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Syntheses of Potential Spin Probes for Biomembranes - Tempo and Proxyl Nitroxides of Lithocholic Acid

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Abstract: The synthesis of a steroidal spin label **10** containing a tempo nitroxide group in the side chain of lithocholic acid **1** is reported. This is the first report of a tempo nitroxide in a steroidal side chain. The methodology involved a Wittig condensation between the steroidal phosphonium ylide **7** and tempone **9**. The synthesis of a proxyl nitroxide **16** with the proxyl group in the side chain of **1** is also described. A mild oxidation of **16** to the corresponding 3-keto spin label **17** in high yield is also achieved.

INTRODUCTION

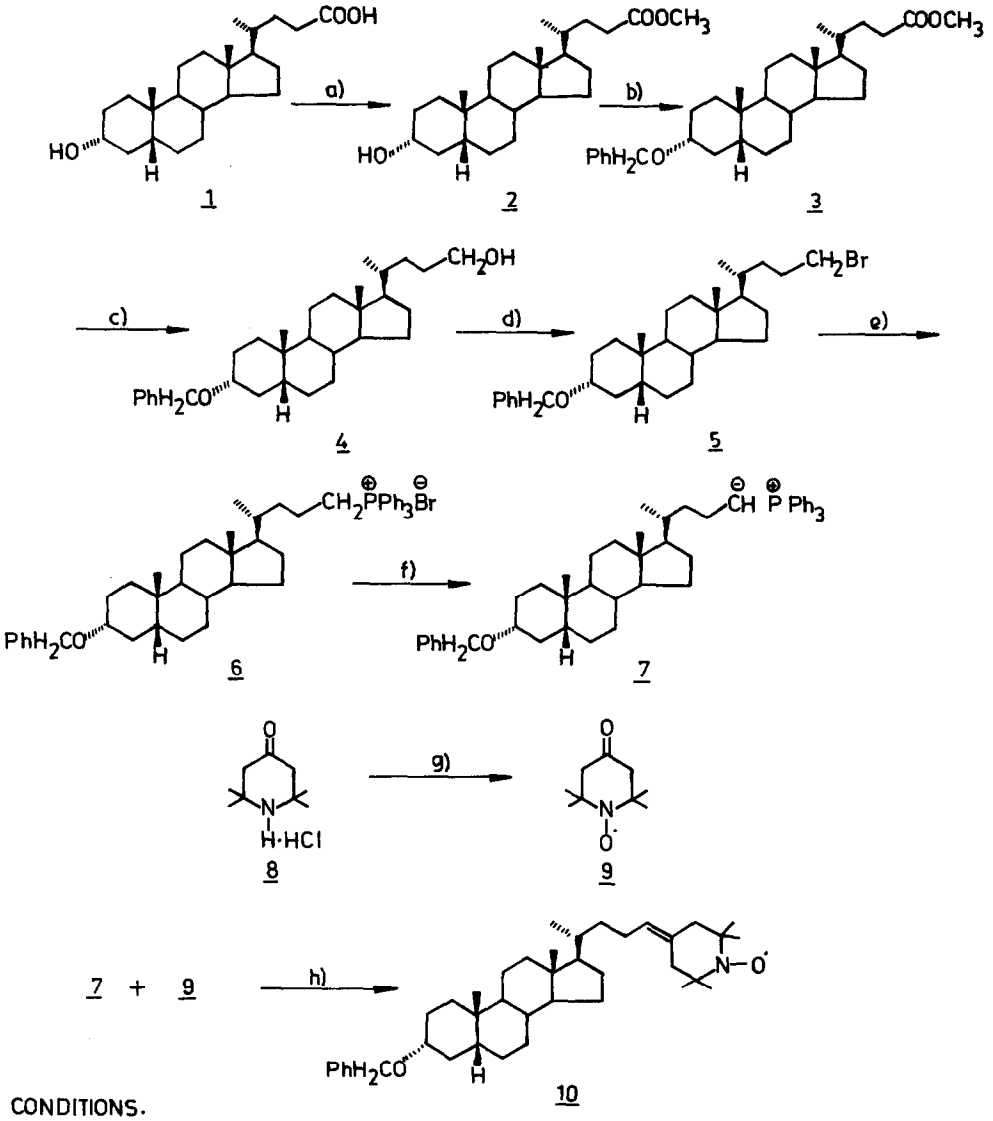
In connection with our work on the syntheses and applications of new steroidal spin probes for biomembranes, we reported[1] the synthesis of a steroidal doxyl (4,4-dimethyloxazolidine-N-oxyl) nitroxide with the doxyl moiety in the side chain of lithocholic acid **1**. Since tempo (2,2,6,6-tetramethylpiperidine-N-oxyl) nitroxides are known[2] to partition efficiently between aqueous and lipid phases, it was felt pertinent to synthesize a steroidal spin probe with the tempo moiety in the side chain of the steroid molecule viz. lithocholic acid **1**. Similarly, inherent differences in polarity between doxyl and proxyl (2,2,5,5 -tetramethylpyrrolidine-N-oxyl) nitroxides[3], which could lead to differences in the mode of binding to biomembranes, prompted us to synthesize proxyl nitroxide in the pendant side chain of lithocholic acid **1**. In this paper, we report the syntheses of a new tempo nitroxide viz. 4'-(3α-benzyloxychol-24-en)-2',2',6',6'-tetramethylpiperidine-1'-oxyl **10**, the proxyl nitroxide, 3'-(3α -hydroxycholan-24-oate)-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl **16** and its 3-keto derivative, 3'-(cholan-3-one-24-oate)-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl **17**. It is worthwhile to mention that this is the first report of a tempo nitroxide in a steroidal substrate.

RESULTS AND DISCUSSION

There are two distinct strategies for the synthesis of steroidal nitroxides. The first method[4,5] involves the construction of the nitroxide moiety via a series of chemical transformations. Our synthesis of the doxyl nitroxide[1] in the side chain of lithocholic acid exemplifies this method. The second method[6] involves the covalent attachment of a molecule containing a nitroxide moiety with a suitable functional group in the steroidal substrate. The syntheses of the tempo nitroxide **10** and proxyl nitroxides **16** and **17** which are described in the sequel utilize the second method.

In the synthesis of the tempo nitroxide, the 4-keto derivative of 2,2,6,6-tetramethylpiperidine-N-oxyl **9** (tempone) was chosen as the synthon containing the nitroxide group. This compound was obtained[7] in high yield from commercially available 2,2,6,6-tetramethyl-4-piperidone hydrochloride **8**. Although Rozantsev *et al*[8] have reported the Grignard reaction of tempone **9** in very low yields, other reports of Grignard reaction of stable nitroxides[9] are known to yield nitroxide reduction products rather than the addition to carbonyl groups. It was therefore worthwhile to investigate the feasibility of Grignard reaction of tempone **9** using the Grignard reagent generated from the C-24 bromo derivative of lithocholic acid. The 3α-benzylated derivative of methyl lithocholate **3** (Scheme-1) served as the starting material for the synthesis of the steroidal synthon. Lithocholic acid **1** was converted to methyl lithocholate **2** which on benzylation[10] yielded the corresponding 3α-benzylated derivative **3**. Reduction of the methyl ester **3** with LAH yielded the C-24 primary alcohol **4** characterized by the -OH absorption band at 3360 cm^{-1} in the IR spectrum. The ^1H NMR spectrum revealed the presence of a two-proton triplet at δ 3.61 characteristic of 24-H_2 instead of the three-proton singlet observed at δ 3.66 corresponding to -COOMe group of **3**. The alcohol **4** was subjected to reaction with triphenylphosphine and tetrabromomethane[11] in dry ether to give the desired bromo compound **5** in almost quantitative yield. The product was characterized by the absence of -OH absorption band in its IR spectrum. The ^1H NMR spectrum exhibited a three-proton multiplet at δ 3.44-3.31 corresponding to the 3β-proton and the 24-H_2. In the mass spectrum of the bromo compound **5** nearly equal intensity molecular ion peaks were observed at m/z 516 and 514. A very small quantity of the dibromo compound[10] formed by the nucleophilic displacement of the 3α-benzyl ether group by $PPh_3Br^+Br^-$ species[12] generated in the reaction was also isolated during the purification of compound **5**.

The next step involved the conversion of the bromo derivative **5** to the corresponding Grignard reagent. Though the reactions involving Grignard addition to carbonyl groups in steroidal substrates are numerous, the reverse mode of reaction which would utilize the addition of the steroidal Grignard reagent to the



CONDITIONS.

a) CH_3OH, H_2SO_4 (few drops), reflux, 2h.
b) NaH, THF, TBAI, $PhCH_2Br$, reflux, 3.5h.
c) LAH, THF, reflux, 3h.
d) PPh_3, CBr_4, K_2CO_3, Et_2O, rt, 12h.
e) PPh_3, DMF, reflux, 48h, Et_2O.
f) PhLi, Et_2O, 1h.
g) $NaWO_4$, 30% aqueous H_2O_2, rt, 2h.
h) Et_2O, rt, 12h, replaced Et_2O with THF, reflux, 6h.

Scheme-1

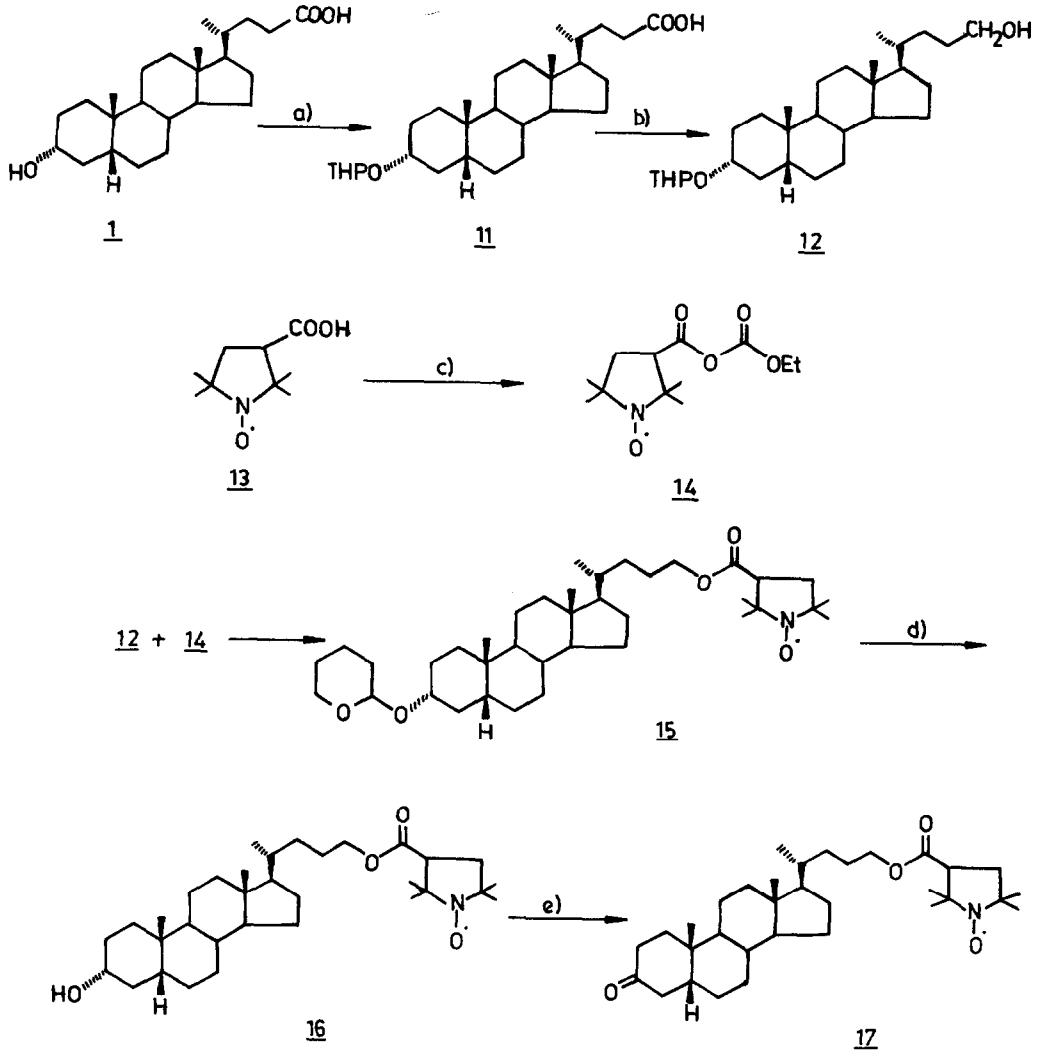
carbonyl compound is not known. The commonly used method involving iodine-activated magnesium turnings in refluxing THF was employed to effect the conversion of **5** to the corresponding Grignard reagent. The starting material however remained unaffected. Subsequent attempts using activated magnesium by entrainment with 1,2-dibromoethane in THF[13] under reflux and highly activated magnesium in powdered state obtained from magnesium(II)chloride-potassium-potassium iodide[13], both at room temperature and at the temperature of refluxing THF, also proved unsuccessful. The extreme inertness of the steroidal bromide towards the formation of the Grignard reagent prompted us to seek an alternative strategy for the attachment of the tempo moiety in the side chain of lithocholic acid **1**. With the steroidal bromide **5** in hand, the obvious alternative of Grignard reaction was a Wittig condensation between tempone **9** and the former. There are few reports of Wittig reaction[14] involving the carbonyl group of tempone. However, the envisaged methodology involving a Wittig reaction between a steroidal phosphonium ylide and **9** constitutes a novel method of synthesizing a steroidal spin label.

The steroidal bromide **5** was converted[15] to the triphenyl phosphonium salt **6** on refluxing with triphenylphosphine in DMF solution for several hours. The salt was isolated in a yield of 74%. Owing to its inherent instability towards moisture, it was immediately subjected to further reaction. The phosphonium ylide **7** was obtained from the phosphonium salt **6** with freshly prepared phenyl lithium[16] in ether. The final step involved *in situ* condensation of the ylide **7** with tempone **9**. The TLC of the reaction mixture showed, among a multitude of other spots, an intense yellow spot. Subsequent purification yielded the desired tempo spin label **10** as a yellow crystalline solid in a yield of 33%. Its paramagnetic nature was confirmed by the nitroxide triplet in the ESR spectrum. The ^{1}H NMR spectrum exhibited line broadening due to the presence of the nitroxide moiety. The reduced N-hydroxy derivative was obtained by *in situ* reduction of the $CDCl_3$ solution of **10** using 1.5 equivalents of phenylhydrazine. However, one drop of D_2O was added to avoid the undesirable interference of >N-H and -NH_2 protons of phenylhydrazine. The well resolved ^{1}H NMR spectrum thus obtained exhibited a one-proton triplet at δ 5.19 (J = 6.95 Hz) corresponding to the single olefinic proton in the side chain of **10**, adjacent to the 23-H_2. The gem dimethyls of the piperidine moiety were observed as two singlets integrating for twelve protons at δ 1.12 and δ 1.10. In the mass spectrum the peaks at m/z 589(M^++1) and 588(M^+) further confirmed the formation of the desired tempo nitroxide **10**. It is also interesting to note that in this novel methodology, the nitroxide moiety remained unaffected under the Wittig condition.

For the synthesis of the spin label containing the proxyl moiety in the side chain of lithocholic acid, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-N-oxyl **13** was chosen as the molecule containing the nitroxide group. Therefore, the obvious mode

of derivatization of the steroidal side chain was via an esterification reaction. For this purpose lithocholic acid was converted to its 3α-tetrahydropyranyloxy derivative **11** and was reduced *in situ* to the corresponding primary alcohol **12** (Scheme-2) by a method described earlier[10]. Vaughan and Osato have reported[17] esterification involving mixed anhydrides. With a view to utilizing this method the mixed anhydride **14** was prepared from 3-carboxyproxyl **13** and ethyl chloroformate in presence of triethylamine. The reaction between compound **14** and the steroidal alcohol **12** afforded the desired proxyl ester **15**. Chromatographic purification of the reaction mixture afforded pure nitroxide ester **15** as a yellow viscous product. The band at 1735 cm^{-1} indicated the desired esterification. The incorporation of the nitroxide moiety was confirmed by the three-line ESR spectrum exhibited by compound **15**. The tentative assignment of the protons in the ^1H NMR spectrum of the product was only possible after *in situ* reduction of the $CDCl_3$ solution of the nitroxide with phenylhydrazine. The ^1H NMR spectrum of compound **15** revealed four additional three-proton singlets corresponding to the presence of the four methyls of the proxyl moiety, besides the methyl signals observed for 18-H_3, 19-H_3 and 21-H_3. The 3'-H of the pyrrolidine moiety was observed as a one-proton dd centred at δ 2.80. In order to confirm the assignments of the ^1H NMR signals the COSY spectrum of the phenylhydrazine reduced product of compound **15** was also recorded. In the COSY spectrum, strong cross peaks were observed indicating coupling between the one-proton dd observed at δ 2.80 and the one-proton dd observed at δ 2.17. The latter signal was, therefore, assigned to one of the two protons at C-4' of the pyrrolidine residue. The second proton at C-4' which was buried in a cluster of peaks between δ 1.94-1.68 in the ^1H NMR spectrum, exhibited strong cross peaks indicating coupling with the protons at δ 2.80 and δ 2.17 in the COSY spectrum. The two one-proton multiplets observed at δ 3.47-3.43 and δ 3.92-3.87 showed strong cross peaks, indicating their mutual coupling and were unambiguously assigned to the two protons -OCH'H" of the tetrahydropyranyl ether group. The third one-proton multiplet at δ 3.60-3.57 which did not show any COSY connectivity was consequently assigned to the 3β-proton of the steroidal skeleton. The MS of the product also showed the $(M^+ + 1)$ peak at m/z 615. The unreacted mixed anhydride **14**, isolated in the purification procedure was characterized with the help of ^1H NMR spectrum of its phenylhydrazine reduced product.

Since the synthesized proxyl nitroxide was to be used as a spin probe for studies in model membranes, it was desirable to have distinct polar and apolar regions in the spin label. This necessitated the deprotection of the 3α-tetrahydropyranyl ether group in **15**. The deprotection to give the 3α-hydroxy derivative **16** was conveniently achieved using methanol and p-toluene sulphonic acid in near quantitative yield.



CONDITIONS.

a) DHP, PTSA (catalytic amount), PhH-THF, rt, 2h.

b) LAH, THF, reflux, 3h.

c) Ethylchloroformate, triethylamine, THF, rt, 48h.

d) PTSA, CH_3OH, rt, 0.5h.

e) Pyridinium chlorochromate, CH_2Cl_2, rt, 2.5h.

Scheme-2

It has been reported[18] that in certain cases 3-keto steroids increase the permeability of lipid bilayers constituting model membranes. Therefore, oxidation of the 3α-hydroxy group in nitroxide **16** to its corresponding 3-keto derivative was desirable. However, the instability of the nitroxide group towards reagents which are commonly employed for the oxidation of the hydroxy group e.g. Jones reagent, dimethylsulphide-oxalyl chloride are documented[19]. In the present case, it was possible to oxidize the nitroxide **16** without affecting the proxyl moiety, under relatively mild condition employing pyridinium chlorochromate[20]. The desired 3-keto derivative **17** was obtained in high yield.

It was pertinent to explore the possibility of synthetically modifying the 3-keto derivative into the α,β-unsaturated Δ^4-derivative, a structural feature commonly encountered in steroidal hormones. This was attempted with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone(DDQ)[21] in refluxing benzene. However, the reaction proved unsuccessful yielding an intractible mixture of non-paramagnetic products. Variations in the reaction conditions viz. increase in reaction time or increase in proportion of DDQ from 1.1 equivalents to 2.2 equivalents for preparing the Δ^4-derivative of **17** also did not help. This was presumably due to the instability of the nitroxide group under DDQ reaction conditions.

The studies involving the synthesized nitroxides as potential spin probes for biomembranes will be reported elsewhere.

EXPERIMENTAL

Melting points are reported uncorrected. Laboratory solvents were predried according to standard procedures before use. Petroleum ether refers to the fraction having b.p. 60°-80°C. Lithocholic acid and 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl were purchased from Aldrich and 2,2,6,6-tetramethyl-4-piperidone hydrochloride was purchased from Fluka and used as such. IR spectra were recorded on Perkin Elmer 688 spectrometer. The 300 MHz ^{1}H NMR spectra, COSY spectrum were recorded on Varian VXR 300S spectrometer and 500 MHz ^{1}H NMR spectra were recorded on Bruker AM 500 spectrometer, as solutions in $CDCl_3$ at ambient temperature with TMS as internal standard. The ^{1}H NMR spectra of nitroxides **10**, **14**, **15**, **16** and **17** were recorded after *in situ* reduction of their $CDCl_3$ solutions with 1.5 equivalents of freshly distilled phenyl hydrazine. Mass spectra were obtained on Shimadzu QP 1000 spectrometer. UV spectra were recorded on Shimadzu UV 260 spectrometer. ESR spectra were recorded at room temperature on Varian E-112 spectrometer operating in the X-band with tetracyanoethylene as internal standard (g_o = 2.00277). Deoxygenated chloroform was used as the solvent for ESR measurements and the concentrations of the nitroxides used were 10^{-5}(M). Elemental analyses were performed on CEST MOD.110 analyser.

2,2,6,6-Tetramethyl-4-piperidone-N-oxyl (9)

A mixture of **8** (2.0 g, 10.4 mmol) in distilled water (25 mL), 30% aqueous H_2O_2 (1.3 mL, 41.7 mmol) and sodium tungstate (0.08 g, 0.25 mmol) were vigorously stirred at rt for 2 h. The solution was then saturated with anhydrous K_2CO_3 and the mixture extracted with ether (5 x 20 mL) until the organic layer became colourless. The combined ether extracts were dried over $MgSO_4$, filtered and concentrated in vacuum. The residue was crystallized from a mixture of cyclohexane and benzene to give low melting, orange, crystalline solid (1.73 g, 98%). The melting point of **9** could not be recorded as it invariably melted during handling at ambient temperature.

IR (Nujol): $\bar{\nu}$ = 1720 (>C=O), 1380, 1365 [$>C(CH_3)_2$], 1320 (>N-Ȯ) cm^{-1}; UV($CHCl_3$): λ_{max} = 244 nm (ε 6824); MS: m/z (rel int) = 170(7.1, M^+), 114(12.0), 83(24.1), 69(7.6), 56(23.5), 44(23.3), 40(100).

Methyl-3α-benzyloxycholan-24-oate (3)

The synthesis of compound **3** from lithocholic acid **1** has already been described[10].

3α-Benzyloxycholan-24-ol (4)

To a stirred solution of the ester **3** (2.98 g, 6.22 mmol) in dry THF (15 mL) was added under nitrogen atmosphere LAH (0.70 g, 18.4 mmol) in small portions. After the addition was complete the mixture was refluxed for 3 h. Next, moist ether (10 mL) was added carefully, followed by addition of water (3 mL). The reaction mixture was filtered, precipitate washed with ethyl acetate. The filtrate was extracted with ether (4 x 20 mL) after saturation with brine and dried over $MgSO_4$. Removal of solvent afforded a colourless viscous product. It was purified by column chromatography on silica gel using 10% ethyl acetate in petroleum ether as eluant to give a colourless semisolid product (2.63 g, 94%).

IR (Neat): $\bar{\nu}$ = 3340 (-OH), 3100, 3000 (aromatic), 1370, 1350 [$>C(CH_3)_2$] cm^{-1}; ^1H NMR($CDCl_3$): δ 7.38-7.23 (m, 5H, Ar-H), 4.55(s, 2H, $-OC\underline{H}_2Ph$), 3.61(t, J = 6.59 Hz, 2H, 24-H_2), 3.40-3.32 (m, 1H, 3β-H), 0.91(d, J = 6.04 Hz, 3H, 21-H_3), 0.90(s, 3H, 19-H_3), 0.64(s, 3H, 18-H_3); MS : m/z (rel int) = 452(35.1, M^+), 435(20.3), 419(11.5), 391(27.0), 361(33.8), 344(95.9), 329(29.7), 290(10.8), 257 (28.4), 230(23.0), 215(86.5), 201(23.0), 161(33.8), 149(31.1), 135(27.0), 121(31.1), 107(50.0), 95(60.8), 91(100).

3α-Benzyloxy-24-bromocholane (5)

To a mixture of the alcohol **4** (1.71 g, 3.8 mmol) and PPh_3 (2.0 g, 7.6 mmol) containing a small amount of K_2CO_3 (0.078 g, 0.57 mmol) in dry ether (15 mL) at 0°C was added portionwise CBr_4 (2.51 g, 7.6 mmol) and the reaction mixture

was stirred at rt for 2 h and finally refluxed for 15 min. The mixture was filtered and the filtrate concentrated to a semisolid residue. The crude product was purified by column chromatography on neutral alumina using 2.5% ethyl acetate in petroleum ether to a thick glassy liquid (1.92 g, 99%) which failed to crystallize.
IR (Neat): $\bar{\nu}$ = 3080, 3070, 3060 (aromatic), 610 (>C-Br) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.37-7.06 (m, 5H, Ar-H), 4.55(s, 2H, $-OC\underline{H}_2Ph$), 3.44-3.31(m, 3H, 3β-H and 24-H_2), 0.91(d, J = 6.56 Hz, 3H, 21-H_3), 0.90(s, 3H, 19-H_3), 0.64(s, 3H, 18-H_3); MS : m/z (rel int) = 516, 514 (both 9.5, M^+), 500(9.5), 465(9.5), 425(20.3), 423(21.6), 408(51.4), 406(51.4), 391(16.2), 257(21.6), 230(16.2), 215(51.4), 161(20.3), 147(21.6), 135(16.9), 121(20.3), 107(35.1), 91(100).

Methyl-(3α-benzyloxy-24-norcholan-23-yl)-triphenylphosphonium bromide (6)

A solution of the bromide **5** (0.5 g, 0.97 mmol) and triphenyl phosphine (0.25g, 0.97 mmol) in dry dimethylformamide (50 mL) was refluxed for 40 h under nitrogen, after which half the solvent was removed by distillation under vacuum. The salt (0.55 g, 74%) which was precipitated by adding dry ether to the DMF solution was filtered off, washed with ether, dried and carefully protected from moisture.

4'-(3α-Benzyloxychol-24-en)-2',2',6',6'-tetramethylpiperidine-1'-oxyl (10)

To a suspension of the salt **6** (0.55 g, 0.72 mmol) in dry ether (10 mL) was added dropwise under nitrogen atmosphere phenyl lithium (prepared according to standard procedure[16] from 0.75 g lithium shavings and 0.55 mL bromobenzene) in ether, when a deep red solution of the ylide **7** was obtained. After stirring for 1 h at room temperature, a solution of tempone **9** (0.12 g, 0.73 mmol in 5 mL ether) was added when colour of the reaction mixture changed to yellow and precipitation was observed. The reaction mixture was stirred at rt overnight, the solvent was evaporated in a stream of dry nitrogen and replaced with dry THF (20 mL). The mixture was refluxed for 5 h, after which the solvent was removed under vacuum. The residue was extracted with petroleum ether (5 x 20 mL), the petroleum ether layer was washed with 9:1 methanol-water (2 x 10 mL), dried over Na_2SO_4 and solvent removed to give a sticky product. The product was chromatographically purified on neutral alumina using 1% ethyl acetate in petroleum ether as eluant to give **10** (0.124 g, 33%) as a yellow crystalline solid.
Mp : 90°C; IR ($CHCl_3$): $\bar{\nu}$ = 3080 (aromatic), 1670, 840 (>C=CH-), 1380, 1360 [$>C(CH_3)_2$] cm^{-1}; UV ($CHCl_3$): λ_{max} = 243.8 nm (ε 6652).
^1H NMR was recorded after *in situ* reduction of the $CDCl_3$ solution of **10** with 1.5 equivalents of $PhNHNH_2$. One drop of D_2O was also added to avoid interference of >NH and $-NH_2$ protons. ^1H NMR ($CDCl_3$): δ 5.2 (t, J = 6.95 Hz, 1H, 24-H), 4.55(s, 2H, $-OC\underline{H}_2Ph$), 3.51-3.36(m, 1H, 3β-H), 1.12(s, 6H, gem methyls), 1.10(s, 6H, gem methyls), 0.92(d, J = 6.3 Hz, 3H, 21-H_3), 0.90(s, 3H, 19-H_3), 0.63(s, 3H,

18-H_3); MS : m/z (rel int) = 589(20.2, M^++1), 588(23.4, M^+), 575(27.6), 574(31.9), 559(40.4), 558(46.8), 499(14.9), 467(14.9), 428(10.6), 255(6.4), 151(17.0), 119(48.9), 118(51.0), 96(76.6), 91(100).

ESR spectrum (10^{-5} M in $CHCl_3$): symmetrical triplet with g_o = 2.00498 and A_o = 15.75 G.

Elemental analysis:

Calculated for $C_{40}H_{62}NO_2$: C 81.57; H 10.61; N 2.38
Found : C 81.61; H 10.56; N 2.29%

3α-Tetrahydropyranyloxycholan-24-ol (12)

The synthesis of compound **12** from lithocholic acid **1** has already been described[10].

3'-(3α-Tetrahydropyranyloxycholan-24-oate)-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (15)

3-Carboxy-2,2,5,5-tetramethylpyrrolidine-N-oxyl **13** (0.17 g, 0.9 mmol) was dissolved in dry tetrahydrofuran (3.5 mL) and a solution of freshly distilled ethyl chloroformate (0.08 mL, 0.9 mmol) and triethylamine (0.14 mL, 1.0 mmol) was added to it. The resultant mixture was stirred for one hour when white precipitation was observed. To this solution, a solution of the protected alcohol **12** (0.26 g, 0.6 mmol) in THF (5.0 mL) was added and the reaction mixture stirred at room temperature for 48 h. The solution was then filtered to remove the precipitated salt and the filtrate concentrated *in vacuo* to give a yellow viscous product. The crude product showed two close spots on TLC. Repeated column chromatographic purification on silica gel using ethyl acetate in petroleum ether as eluant afforded the unreacted mixed anhydride **14** (0.012 g, 5%) and the nitroxide ester **15** as a yellow viscous liquid (0.13 g, 37%).

IR (Neat): $\bar{\nu}$ = 1735 (-OCOR), 1385, 1380 [$>C(CH_3)_2$], 1365 (>N-O·), 1080, 985 (-THP ether) cm^{-1}; UV ($CHCl_3$): λ_{max} 244.6 nm (ϵ 8341); ^1H NMR ($CDCl_3$): δ 4.70-4.69 (m, 1H, -OCHO), 4.16-4.00(m, 2H, 24-H_2), 3.92-3.87(m, 1H, -OC<u>H</u>'H"), 3.60-3.57 (m, 1H, 3β-H), 3.47-3.43(m, 1H, -OCH'<u>H</u>"), 2.80(dd, J = 11.0, 8.24 Hz, 1H, 3'-H), 2.17(dd, J = 13.13, 11.0 Hz, 1H, 4'-<u>H</u>'H"), 1.93-1.68(m, 4'-H'<u>H</u>"), 1.38(s, 3H, gem methyl), 1.25(s, 3H, gem methyl), 1.21(s, 3H, gem methyl), 1.07(s, 3H, gem methyl), 0.88(d, J = 5.65 Hz, 3H, 21-H_3), 0.87(s, 3H, 19-H_3), 0.60(s, 3H, 18-H_3); MS : m/z (rel int) = 615(22.5, M^++1), 585(6.5), 531(15.0), 515(15.0), 499(26.5), 109(13.1), 101(10.2), 95(21.5), 85(100).

ESR spectrum (10^{-5} M in $CHCl_3$) : symmetrical triplet with g_o = 2.00573 and A_o = 14.0G.

Carbethoxyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-carboxylate (14)

IR ($CHCl_3$): 1730 (-$\overset{O}{\overset{\|}{C}}$-O-$\overset{O}{\overset{\|}{C}}$-), 1380, 1370 [>$C(CH_3)_2$], 1360 (>N-$\dot{O}$) cm^{-1}; UV ($CHCl_3$): λ_{max} = 244.4 nm (ε 6530); ^1H NMR ($CDCl_3$): δ 4.24-4.09(m, 2H, -OC$\underline{H}_2$-CH_3), 2.78 (dd, J = 11.0, 8.24 Hz, 1H, 3'-H), 2.16(dd, J = 13.12, 11.0 Hz, 1H, 4'-$\underline{H}$'H"), 1.80(dd, J = 13.12, 8.24 Hz, 1H, 4'-H'$\underline{H}$"), 1.37(s, 3H, gem methyl), 1.26(t, J = 7.02 Hz, 3H, -$OCH_2C\underline{H}_3$), 1.25(s, 3H, gem methyl), 1.20(s, 3H, gem methyl), 1.06 (s, 3H, gem methyl); MS : m/z (rel int) = 228(27.6, M^+-NO), 182(29.2, M^+-NO-OC_2H_5), 118(24.2), 117(23.9), 85(50.2), 71(59.1), 57(100).

ESR spectrum (10^{-5} M in $CHCl_3$) : symmetrical triplet with g_o = 2.0060 and A_o = 14.50G.

3'-(3α-Hydroxycholan-24-oate)-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (16)

The protected proxyl ester **15** (0.145 g, 0.23 mmol) was suspended in methanol (30 mL) followed by addition of p-toluene sulphonic acid (0.04 g, 0.23 mmol). The reaction mixture was stirred at room temperature for 2 h, while the progress was followed by TLC. It was then concentrated in vacuum at a temperature below 40°C. Dilution with CH_2Cl_2 (100 mL), washing successively with 5% $NaHCO_3$, water and brine and drying over anhydrous sodium sulphate followed by evaporation of solvent yielded a yellow product. It was purified on silica gel column using 12% ethyl acetate in petroleum ether to give a yellow crystalline product (0.115 g, 92%). Mp : 157°C; IR (KBr): $\bar{\nu}$ = 3450 (-OH), 1735 (-OCOR), 1380, 1370 [>$C(CH_3)_2$], 1360 (>N-$\dot{O}$) cm^{-1}; UV ($CHCl_3$): λ_{max} = 244.2 nm (ε 4337); ^1H NMR ($CDCl_3$) : δ 4.11-4.00(m, 2H, 24-H_2), 3.66-3.57(m, 1H, 3β-H), 2.76(dd, J = 11.1, 8.1 Hz, 1H, 3'-H), 2.15(dd, J = 12.97, 11.1 Hz, 1H, 4'-$\underline{H}$'H"), 1.98-1.67(m, 4'-H'$\underline{H}$"), 1.35(s, 3H, gem methyl), 1.24(s, 3H, gem methyl), 1.17(s, 3H, gem methyl), 1.04 (s, 3H, gem methyl), 0.92(d, J = 4.6 Hz, 3H, 21-H_3), 0.91(s, 3H, 19-H_3), 0.64 (s, 3H, 18-H_3); MS : m/z (rel int) = 530(100, M^+), 512(13.5), 500(40.5), 482(6.8), 344(14.9), 215(33.8), 175(13.5), 161(16.2), 147(17.6), 121(21.6), 107(33.8), 91(64.9), 81(37.8).

ESR spectrum (10^{-5} M in $CHCl_3$) : symmetrical triplet with g_o = 2.00572 and A_o = 14.80G.

Elemental analysis:

Calculated for $C_{33}H_{56}NO_4$: C 74.67; H 10.63; N 2.63
Found: C 74.04; H 10.20; N 2.39%

3'-(Cholan-3-one-24-oate)-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (17)

A mixture of **16** (0.074 g, 0.14 mmol) and pyridinium chlorochromate (PCC) (0.072 g, 0.33 mmol) was stirred in CH_2Cl_2 (10 mL) at rt for 2.5 h, when TLC

indicated no starting material. CH_2Cl_2 was removed from reaction mixture in vacuum and the residue impregnated in silica gel. The impregnated material was loaded on a silica gel column and purified chromatographically using 17% ethyl acetate in petroleum ether. The pure compound which was obtained as a yellow solid was recrystallized from ethyl acetate (0.051 g, 70%).

Mp : 121°C; IR ($CHCl_3$): $\bar{\nu}$ = 1735 (-OCOR), 1717 (>C=O), 1380, 1370 [$>C(CH_3)_2$], 1350 (>N-O) cm^{-1}; UV ($CHCl_3$): λ_{max} = 245.2 nm (ϵ 2542); ^1H NMR ($CDCl_3$): δ 4.12-4.01(m, 2H, 24-H_2), 2.79(dd, J = 9.0, 8.5 Hz, 1H, 3'-H), 2.21-2.13(m, 1H, 4'-H'H"), 1.98-1.65(m, 1H, 4'-H'H"), 1.38(s, 3H, gem methyl), 1.26(s, 3H, gem methyl), 1.21(s, 3H, gem methyl), 1.07(s, 3H, gem methyl), 0.99(s, 3H, 19-H_3), 0.93(d, J = 6.10 Hz, 3H, 21-H_3), 0.67 (s, 3H, 18-H_3); MS : m/z (rel int) = 528 (100, M^+), 498(17.0), 107(7.8).

ESR spectrum (10^{-5} M in $CHCl_3$) : symmetrical triplet with g_o = 2.00572 and A_o = 14.50G.

Elemental analysis:

Calculated for $C_{33}H_{54}NO_4$: C 74.95; H 10.29; N 2.64

Found : C 75.06; H 10.25; N 2.50%

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