 3α-hydroxyl group similar to that in the parent molecule, lithocholic acid and, hence, could ensemble in the membrane layers like a bile steroid. The cleavage of the tetrahydropyranyl ether group was achieved in a yield of 70% by using *p*-toluenesulphonic acid in methanol. The IR spectrum of product **7** showed the presence of a broad band at 3320 cm^{-1} indicating the desired deprotection had occurred. Peaks corresponding to the THP group were absent in the ^1H NMR spectrum of phenyl hydrazine reduced product **7** whereas the 3α-H peak appeared at 3.62 ppm. The mass spectrum of the nitroxyl **7** exhibited the M + 1 peak at *m/z* 617 ($C_{37}H_{62}O_6N$) and the ESR spectrum showed a symmetrical triplet with $g_0 = 2.0060$ and $A_0 = 15.375$ G. These data confirmed that compound **7** is 3α-hydroxycholan-24-yl-(2″,2″,6″,6″-tetramethyl-*N*-oxyl) piperidyl butan-1′,4′-dioate.

The TEMPO derivative **7** of 24-hemisuccinoyl caprostan-3α-ol was then incorporated in the model membrane DPPC liposomes to study its application in probing the

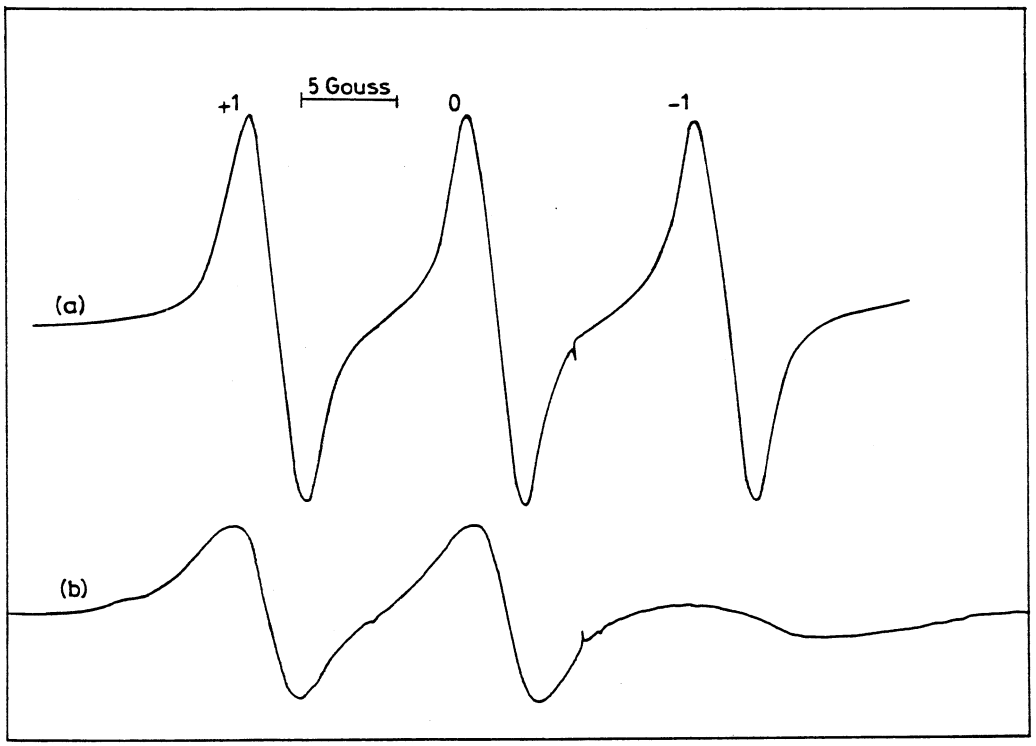

Fig. 1. ESR spectra of the nitroxyl **7** in (a) rapidly tumbling solution state (10^{-5} M in $CHCl_3$) and (b) lipid matrix (DPPC liposomes), SL:DPPC 0.8:80.0 mM.

fluidity and permeability properties of the model membrane as such and in presence of certain drugs.

The incorporation of the nitroxyl **7** in liposomes was ascertained by comparing the ESR spectra of the nitroxyl in rapidly tumbling solution state ($CHCl_3$) with that in the lipid matrix, i.e. DPPC liposomes (Fig. 1). The hyperfine coupling constants of the SLs in multi-lamellar vesicles was found to be 15.26 G although in rapidly tumbling solution phase was found to be 15.375 G. The differences observed in the line shapes of the highest and lowest field lines in the ESR spectrum of these SL in liposomes is due to the anisotropy of the nuclear hyperfine coupling tensor and g-tensor of the radicals in the lipid [13]. The absence of composite peaks along with the changes in the line shape and hyperfine coupling constant suggested the complete incorporation of the SL **7** into the DPPC liposomes.

Differential scanning calorimetry of pure DPPC vesicles showed the phase transition temperature at 41°C whereas those incorporated with steroid **7** showed the same at 40°C that is within the error limits of the instrument. This showed the incorporation of the SL **7** did not cause any significant perturbation to the membrane system.

Further evidences for the incorporation of the SL **7** in the liposomes and the mode of localization came from the ^1H and ^{31}P NMR spectroscopy [17,18]. It was observed that on incorporation of the nitroxyl **7**, the ^1H NMR resonances of different regions underwent line broadening to different extents. The half-line widths of the ^1H and ^{31}P NMR resonances of pure DPPC (W_L) and those incorporated with SL (W_{LS}) are tabulated (Table 1). Among the peaks in the ^1H NMR spectra, the broadening observed for the characteristic -NMe_3 constituting the polar head group of the lipid and the terminal-CH_3 group constituting the other extreme end viz. nonpolar hydrocarbon region of the lipid were taken into consideration. The -NMe_3 peak showed more broadening due to the proximity of the TEMPO group to the polar head group of the lipid. The ^{31}P resonances also indicated similar possibility.

Phase transition behavior of DPPC dispersions were studied by using the SL **7** as ESR-sensitive probe. The empirical parameter h_{+1}/h_0 (ratio of the signal heights of the low field line to the central line in the ESR spectrum of the SL) was plotted as a function of temperature to monitor the phase transition characteristics (Fig. 2). This parameter (h_{+1}/h_0) showed an initial gradual increase, followed by a sudden large increase in the transition of the lipid from gel to liquid crystalline state at 40°C, close to the values reported earlier by using other techniques. The alterations in

Table 1
Line broadening of NMe_3 and Terminal Me ^1H and ^{31}P NMR signals caused by incorporation of spin label **7** in DPPC

		Half-line widths (Hz)		
		N^+Me_3	Terminal Me	^{31}P
W_L	Pure DPPC	3.36505	1.3206	1.9362
W_{LS}	7 + DPPC	5.50946	1.542	2.7107
W_{LS}/W_L		1.6372	1.151	1.4

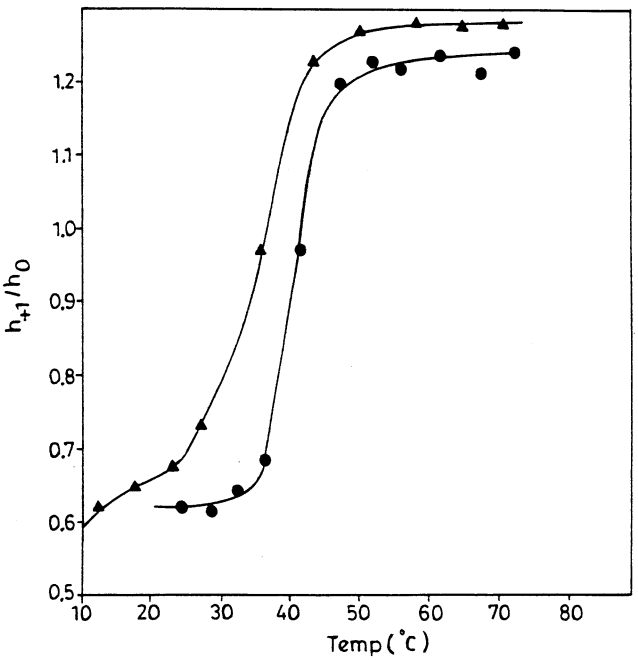

Fig. 2. Plot of temperature (°C) v/s spectral parameter h_{+1}/h_0 of the SL **7** incorporated into pure DPPC liposomes, SL:DPPC 1:100 (●) and in presence of diltiazem, SL:DPPC:Drug 1:100:40 (▲).

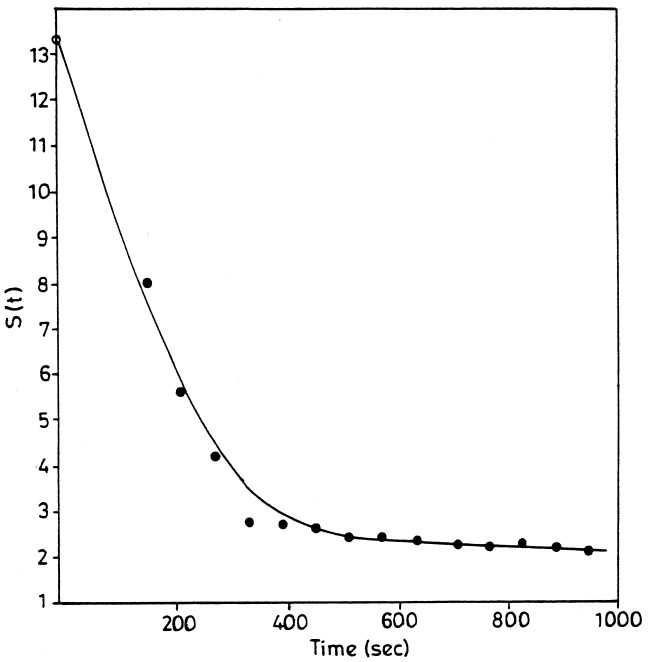

Fig. 3. Plot of time (s) v/s the signal height $S(t)$ of the ESR spectrum of the SL **7** incorporated into DPPC liposomes in presence of epinephrine (SL: DPPC:Drug 1:100:20).

the phase transition temperature induced by vasodilating drug, diltiazem was also studied. In the presence of diltiazem, the transition temperature of DPPC was observed to be lowered to 36°C (Fig. 2). However, the sigmoidal nature of the curve is retained. This showed that the drug has an affinity to bind loosely to the hydrophobic region of the lipid [4,19].

Finally, the permeation of epinephrine, an adrenergic neurotransmitter, in model membrane DPPC was studied utilizing ESR properties of the new TEMPO probe **7**. Most of the actions of this anti-anesthetic drug molecule, known to inhibit glycogen breakdown and increase sodium transport across the cell membrane of erythrocytes, are triggered when it complexes with appropriate receptors located in the plasma membrane with their binding sites oriented externally causing a local change in the membrane [20].

The nitroxyl spin labels are known to undergo reduction by reducing agents such as ascorbic acid. Making use of this fact, the rate of drug permeation in outer and inner monolayers has been determined earlier [21]. Epinephrine possess the property of reacting with the spin label, thereby causing loss of its paramagnetism. When this drug was introduced into the DPPC system labeled with nitroxyl **7**, it diffused into the bilayer. The spin label molecules present in the outer monolayer were readily accessible and therefore underwent reduction at a faster rate as compared to those residing in the inner region. As a result, the ESR signal height decreased with time. The plot of signal height with time showed an exponential decay (Fig. 3).

The ESR signal heights (S_0) and (S_i) due to the SL present in the outer and the inner monolayers can, in principle, decay with different rate constants, say k_0 and k_i, respectively. Under these conditions the decay of the signal intensity is expected to show a behavior as given by the equation:

$$S(t) = S_0(0)e^{-k_0 t} + S_i(0)e^{-k_i t}$$

where $S(t)$ is the ESR signal height due to the total SL present at time t and $S_0(0)$ and $S_i(0)$ are signal heights due to initial SL concentration in the outer and inner monolayers, respectively [21,22]. From these results, half-life times for the reduction of the SLs, that in turn reflect the rates of permeation of the drugs, for the outer and inner monolayer were calculated to be 19.05 s and 68.98 s, respectively. These results showed that the drug molecules did not protrude deep into the lipid hydrophobic core of the membrane but were bound on the surface and diffused laterally at a moderate rate [23].

In conclusion, a novel steroidal SL—3α-hydroxycholan-24-yl-(2″,2″,6″,6″-tetramethyl-*N*-oxyl) piperidyl butan-1′,4′-dioate—was synthesized and was successfully incorporated in DPPC liposomes. The paramagnetic properties of this SL could be used to monitor phase transition in DPPC as such and in the presence of diltiazem. The study involving the permeation of epinephrine in DPPC by using ESR properties of the synthesized spin labels showed that the results were congruent with the literature reports. Thus, the new caprostanol TEMPO derivative shows good potential as a spin probe in membrane structure studies.

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