A new membrane probing steroidal spin label: Synthesis and applications

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The applicability of a new steroidal spin label, 3-oxo-androstan-17\beta-yl-(2',2',6',6'-tetramethyl-N-oxy) piperidyl butan-1,4-dioate, in studying the phase transition properties of model membrane 1-\alpha-dipalmitoyl phosphatidyl choline (DPPC) in the presence and absence of drugs has been explored. Its synthesis and characterization has been described herein. Besides, the localization of this spin label in lipid liposomes has been studied using electron spin resonance (ESR), differential scanning calorimetry (DSC) and \(^1\)H and \(^3\)P NMR spectroscopic techniques. The label has also been used to study the permeability of epinephrine into membrane. The results show that the spin label has a good potential as a spin probe in the study of biomembranes.

Bilayers prepared from pure lipids exhibit a characteristic biphasic transition from a gel to liquid crystalline phase\(^1\). Many of the membrane bound enzymes are switched on or off as the system goes from gel to liquid crystalline phase and vice versa\(^2\). The permeability across biomembranes is directly linked to the mobility of membranes\(^3\). The drug-membrane interactions play a vital role in the biological action of drugs\(^4,5\). The ability of the drug molecule to reach the receptor site in the system is ultimately connected to the transport of the drug across biological membranes\(^6\). Certain drugs can act directly on the membranes. Examples of this class are neurotransmitter drug epinephrine and coronary vasodilating drug diltiazem. The drug-membrane interaction can be monitored with the help of spin labels. Membrane structure studies have been carried out by labeling them with steroidal nitroxides, steroids being appropriate carrier molecules since they are natural components of membranes and are easily incorporated without causing perturbations. The synthesis and applications of numerous rigid DOXYL and PROXYL spin labels of cholesterol and androstanolone have been reported in the literature\(^7,9\). However, the reports on the synthesis of their TEMPO nitroxyl derivatives are few\(^10,12\).

In continuation of our efforts in the synthesis and applications of such steroidal nitroxyls\(^1,18\), we describe here the synthesis and application of yet another new TEMPO spin label derivatized from androstanolone. The localization of the spin label in the multilamellar DPPC vesicles was determined with the help of ESR, DSC, \(^1\)H NMR and \(^3\)P NMR spectroscopic techniques. The effect of diltiazem on phase transition property and the epinephrine permeation in model membranes were studied using the spin label \(3\), with the help of ESR spectroscopy.

**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. Androstan-17\beta-ol-3-one, epinephrine and DPPC were purchased from Sigma Aldrich Chemical Company. Diltiazem was received as a gift from Istituto di Science Fisiche, Univ. Di Ancona, Italy. 4-Hydroxy TEMPO was prepared from 2,2,6,6-tetramethylpiperidine-4-one monohydrochloride which was purchased from Fluka. 4-Oxo TEMPO was obtained by treating 2,2,6,6-tetramethylpiperidine-4-one with sodium tungstate and was converted to the corresponding alcohol by reduction with lithium aluminium hydride. The elemental analysis was done on CEST 1106. IR spectra were recorded on a Nicolet Impact 400 FTIR spectrophotometer. A Hewlett Packard MS Engine 5989-A spectrometer was used to record the mass spectra. \(^1\)H NMR and \(^3\)P 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 nitroxides 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 \(^3\)P NMR for determination of the localization of the spin label (SL) in DPPC (1:5 molar ratio) was taken in 0.6 ml. The DSC 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 = 2.00277). Deoxygenated chloroform was used as the solvent for the ESR measurements, the concentrations of the nitroxides being c.a. 10\(^{-5}\) M.

17β-Hemisuccinyl oxy androstan-3-one (2)

Androstanolone (1; 0.2 g, 0.689 mmol) was dissolved in pyridine (2 ml) in a round bottom flask to which succinic anhydride (0.068 g, 0.68 mmol) was added and stirred for 4 hr (monitored by TLC). After completion of the reaction, ethylacetate was added and the reaction mixture was filtered through celite. The filtrate was washed with dilute HCl, water and brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. Silica gel column chromatography using ethylacetate/petroleum ether eluant afforded the pure hemisuccinate 2 in 79.5% yield (0.2 g). mp: 146-147°C; IR(KBr): ν 3450, 1714, 1651 cm\(^{-1}\); Elemental analysis: Calculated for C\(_{23}\)H\(_{29}\)O\(_5\): C, 70.68; H, 8.69%; Found: C, 70.68; H, 8.69%.

\(^1\)H NMR (300 MHz, CDCl\(_3\)) δ: 4.26 (dd, J=7.8, 9 Hz, 1H, 17α-H), 2.65 (m, 4H, succinyl-(H\(_2\))), 1.02 (s, 3H, 18-H), 0.80 (s, 3H, 19-H).

\(^13\)C NMR (75 MHz, CDCl\(_3\)) δ: 212.3 (C–3), 177.2 (-COO\(_{-}\)), 172.2 (-COOH), 83.2 (C–17), 12.0 (C–19), 11.4 (C–18).

3-Oxo-androstan-17-yl-(2\(^\prime\),2\(^\prime\),6\(^\prime\),6\(^\prime\)-tetramethyl-N-oxyl) piperidyl butan-1\(^{\prime}\)-dioate (3)

Compound 2 (0.1 g, 2.564 mmol) was dissolved in dry THF (50 ml) and to it 4-hydroxy-tempo (0.53 g, 3.07 mmol) in THF (5 ml), 1,3-dicyclohexylcarbodiimide (0.53 g, 2.56 mmol) and 4-dimethylaminopyridine (0.31 g, 2.56 mmol) were added under nitrogen atmosphere. The reaction mixture was stirred overnight followed by filtration of insoluble urea and evaporation of the solvent. The crude product obtained was purified by silica gel column chromatography to get the required compound 3 in 72% yield.

MS: m/z: 515, 471, 446, 389, 272, 254, 230, 212, 198, 181, 124, 118, 100, 67, 55; IR(KBr): ν 2936, 2855, 1735, 1711, 1657, 1449, 1375 cm\(^{-1}\); Elemental analysis: Calculated for C\(_{29}\)H\(_{35}\)O\(_5\)N: C, 70.56; H, 9.25; N, 2.57%; Found: C, 70.43; H, 9.18; N, 2.34%.

\(^1\)H NMR (CDCl\(_3\) with 1.5 equivalents of PhNHNNH\(_2\)) δ: 4.61 (m, 1H, 17α-H), 4.04 (m, 1H, 4\(^\prime\)-H), 2.66 [m, 4H, succinyl-(H\(_2\))], 1.32 (s, 6H, gemdimethyls), 1.26 (s, 6H, gemdimethyls), 0.92 (s, 3H, 19-H), 0.80 (s, 3H, 18-H).

ESR spectrum (10\(^{-5}\) M in CHCl\(_3\)): symmetrical triplet with g\(_0\) = 2.0057 and A\(_0\) = 15.62 G

Multilamellar dispersions of DPPC were prepared following Hill’s method. 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 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.

For unilamellar dispersions of DPPC, a lipid dispersion of DPPC in 10 mM phosphate buffer with spin label 3 was prepared following 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. 35W) at 40°C. Sonication for 30 min produced clear homogeneous solution which was used for the ESR experiments.

For permeation studies with epinephrine, the temperature was kept at 50°C, which 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 sec. The scan time for each spectrum was 4 min. 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 and 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 50 µl of the sonicated preparation, required amount of drug solution in buffer was added. The 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 spin label was 0.01 mM. The concentration of the lipid: drug was maintained at 1:0.2. The quencher solutions were prepared in 10 mM phosphate buffer, pH 7.2.

Results and Discussion

17β-Hydroxy-androst-3-one (1) was converted to its hemisuccinate 2 (Scheme 1), whose formation was confirmed by the presence of two carbonyl peaks at 1734 and 1650 cm⁻¹ in IR spectrum. The mass spectrum of product 2 exhibited a molecular ion peak at m/z 390 (C₂₃H₄₂O₄). The ¹H NMR spectrum of compound 2 showed a multiplet at 2.70 ppm, characteristic of the succinyl methylene protons along with a doublet of doublet (J=7.8, 9 Hz) at 4.26 ppm for 17α-H. The ¹³C NMR spectrum of hemisuccinate 2 showed the presence of three carbonyl resonances, C-3 at 212.3 ppm, the ester carbonyl at 177.2 and the acid carbonyl at 172.2 ppm. The hemisuccinate 2 was condensed with 4-hydroxy TEMPO in the presence of dicyclohexylcarbodiimide (DCC) and N,N-dimethylamino pyridine (DMAP) to obtain compound 3. The product formation was indicated by the absence of the characteristic acid carbonyl peak and the appearance of ester carbonyl peaks at 1735 and 1711 cm⁻¹ in the IR spectrum. The mass spectrum showed peak at m/z 514 (M-30) due to loss of NO moiety from the M⁺ peak (C₁₂H₈O₆N)⁹⁻. The ¹H NMR spectrum of compound 3, after reduction of the nitroxide with 1.5 equivalents of phenyl hydrazine showed additional multiplet for 4'-H at 4.04 ppm along with two singlets at 1.32 and 1.26 ppm for the gem dimethyls of the TEMPO moiety. These data confirmed that nitroxide 3 was androst-3-ene-17-yl-(2"-2",6"-6",6"-tetramethyl-N-oxyl)piperidyl butan-1',4'-dioate. The ESR spectrum showed a symmetrical triplet with gₓ=2.0057 and Aₓ=15.62 G.

The incorporation of the TEMPO derivative of 17β-hemisuccinylloxy androst-3-one, which is a metabolite of progesterone, in the model membrane DPPC and its application in probing the fluidity and permeability properties of model DPPC bilayers in the presence and absence of certain drugs was studied using ESR spectroscopy. The localization of the spin label 3 in liposomes was ascertained by comparing the ESR spectra of the nitroxide in rapidly tumbling solution state (CHCl₃) with that in the lipid matrix i.e. DPPC liposomes (Fig. 1). The hyperfine coupling constants of the spin label in multilamellar vesicles was found to be 15.11 G, while that in rapidly tumbling solution phase was found to be 15.62 G. The differences observed in the line shapes of the highest and lowest field lines in the ESR spectrum of the spin label in liposomes is due to the anisotropy of the nuclear hyperfine coupling tensor and g tensor of the
radicals in the lipid\textsuperscript{20}. Hence, the absence of composite peaks along with the changes in the line shape and hyperfine coupling constant suggest complete incorporation of the spin label into the liposomes.

The DSC of pure DPPC vesicles showed the phase transition temperature at 41\degree{C} while those incorporated with spin label 3 showed at 39.8\degree{C} which is within the error limits of the instrument. This indicated the incorporation of 3 and also proved that it did not cause much perturbation to the system.

Further evidences for the incorporation of 3 in the liposomes and the mode of localization came from the \textsuperscript{1}H NMR, \textsuperscript{31}P NMR spectroscopy\textsuperscript{21,22}. As the spin label is a paramagnetic moiety, \textsuperscript{1}H NMR signals arising from protons in close proximity (<10 Å) of the nitroxide group experience broadening on account of dipole-dipole interactions. It was observed that on incorporation of the spin label the \textsuperscript{1}H NMR resonances of different regions underwent line broadening to different extents. The peak widths of the \textsuperscript{1}H NMR resonances of pure DPPC and those incorporated with spin label are tabulated (Table 1).

![ESR spectra](image)

**Fig. 1** — ESR spectra of the nitroxide 3 in (a): rapidly tumbling solution state (10^{-4} M in CHCl\textsubscript{3}) and (b): lipid matrix (DPPC liposomes), SL:DPPC 0.8:80.0 mM.

| Table 1 — Line broadening of NMe\textsubscript{3} and terminal Me \textsuperscript{1}H and \textsuperscript{31}P NMR signals caused by incorporation of spin label 3 in DPPC |
|-----------------|-----------------|-----------------|-----------------|
| Half line widths (Hz) | N'Me\textsubscript{3} | Terminal Me | \textsuperscript{31}P |
| W\textsubscript{L}, Half line width of pure DPPC solution | 3.36505 | 1.3206 | 1.9362 |
| W\textsubscript{LS}, Half line width of DPPC solution containing the spin label | 3.83484 | 1.4000 | 2.8442 |
| W\textsubscript{LS}/W\textsubscript{L} | 1.1396 | 1.060 | 1.46896 |

Among the peaks in the \textsuperscript{1}H NMR spectra the broadening observed for the characteristic NMe\textsubscript{3}, constituting the polar head group of the lipid and the terminal-Me group constituting the other extreme end viz. nonpolar hydrocarbon region of the lipid have been considered.

It was observed that both NMe\textsubscript{3} and terminal Me peaks underwent broadening to different extents. The NMe\textsubscript{3} peak showed more broadening due to the proximity of the TEMPO group to the polar head group of the lipid. The increase in the \textsuperscript{31}P resonance line widths further confirm this\textsuperscript{21,22}.

Phase transition behaviour of DPPC membranes was studied using spin label 3 as ESR sensitive probe.
The alterations in the phase transition temperature induced by vasodilating drug, diltiazem has also been studied. The empirical parameter $h_{l}/h_{0}$ (ratio of signal heights of the low field line to the central line in the ESR spectrum of spin label 3) has been plotted as a function of temperature to monitor the phase transition characteristics (Fig. 2). This parameter ($h_{l}/h_{0}$) showed an initial gradual increase, followed by a sudden large increase at a particular temperature which corresponds to the transition of the lipid from gel to liquid crystalline state. The transition temperature was obtained at 40.5°C which is close to the values reported earlier by using other techniques. In the presence of the drug the transition temperature lowers down to 34.5°C thus increasing the fluidity of the membrane. However, the sigmoidal nature of the curve is retained showing that the drug has affinity to bind loosely to the hydrophobic region of the lipid. This is in agreement with the literature reports that high solubility of the drugs in the hydrophobic phase of lipid results in the large decrease of the phase transition of lipids. Thus, spin label 3 has the ability to report the phase transition of pure lipids and the lipids in the presence of drugs.

The permeation of epinephrine in model DPPC membranes has been studied using ESR spin labeling technique. Adrenergic neurotransmitter epinephrine, often used as antianesthetic and glycogen breakdown inhibitor is known to bring about cardiac and respiratory simulations and increased sodium transport across the cell membrane of erythrocytes. Most of the actions are triggered when the drug molecule complexes with appropriate receptors that are located in the plasma membranes with their binding sites oriented externally. Binding of neurotransmitters on the outer source of the cell membrane causes a local change in the membrane which leads to the modifications of the cell functions and consequently regulates the biochemical pathways.

The spin label on dissolution in the lipid phase of the water/lipid system gets incorporated into unilamellar vesicles. The label is dispersed both in the outer and inner monolayers of the lipid bilayer. Nitroxide spin labels are known to undergo reduction by reducing agents such as ascorbic acid. Epinephrine possesses the property of reacting with the spin label, thereby causing loss of its paramagnetism. When the drug is introduced into the system, it diffuses into the bilayer. The spin label molecules present in the outer monolayer are readily accessible and therefore undergo reduction at a faster rate as compared to those residing in the inner region. Consequently, the ESR signal height decreases with time and the plot of signal height shows an exponential decay with time (Fig. 3).

Fig. 2—Plot of temperature vs spectral parameter $h_{l}/h_{0}$ of the spin label 3 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 vs signal height $S(t)$ of the ESR spectrum of the spin label 3 incorporated into DPPC liposomes in presence of epinephrine (SL:DPPC:Drug 1:100:20).
The number of spin labels present in the outer and the inner monolayers are related to the relative surface areas $4\pi r_0^2$ and $4\pi r_i^2$ where $r_0$ and $r_i$ are the radii of the outer and inner monolayers respectively. The ESR signal heights ($S_0$) and ($S_i$) due to the spin label present in the outer and the inner monolayers would, in principle, decays 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 behaviour as given by the equation

$$S(t) = S_0(0)e^{-k_0t} + S_i(0)e^{-k_it}$$

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.

Assuming a value of 50Å for the thickness of the bilayer and 250Å for the outer diameter of the sonicated vesicles, the value of $S_0(0)/S_i(0)$ can be calculated. Further $S_i(0)$, $k_0$ and $k_i$ can be determined by the least square fitting of the data. From these results, half-life times for the reduction of the spin labels which in turn reflect the rates of permeation of the drugs for the outer and inner monolayer have been calculated to be 19.88 and 68.82 s, respectively.

The oxidation of the drug requires its contact with spin label dispersed on the two sides of the lipid bilayer. The rate $k_0$ is thus related to the lateral diffusion of the drug on the outer surface of the lipid bilayer. The rate $k_i$ on the other hand depends upon the lateral diffusion and the transport of the drug across the bilayer since flip-flop motion of the spin label itself is highly restricted. The values of $k_0$ and $k_i$ thus indicate a reasonable rate of lateral and transmembrane diffusion of the drug.

These results show that the drug molecules do not protrude deep into the lipid hydrophobic core of the membrane but are bound on the surface and can diffuse laterally at a moderate rate. The diffusion process enables the drug to move through the membrane structure and to reach the receptor sites.

In conclusion, a novel steroidal spin label, 3-oxo-androstan-17β-yl-(2".2".6".6"-tetramethyl-N-oxyl) piperidyl butan-1',4'-dioate was synthesized and was successfully incorporated in DPPC liposomes. The ESR signals of this spin label were utilized to determine the phase transition in DPPC as such and in presence of diltiazem. The study involving the permeation of epinephrine in DPPC using ESR properties of the synthesized spin labels showed that the drug diffuses laterally on the surface of the membrane. Thus, the new androstan TEMPO derivative shows potential as a good spin probe in membrane structure studies.

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References
3 Paphadjopoulous D, Jacobson K, Nir S & Isac T (1973) Biochim Biophys Acta 311, 330-334
10 Weiner H (1969) Biochemistry 8, 526-533
12 Waggoner A S, Keith A D & Griffith O H (1968) J Phys Chem 72, 4129-4132
22 Brockerhoff H (1974) Lipids 9, 645-650
23 Rosenberg P H, Janson J E & Gripenberg J (1977) Anaesthesiology 46, 322-326