Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 2753–2758

BIOORGANIC & MEDICINAL CHEMISTRY

1-[2-Hydroxy-3-octadecan-1′-oate]propyl-2″,2″,5″,5″-tetramethyl Pyrolidine-*N*-oxyl-3″-carboxylate as a Potential Spin Probe for Membrane Structure Studies

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Received 27 April 1999; accepted 7 July 1999

Abstract—The synthesis of a new minimum steric perturbing proxyl nitroxide, which is a derivative of glycerol and contains a stearic acid moiety, has been carried out. Its localization in model membrane L-α-dipalmitoyl phosphatidyl choline (DPPC) was ascertained with the help of ESR, DSC, ^{1}H and ^{31}P NMR techniques. The nitroxide was used for detecting the changes in the phase transition temperature of the model membranes in the presence and absence of drugs. The permeation of the vasodilating drug epinephrine has also been studied using this spin label. The results prove the potential applicability of the new spin probe in the spin labeling of biomembranes.

Introduction

Nitroxide labeled lipids and phospholipids have been used extensively in ESR studies of the structure and function of biological membranes.[1–5] Despite the proven usefulness of the technique, key studies are sometimes precluded by the unavailability of site specific labels having suitable spectral, physical or chemical properties.[6] Also, there is a constant concern that the spin labeled biological system does not accurately reflect the behavior of its naturally occurring analogue owing to the steric bulk of the nitroxide moiety.[7] Molecules such as proteins, sugars, steroids and lipids are embedded in membrane bilayers at random and can serve well as parent molecules for nitroxide probes. These labels can be embodied in the organized system without causing perturbations.

Our group has been involved in the synthesis of various steroid based nitroxide spin labels.[8–10] We, herein, describe the synthesis and applications of a new non-steroidal minimum steric perturbing proxyl nitroxide lipid spin label. This nitroxide is a derivative of glycerol and contains a stearyl moiety which, being a constituent of the membranes, is highly suitable for incorporation into membranes. The nitroxide synthesized has been employed as an ESR sensitive probe to monitor the changes in fluidity and permeability of model membranes made up of L-α-dipalmitoyl phosphatidyl choline (DPPC) as such and in the presence of various drugs.

Results and Discussion

For the synthesis of the lipid nitroxide, mannitol (**1**) was first protected and cleaved to be converted into 1,2-isopropylidene glycerol (**3**) which showed an M−15 peak at $m/z = 117$ in its mass spectrum and a hydroxy peak at 3450 cm^{-1} in its IR spectrum. The ^{1}H NMR spectrum of this compound showed peaks which were comparable with those reported in the literature.[12] Isopropylidine glycerol (**3**) hence obtained was condensed with stearic acid. Product **4** obtained was connoted by the appearance of an ester peak at 1738 cm^{-1} in the IR spectrum and confirmed by the presence of multiplets at 4.31 (2-H) and 4.05–4.20 ppm ($1H_2$ and 3-H) in the ^{1}H NMR spectrum. The multiplets at 1.62 ppm were assigned to the two 17′ protons, those at 1.25 ppm to the 28 protons (3′-H_2–16′-H_2) of the stearyl group and the triplet at 0.88 ppm ($J = 6.9$ Hz) to the terminal methyl protons of the same. The ^{13}C NMR spectrum of compound **4** also showed the presence of an ester carbonyl peak at 172 ppm confirming it to be isopropylidene glyceryl stearate.

Key words: 1-[2-Hydroxy-3-octadecan-1′-oate]propyl-2″,2″,5″,5′-tetramethyl pyrolidine-*N*-oxyl-3″-carboxylate; proxyl stearate; spin label; model membrane; phase transition.

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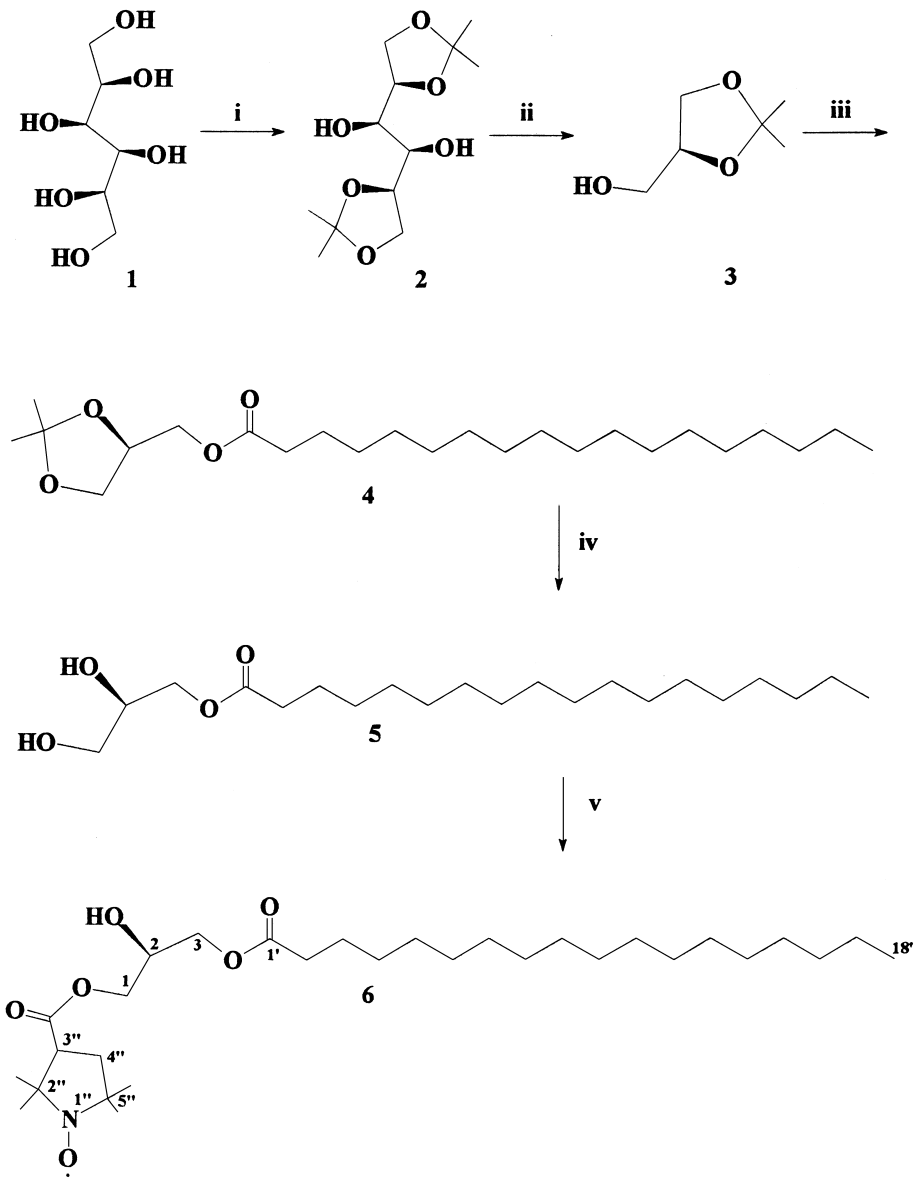
0968-0896/99/$ - see front matter
PII: S0968-0896(99)00216-3



Scheme 1. Synthesis of 1-[2-hydroxy-3-octadecan-1′-oate]propyl-2″,2″,5″,5″-tetramethyl pyrolidine-*N*-oxyl-3″-carboxylate (**6**). Conditions: (i) acetone, $ZnCl_2$, K_2CO_3; (ii) $NaIO_4$, $NaBH_4$; (iii) stearic acid, DMAP, DCC; THF; (iv) Dowex 50W×8; (v) 3-carboxy proxyl, DCC, DMAP, THF, 16 h.

Product **4**, hence formed, was then deprotected to yield the corresponding diol **5** which showed a broad peak at 3320 cm^{-1} in its IR spectrum. The elemental analysis and M^+ peak at *m/z* 358 established a molecular formula of $C_{21}H_{42}O_4$ for compound **5**. The ^1H NMR spectrum of compound **5** was deficient of the isopropyl methyl singlets observed in the case of its parent compound **4** at 1.44 and 1.37 ppm. The C-2 and C-3 signals were shifted upfield and appeared at 65.1 and 63.2 ppm, respectively, in the ^{13}C NMR spectrum of diol **5**, further establishing its formation.

The primary hydroxy group in compound **5** was then condensed selectively with 3-carboxy proxyl in presence of 4-dimethyl amino pyridine (DMAP) and dicyclohexyl carbodiimide (DCC) to obtain spin label **6**. The formation of the nitroxide **6** was indicated by the presence of two ester peaks at 1728 and 1650 cm^{-1} along with a hydroxy peak at 3457 cm^{-1} in its IR spectrum. The mass spectrum of this compound showed M^+ peak at *m/z* 526 ($C_{30}H_{56}NO_6$).

The ^1H NMR of compound **6** obtained after reduction with phenylhydrazine showed four singlets at 1.35, 1.27, 1.21 and 1.06 ppm for the gemdimethyls of proxyl moiety, confirming the product to be 1-[2-hydroxy-3-octadecan-1′-oate]propyl-2″,2″,5″,5″-tetramethyl pyrolidine-*N*-oxyl-3″-carboxylate. The ESR spectrum of the proxyl compound **6** showed a symmetrical triplet with $g_0 = 2.0055$ and $A_0 = 14.62$ G.

The incorporation of the nitroxide **6** in liposomes was ascertained by comparing the ESR spectra of the nitroxide in rapidly tumbling solution state ($CHCl_3$) with that in DPPC liposomes (Fig. 1). The hyperfine coupling constant of SL in multilamellar vesicles was 14.50 G

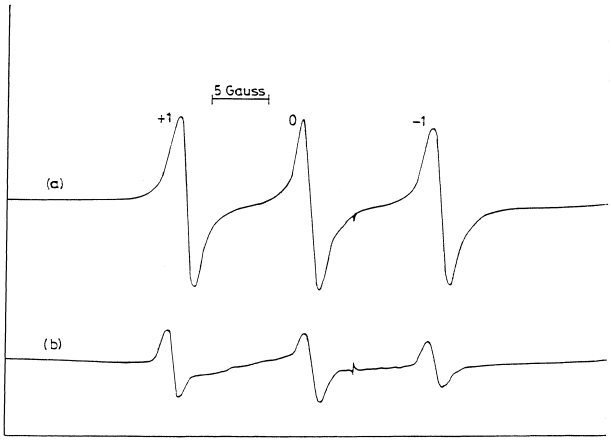


Figure 1. ESR spectra of the nitroxide **6** in (a) solution state ($CHCl_3$) and (b) DPPC liposomes.

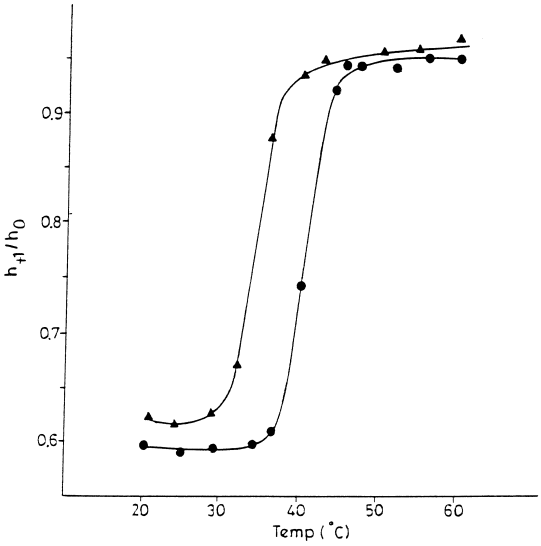


Figure 2. Plot of temperature (°C) vs spectral parameter h_{+1}/h_o of the spin label **6** incorporated into pure DPPC liposomes (●) and in the presence of diltiazem (▲).

while that in rapidly tumbling solution phase was found to be 14.62 G. The differences observed in the line shapes of the highest and lowest field lines in the ESR spectrum of the SL in liposomes is due to the anisotropy of the nuclear hyperfine coupling tensor and *g* tensor of the radical in the lipid.[13] These results suggested that the spin label **6** was completely incorporated into the liposomes.

The DSC of DPPC incorporated with compound **6** showed a phase transition temperature of 40°C, suggesting that the incorporation did not cause any significant perturbation to the system. The localization of spin label **6** in DPPC liposomes was also evident from the ^1H and ^{31}P NMR spectral studies. It was observed that, on incorporation of the spin label, the ^1H NMR resonances of different regions underwent line broadening to different extent.

The NMe_3 peak showed more broadening due to the possible proximity of the proxyl group as well as the hydrogen bonding of the hydroxy group of the SL with the polar head group of the lipid, while the broadening of the terminal Me signal was markedly less (Table 1). The increase in the ^{31}P line widths also indicated the same.[14–16]

In order to explore the potential of nitroxide **6** as a 'reporter group', phase transition temperature of DPPC dispersions incorporated with compound **6** as an ESR sensitive probe was determined. Alterations in the phase transition temperature induced by a vasodilating drug, diltiazem, were also studied (Fig. 2). The transition temperature obtained for pure DPPC incorporated with spin label **6** was in agreement with the values reported by other techniques. In the presence of diltiazem, the sigmoidal curve indicated a transition temperature of 36°C. This showed that the drug was bound to the hydrophobic region in DPPC liposomes.[17,18] Hence, it can be concluded that the proxyl glyceryl stearate has the ability to report the phase transition of pure lipids and the lipids incorporated with external agents.

The permeation of epinephrine in model DPPC membranes was studied using ESR spin labeling technique. The adrenergic neurotransmitter epinephrine, known to exhibit cardiac and respiratory stimulations by complexing with appropriate receptors that are usually located in the plasma membranes, is also capable of quenching paramagnetism of a spin label.[19,20] This made it possible to study the dynamics of the permeation process in DPPC model membrane by monitoring the ESR signal heights of the spin label **6** incorporated in the system. The spin label dissolved entirely into the lipid phase of the water/lipid system and when incorporated into unilamellar vesicles the label was dispersed both in the outer and inner monolayers of the lipid bilayer. When epinephrine was introduced into the DPPC system, it diffused into the bilayer and quenched the paramagnetism of the spin label molecules present in the outer monolayer at a faster rate as compared to those residing in the inner region. As a result, the ESR signal height decreased with time and the plot of signal height with time showed an exponential decay (Fig. 3). The half-life times for the reduction of the spin label in

Table 1. Comparative line widths of the ^1H and ^{31}P resonances of the DPPC liposomes as such and in presence of spin label **6**

				Half line widths (Hz)			
		$N+Me_3$		Terminal Me			^{31}P
W_L	Pure DPPC	3.36505	W_{LS}/W_L	1.3206	W_{LS}/W_L	1.9362	W_{LS}/W_L
W_{LS}	6 + DPPC	4.12795	1.2715	1.3846	1.0484	2.4542	1.2675

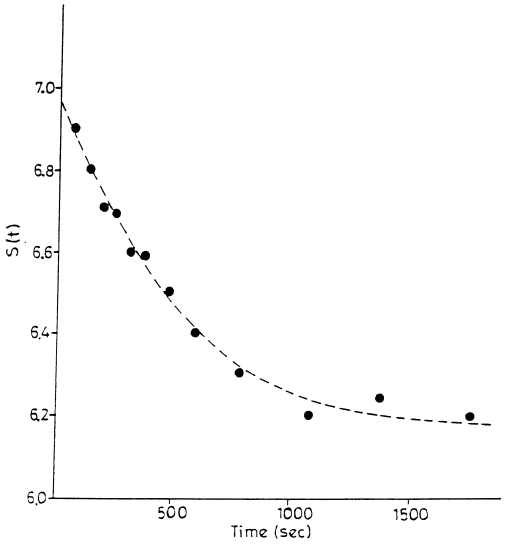


Figure 3. Plot of time (s) vs the signal height $S(t)$ of the ESR spectrum of the spin label **7** incorporated into DPPC liposomes in the presence of epinephrine.

the outer and inner reflect the rates of permeation of the drug for the outer and inner monolayer. These half times were calculated to be 28.88 s and 69.33 s, respectively, according to the equation[14]

$$S(t) = S_0(0)e_0^{-kt} + S_i(0)e_i^{-kt}$$

where $s(t)$ is the ESR signal height due to the total spin label present at time t and $S_0(0)$ and $S_i(0)$ are signal heights due to initial spin label concentration in the outer and inner monolayers, respectively. These results showed that the diffusion process enables the drug to move through the membrane structure by lateral diffusion at a moderate rate to reach its receptor sites.[21,22]

Conclusively, 1-[2-hydroxy-3-octadecan-1(-oate]propyl-2″,2″,5″,5″-tetramethyl pyrolidine-*N*-oxyl-3″-carboxylate (**6**) was synthesized by selective esterification of the primary hydroxy group of glyceryl stearate. To ascertain its utility as a spin probe, this new proxyl spin label was incorporated in DPPC liposomes. The ESR studies indicated that this spin label can be successfully used for observing phase transition in pure DPPC and in the presence of drugs, as well as in determining the permeation of drugs through membranes, and, hence, shows a strong potential for probing biomembrane properties.

Experimental

Melting points are reported uncorrected. All solvents were predried according to standard procedures. Petroleum ether refers to the fraction having bp 60–80°C. 3-Carboxy proxyl, DPPC and epinephrine were purchased from Sigma-Aldrich Chemical Company. Diltiazem was received as a gift from Iswtituto di Science Fisiche, Unversity di Ancona, Italy. Elemental analysis was carried out on CEST 1106, IR spectra on a Nicolet Impact 400 FT IR spectrophotometer, while 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 using about 5 mg of the sample dissolved in 0.6 mL of the solvent. The ^{1}H NMR spectra of nitroxides were recorded after in situ reduction of their $CDCl_3$ solutions with 1.5 equivalents of freshly distilled phenyl hydrazine ($PhNHNH_2$). The ^{1}H NMR and ^{31}P NMR for determination of the localization of the spin label in DPPC (2:10 mmol) was taken in 0.6 mL of $CDCl_3$. The DSC experiments were carried out on a 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 ($g_o = 2.00277$). Deoxygenated solvents were used for ESR measurements. The concentrations of the nitroxides were maintained ca. 10^{-5} M. Multilamellar dispersions of DPPC were prepared using Hill's method[11] in the following way.

Chloroform solutions of the required spin label and the lipid were taken in a small tube. The solvent was evaporated slowly with a stream of nitrogen gas to get a thin film on the walls of the tube. Traces of solvent were removed by drying under vacuum for three hours. The dried film was then hydrated with the 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 spin label 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.

To prepare unilamellar vesicles, a lipid dispersion of DPPC in 10 mM phosphate buffer with spin label **6** was prepared according to the above mentioned method. After a vortex of 10 min at 50°C, the dispersion was subjected to sonification 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, which was used for the ESR experiments.

For phase transition experiments using diltiazem, the multilamellar dispersion was hydrated as usual using an 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:spin label were maintained at 40:100:1.

For permeation studies with epinephrine, at time $t = 0$, to 50 μL of the sonicated preparation, the required amount of drug solution in buffer was added. Subsequently, the decay of the ESR signal with time was monitored. The samples were taken in 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 spin label was 1 mM. The molar ratios of the drug: lipid:spin label were maintained at 20:100:1. The quencher solutions were prepared in 10 mM phosphate buffer (pH 7.2). The temperature was kept at 50°C 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×1G 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 and was not altered throughout the experiment to give a strong signal whose decay could be monitored with time. Only the first line in the ESR spectrum and its decay with time were recorded.

1,2:5,6-Di-*O*-isopropylidene-D-mannitol (2). Acetone (500 mL) was saturated with anhydrous zinc chloride (150 g, 1.1 mol) and the milky solution so formed was allowed to settle down. The saturated solution was transferred in two portions under N_2 pressure to a round bottom flask containing D-mannitol (**1**, 72 g, 395 mmol). The flask was fitted with a mechanical stirrer with a calcium chloride drying tube and its contents stirred at room temperature till the solution became clear (2 h). The mixture was poured in saturated K_2CO_3 solution (400 mL), stirred for half an hour and filtered. The residue and filtrate were extracted with $CHCl_3$ (300 mL×3), dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum to a crude solid (93.2 g). The solid thus obtained was again dissolved in hot hexane: dichloromethane (9:1) and kept overnight for crystallization. The crystals formed were filtered off, washed with cold hexane and dried to a constant weight in vacuo over P_2O_5. Yield: 88 g (85%). Mp: 120°C (lit. 120–121°C). MS: *m*/*z* 247 ($M^+ - 15$). IR (KBr): ν 3450–3300, 1395, 1385 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 4.23–4.17 (m, 2H, 2-H, 5-H), 4.01 (dd, *J* = 8.2, 6.6 Hz, 2H, 3-H, 6-H), 3.76 (dd, *J* = 8.2, 6.6 Hz, 2H, 1-H, 6-H), 3.67 (dd, *J* = 11.7, 3.9 Hz, 1-H, 3H/4H), 3.56 (dd, *J* = 11.5, 5.3 Hz, 1H, 3H/4H), 2.5 (bs, 2H, 3-OH and 4-OH), 1.41 (s, 6H, $>C(CH_3)_2$), 1.35 (s, 6H, $>C(CH_3)_2$).

1,2-Isopropylidine glycerol (3). To an ice-cold solution of $NaIO_4$ (15 g, 70 mmol) in 250 mL distilled water, 1,2:5,6-*O*-isopropylidene-D-mannitol (2; 15 g, 57 mmol) was added in four portions with constant stirring till the solution became clear. Ethanol (500 mL) was added and stirred for half an hour at 0°C. The reaction mixture thus obtained was filtered and the residue was washed with precooled ethanol. To the cold filtrate, $NaBH_4$ (5 g, 131.6 mmol) was added at 0°C in portions over a period of half an hour and left stirring for 2 h at room temperature. The reaction mixture was then neutralized with NH_4Cl to pH 7 and concentrated under reduced pressure to a final volume of 250 mL. The turbid solution was saturated with NaCl and evaporated to dryness in vacuo at room temperature. It was distilled to 6.6 g (88%) of a colorless oil at 70°C/8–9 mm of Hg. MS: *m*/*z*: 117 (M−15, 20%), 114 (M−18, 24), 101 (20). IR (neat): ν 3450, 1390, 1380, 1050, 840 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 4.27–4.19 (m, 1H, 2-H), 4.03 (ddd, *J* = 1.0, 4.1, 7.8, 1H, 1-H), 3.78 (ddd, *J* = 1.0, 3.8, 7.8, 1H, 1-H), 3.68 (dd, *J* = 4.0, 11.6, 1H, 3-H), 3.58 (dd, *J* = 3.6, 11.6, 1H, 3-H), 1.44 (s, 3H, $>C(CH_3)$), 1.36 (s, 3H, $>C(CH_3)$).

2,3-Isopropylidene glyceryl stearate (4). Stearic acid (0.75 g, 2.65 mmol) was dissolved in dry THF (20 mL) to which DCC (0.54 g, 2.65 mmol), DMAP (0.32 g, 2.65 mmol) and isopropylidine glycerol (**3**; 0.42 g, 3.19 mmol) were added. The reaction mixture was stirred for 24 h, the insoluble urea filtered off and the solvent removed in vacuo. The resulting oil on purification over silica gel column yielded product **4** (1.6 g) as a white solid having low melting point in 70% yield. $[\alpha]_D = -22.222$ (0.045% in $CHCl_3$). MS: *m*/*z* 398 (M^+, 22%), 297 (54), 265 (12). IR (KBr): ν 2924, 2850, 1738, 1374, 1385 cm^{-1}. Elemental analysis: calculated for $C_{24}H_{46}O_4$: C, 72.31; H, 11.63%. Found: C, 72.23; H, 11.39%.

^1H NMR ($CDCl_3$, 300 MHz): δ 4.31 (m, 1H, 2-H), 4.05–4.2 (m, 3H, 1-H_2, 3-H), 3.74 (dd, *J* = 6.3, 8.4, 1H, 3-H), 2.34 (t, *J* = 7.5, 2H, 2′-H_2), 1.62 (m, 2H, 17′-H_2), 1.44, 1.37 (3, gemdimethyl), 1.25 (m, 28H, 3′-H_2-16′-H_2), 0.88 (t, *J* = 6.9, 3H, 18′-H_3). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 172 (C-1′), 108 (C-2′), 72 (C-1), 64.7 (C-2), 62 (C-3), 12 (C-18′).

Glyceryl stearate (5). Compound **4** (0.1 g, 0.25 mmol) was dissolved in methanol (60 mL) and Dowex 50W×8 (1–2% w/w) was added to it. The reaction mixture was stirred for 4 h, filtered and the solvent was evaporated under reduced pressure. The crude product on purification over silica gel column yielded the required diol **5** (0.05 g) in 56% yield. Mp: 70–72°C. $[\alpha]_D = -36.363$ (0.055% in $CHCl_3$). MS: *m*/*z* 358 (M^+, 6%), 340 (M−18, 15%), 322 (12%), 265 IR (KBr): ν 3320, 2924, 2850, 1738, 1374, 1385 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{42}O_4$: C, 70.35; H, 11.81%. Found: C, 70.29; H, 11.77%.

^1H NMR ($CDCl_3$, 300 MHz): δ 4.19 (m, 2H, 1-H_2,), 3.94 (m, 1H, 2-H), 3.65 (m, 2H, 3-H_2), 2.35 (t, *J* = 7.5, 2H, 2′-H_2), 1.63 (m, 2H, 17′-H_2), 1.25 (m, 28H, 3′-H_2–16′-H_2), 0.88 (t, *J* = 6.7, 3H, 18′-H_3). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172 (C-1′), 70.2 (C-1), 65.1 (C-2), 63.2 (C-3), 13.9 (C-18′).

1-[2-Hydroxy-3-octadecan-1′-oate]propyl-2″,2″,5″,5″- tetramethyl pyrolidine-*N*-oxyl-3″-carboxylate (6). The diol **5** (0.1 g, 0.28 mmol) obtained was dissolved in dry THF (10 mL) and to this solution was added 3-carboxy proxyl (0.05 g, 0.28 mmol), DCC (0.06 g, 0.28 mmol) and DMAP (0.034 g, 0.28 mmol). The reaction mixture was stirred for 30 h followed by filtration of the insoluble urea formed and the removal of the solvent under reduced pressure. The crude residue was column chromatographed to get the spin label **6** (0.75 g) in 53.5% yield. Mp = 204°C. $[\alpha]_D = -28.282$ (0.055% in $CHCl_3$). MS: *m*/*z* 526 (M^+, 4%), 496 (M−30, 12), 391 (25). IR (KBr): ν 2929, 2855, 1728, 1650, 1456, 1373 cm^{-1}. Elemental analysis: calculated for $C_{30}H_{56}O_6N$: C, 68.40; H, 10.72; N, 2.66%. Found: C, 68.29; H, 10.57; N, 2.56%.

^{1}H NMR ($CDCl_3$ with 1.5 equivalents of $PhNHNH_2$): δ 4.16 (m, 2H,1-H_2,), 3.74 (m, 1H, 2-H), 3.51 (m, 2H, 3-H_2), 2.35 (m, 2H, 2′-H_2), 1.26 (bs, 28H, 3′-H_2–16′-H_2), 0.88 (t, $J = 6.9$, 3H, 18′-H_3), 1.35 (s, 3H, gemdimethyl), 1.27 (s, 3H, gemdimethyl), 1.21 (s, 3H, gemdimethyl), 1.06 (s, 3H, gemdimethyl). ESR spectrum (10^{-5} M in $CHCl_3$): symmetrical triplet with $g_0 = 2.0055$ and $A_0 = 14.62$ G.

Acknowledgements

We thank C.S.I.R., New Delhi and B.R.N.S., Trombay for the financial assistance. The spectral facilities provided by R.S.I.C., I.I.T., Mumbai and National Facility for High Field NMR, T.I.F.R., Mumbai are gratefully acknowledged. We also wish to thank Prof. Nand Kishore, I.I.T., Mumbai for providing the DSC facility.

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