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Synthesis of Potential Pregnenolone and Progesterone Spin Probes for Biomembranes and Immunoassays

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Abstract: The synthesis and characterization of four new steroidal spin labels, *viz.*, 3'-[[(pregn-5-en-3β-ol-20S-yl)methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl,3'-[[(pregn-4-en-3-one-20S-yl)methyl]oate]-2',2',5',5'-tetra methyl pyrrolidine-1'-oxyl, 3"-[(3β-acetoxypregn-5-en-20-one-16α-yl)prop-2'ξ-ol-3'ξ-oate]-2",2",5",5"-tetramethyl pyrrolidine-1"-oxyl and 3"-[(pregn-5-en-3,20-dione-16α-yl)prop-2'ξ-ol-3'ξ-oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl has been described which involves functionalization of the parent hormone at C-20 and C-16. The key step to all the products was the condensation of 3-carboxyproxyl with the derivatized synthons. © 1999 Elsevier Science Ltd. All rights reserved.

In connection with an ongoing project on the synthesis and applications of new steroidal spin probes for biomembranes,[1-4] we herein report the synthesis of nitroxide spin labels of pregnenolones and progesterones derivatized at the C-20 and C-16 positions. Our cardinal interest was to devise a methodology to synthesize spin labeled steroids containing α,β-unsaturated ketone moieties, which are structural features of several hormonal steroids, from commonly and cheaply available starting materials. Substitutions were targeted at C-20 and C-16 since these positions, being remote from the active site of the parent hormone progesterone,[5-9] offered potential sites for attachment of the paramagnetic moiety. The C-20 functionalization has been carried out by introducing a hydroxymethyl moiety at that position on pregnenlone while functionalization at C-16 has been accomplished via 1,4-allylation of 16-dehydropregnenolone-3-acetate (16-DPA).

In order to functionalize C-20 of pregnenolone (**1**), its 3β-hydroxy group was protected by reaction with dihydropyran (Scheme 1). The tetrahydropyranyl ether **2** was then subjected to methylenation at C-20 by Wittig reaction with methyl triphenyl phosphonium iodide using KO*t*Bu/*t*BuOH[10,11] as the base to give the desired product **3** in almost quantitative yields. A weak M+1 peak at m/z 399 in its mass spectrum and the absence of a C=O stretching band in its IR spectrum suggested its formation. A terminal alcohol could now be easily obtained by selective hydroboration of the 20(22) double bond. Midland and Kwon have studied the hydroboration reaction on various 20(22)-ene steroids and have found that an anti-Cram product is predominantly formed in such reactions.[12] Hydroboration of 3-(2'-tetrahydropyranyloxy)pregn-5,20(22)-diene (**3**) was carried out by 9-borabicyclononane (9-BBN) followed by oxidation with H_2O_2/NaOH. The mass

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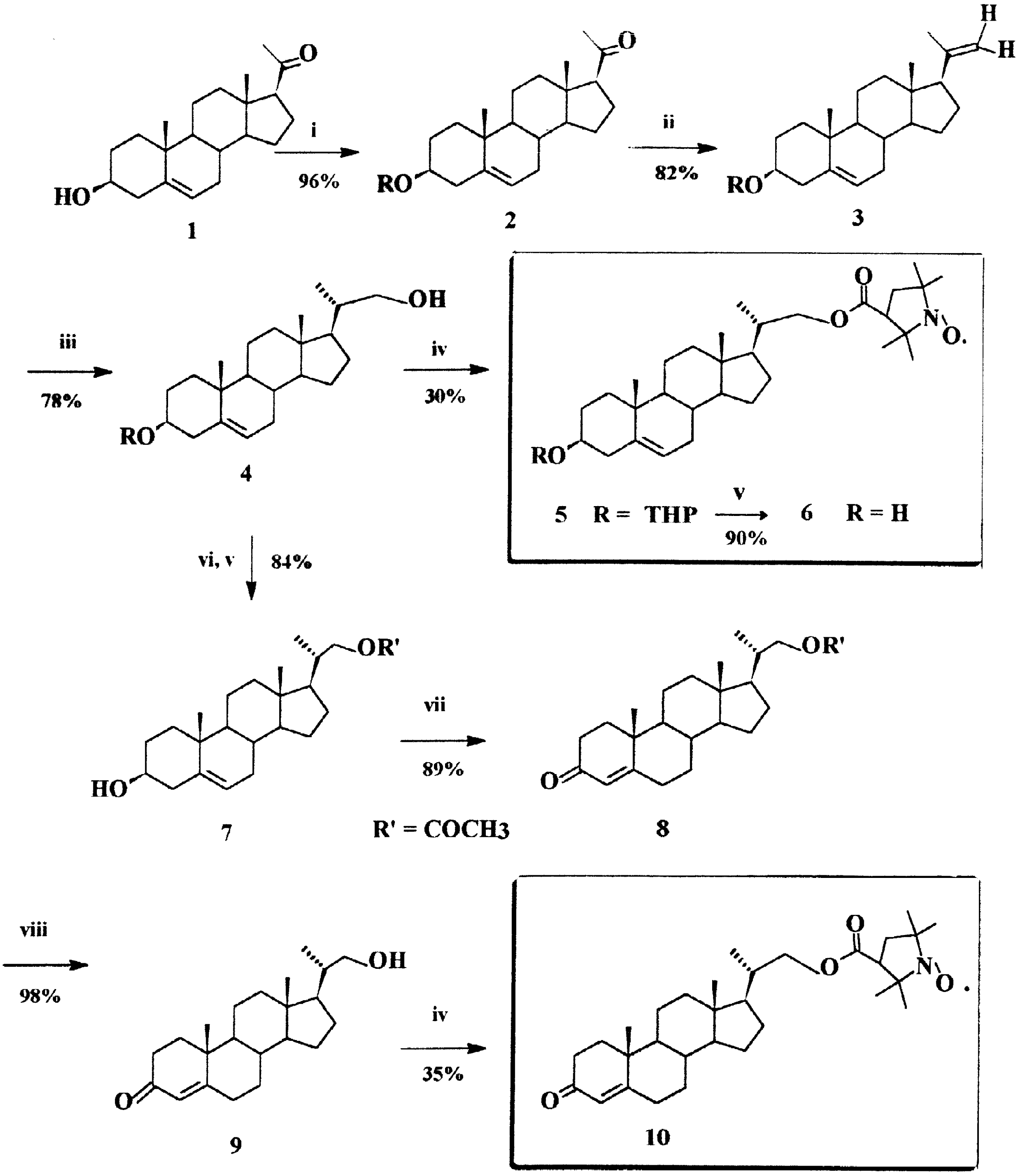
spectrum of the column purified compound 4 did not show any M^+ peak but the M-18 peak, characteristic of a primary alcohol, was observed at m/z 398 as the base peak



i) DHP, p-TSA, dichloromethane; ii) CH_3PPh_3I, KO*t*Bu/*t*BuOH, dry THF; iii) 9-BBN, dry THF, H_2O_2/NaOH, EtOH; iv) DMAP, DCC, 3-carboxyproxyl, dry ether; v) p-TSA, aqueous methanol, vi) acetic anhydride, pyridine; vii) PCC, dichloromethane, viii) 10% aqueous KOH, methanol.

Scheme I

Its IR spectrum also showed the presence of a hydroxy group. A signal integrating to one proton at 3.64 ppm appeared as a double doublet (J=3.3, 10.8 Hz) while another double doublet (J=7.5, 10.8 Hz) was observed at 3.37 ppm in the ^{1}H NMR spectrum. These signals were assigned to the two C-22 protons. Further, the 21-CH_3 appeared as a doublet (J=5.7 Hz) at 1.05 ppm. The C-22 signal at 71.2 ppm in the ^{13}C NMR spectrum also inferred that the compound **4** was the tetrahydropyranyl ether of 20S-(hydroxymethyl)pregn-5-en-3β-ol. The primary alcohol **4** thus obtained was condensed with 3-carboxyproxyl in the presence of DCC and DMAP to give the desired product.

Chromatographic purification of the reaction mixture after filtration, afforded pure nitroxide ester **5** as a yellow viscous product. The IR band at 1736 cm^{-1} indicated the successof the esterification. The presence of the nitroxide moiety was confirmed by the three line ESR spectrum The tentative assignment of the protons in the ^{1}H NMR spectrum was possible after *in situ* reduction of the $CDCl_3$ solution of the nitroxide with 1.5 equivalents of phenyl hydrazine.[13,14] Deshielding of the C-22 protons by ca. 0.3 ppm in the ^{1}H NMR spectrum of the reduced nitroxide **5** indicated formation of an ester. The spectrum also revealed four additional singlets at 1.38, 1.26, 1.20 and 1.07 ppm corresponding to the four gem-methyls of the proxyl moiety, besides the 18 and 19 methyl signals at 0.70 and 1.01 ppm of the parent steroid, thus confirming compound **5** to be 3'-[[[(3β-tetrahydropyranyloxy)pregn-5-en-20S-yl]methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl.

The tetrahydropyranyloxy nitroxide **5** was then subjected to hydrolysis with *p*-toluenesulphonic acid in aqueous methanol to obtain the 3β-hydroxy steroidal proxyl **6** as indicated by its M^+ peak at m/z 501. The IR spectrum of compound **6** showed a characteristic hydroxy signal at 3427 cm^{-1}. The ^{1}H NMR spectrum indicated typical patterns of the proxyl moiety *viz*., four gem-methyl resonances which appeared as singlets at 1.65, 1.57, 1.48 and 1.25 ppm. This confirmed that compound **6** was 3'-[[(pregn-5-en-3β-ol-20S-yl)methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl.

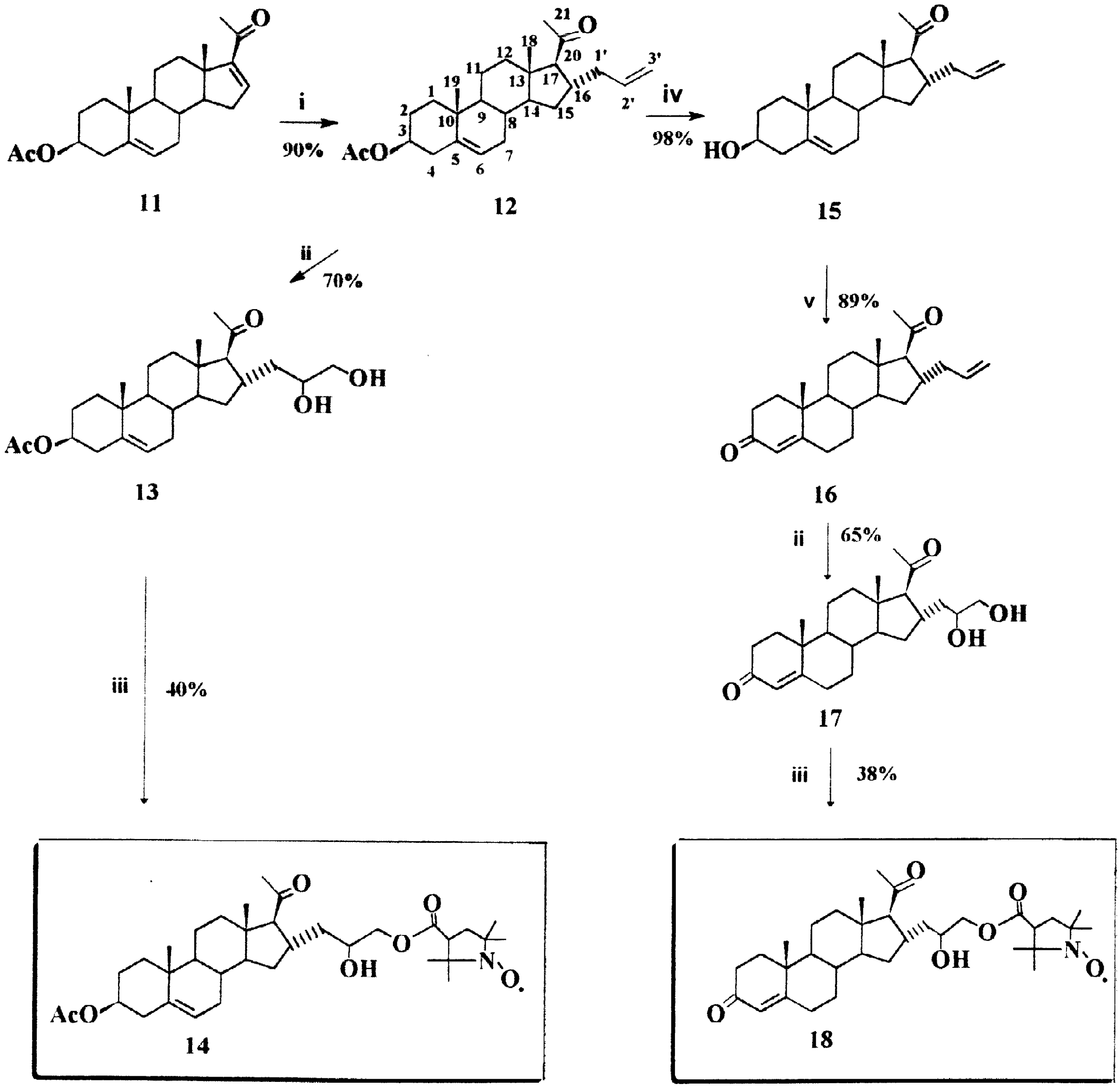
3β-Tetrahydropyranyloxy-20S-(hydroxymethyl)pregn-5-ene (**4**) was also converted into its acetate using 0.5 equivalents of acetic anhydride in pyridine. The reaction was worked up carefully so as to elude an *in situ* generation of a diacetate. The acetylated compound was then subjected to mild acidic conditions with *p*-toluenesulphonic acid to obtain compound 7 which showed the M^+ peak at m/z 374 in its mass spectrum. The IR spectrum indicated the presence of a hydroxy group as well as a carbonyl group. The ^{1}H NMR spectrum revealed that the two double doublets for the C-22 protons were deshielded by ca. 0.3 ppm as compared to their resonances in compound 4 and appeared at 4.07 (J=3.6, 10.8 Hz) and 3.77 ppm (J=7.5, 10.8 Hz). A singlet integrating to three protons was observed at 2.06 ppm and assigned to acetyl-H_3. The ^{13}C NMR spectrum showed a signal for the carbonyl carbon of the acetyl group at 171.5 ppm while its corresponding methyl signal appeared at 21.0 ppm. The signal at 71.8 ppm was assigned to C-3 while that at 69.6 ppm was assigned to C-22. This confirmed the structure of compound 7 to be 20S-(acetoxymethyl)pregn-5-en-3β-ol.

Oxidation of 20S-(acetoxymethyl)pregn-5-en-3β-ol (**7**) with pyridinium chlorochromate (PCC) followed by workup with oxalic acid yielded of 20S-(acetoxymethyl)pregn-4-en-3-one (**8**) as one of the products.[15] Compound **8** was further hydrolyzed by heating to reflux with KOH in methanol. The resultant primary alcohol **9** showed in its mass spectrum, M^+ peak at m/z 330 ($C_{22}H_{34}O_2$). Its IR spectrum indicated the presence of a hydroxy group as well as a conjugated keto group. The ^1H NMR spectrum of the same showed an olefinic signal at 5.73 ppm for 4-H which established the presence of a conjugated ketone while the upfield shift of the resonances of both the protons at C-22 by ca. 0.4 ppm verified the hydrolysis of the acetyl ester. Similarly, the ^{13}C NMR spectrum of compound **9** showed only one carbonyl signal at 199.7 ppm for C-3 while C-22 appeared at 67.9 ppm. These data confirmed that the structure of compound **9** was 20S-(hydroxymethyl)pregn-4-en-3-one. Compound **9** was then condensed with 3-carboxyproxyl in the presence of DCC and DMAP. The resulting product **10** showed a peak at 1736 cm^{-1} in its IR spectrum indicating the presence of the ester carbonyl other than one at 1680 cm^{-1} for the enone moiety. The mass spectrum showed M^+ peak at m/z 499 and ^1H NMR after reduction of the paramagnetic moiety with phenyl hydrazine showed two deshielded double doublets at 4.07 and 3.77 ppm for 22-H_2 which indicated the proxyl ester formation. Singlets at 1.25 (6H), 1.12 and 1.06 ppm for the gem-methyls of the proxyl moiety also confirmed that product **10** was 3'-[[(pregn-4-en-3-one-20S-yl)methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl.

16-Alkylated steroids are biologically important *e.g.* it has been observed that a 16-methyl group prolongs the activity of 16α- as well as 16β-methylprogesterone while 9α-fluoro-16α-methylprednisolone enhances the glucocorticoid activity and completely counteracts the potent salt retaining effect.[16] Hence, functionalisation of ring D was conceived by the introduction of an alkyl group at position 16 of 16-dehydropregnenoloneacetate (16-DPA; **11**) which, being cheap and readily available, was an ideal starting material. Alkylation (alkyl = Me, Et, iPr, etc) at position 16 of a steroid has been achieved by Cu(I) catalysed 1,4-addition, but attempted 1,4-allylations under these conditions resulted exclusively in the 1,2-addition product.[17,18] For conjugate addition of the allyl group to α,β-ethylenic ketones, the best system reported so far is allytrimethylsilane (ATMS)/ $TiCl_4$.[19] Such Sakurai reactions are usually carried out at -79°C. Hence ATMS was reacted with 16-DPA in dry dichloromethane, but no reaction was observed even after several hours. When the reaction was repeated at 0° C, the required 1,4-addition product **12** was obtained in a yield of 20%. At room temperature, 60% yield of the adduct was obtained which increased to 90% on using excess of ATMS (Scheme 2).

The mass spectrum of compound **12** showed a weak M^+ peak at m/z 398 ($C_{26}H_{38}O_3$). The IR spectrum showed signals at 3026 and 1660 cm^{-1} indicating the presence of an olefinic group. A peak at 1709 cm^{-1} showed the presence of a non-conjugated ketone. In its ^1H NMR spectrum, a multiplet integrating to one proton appeared at 5.67 ppm and another integrating to two protons appeared between 4.90 and 5.01 ppm which were assigned to 2'-H and 3'-H_2, respectively. Disappearance of the olefinic 16-H signal observed at 6.93

ppm in the case of the starting material **11** suggested the formation of a 1,4-adduct. The 16β-H signal, in this case, was located at 2.34 ppm as a multiplet.



i. ATMS, TiCl$_4$, dry dichloromethane; ii. AD-mix-β, *t*BuOH, H_2O; iii. DMAP, DCC, 3-carboxyproxyl, dry ether; iv. 10% aqueous KOH, methanol; v. PCC, dichloromethane.

Scheme 2

The ^{13}C NMR spectrum of compound **12** showed a carbonyl signal at 208.9 ppm for C-20 indicating the presence of a non-conjugated ketone. This implied the absence of 16(17) double bond and confirmed formation of the 1,4 adduct. The tertiary carbon signal at 137.4 ppm was assigned to the allylic C-2' whereas the signal at 115.5 ppm was assigned to olefinic methylene C-3'. These spectral data confirmed that compound **12** was 3β-acetoxy-16α-prop-2'-enylpregn-5-en-20-one.

Compound **12** was treated with a highly selective hydroxylation agent AD-mix-β which is known to give a cis diol in excellent enantiomeric excess, the experimental procedure being simple and the catalyst being recoverable.[20] The mass spectrum of compound **13** indicated a M^+ peak at m/z 432 while the IR spectrum showed the presence of hydroxy as well as keto groups. The ^{1}H NMR of compound **13** showed only one olefinic resonance at 5.37 ppm for 6-H. Disappearance of the other olefinic signals and the appearance of deshielded signals at 3.60 (2H) and 3.37 ppm (1H) for 2'-H and 3'-H_2 confirmed that the compound **13** was 3β-acetoxy-16α-prop-2',3'-diol-1'-ylpregn-5-en-20-one. Diol **13** was then treated as usual with 3-carboxyproxyl in the presence of DCC and DMAP. Selective esterification of the primary alcohol yielded 40% of the nitroxide **14**. The IR spectrum of compound **14** [m/z 601 (M^+), $C_{35}H_{54}O_7N$] showed the presence of hydroxy and keto groups. The ^{1}H NMR spectrum of the *in situ* reduced nitroxide showed that the 3'-H_2 signals were deshielded by about 0.6 ppm as compared to those in the starting material **13** and appeared at 4.20 and 4.10 ppm. Besides, four resonances (s) at 1.75, 1.65, 1.35 and 1.25 ppm corresponding to 2',2' 5' and 5' methyl groups of the proxyl moiety confirmed compound **14** to be 3"-[(3β-acetoxypregn-5-en-20-one-16α-yl)prop-2'ξ-ol-3'ξ-oate]-2",2",5",5"-tetramethylpyrrolidine-1"-oxyl.

3β-Acetoxy-16α-prop-2'-enylpregn-5-en-20-one (**12**) was also hydrolyzed under alkaline conditions to obtain the 3β-hydroxy derivative **15** in 98% yield. The M^+ peak at m/z 356 in its mass spectrum indicated that hydrolysis had occurred. The IR spectrum indicated the presence of a hydroxy group and a single carbonyl function. The 3α-H resonance signal in compound **15** was shielded as compared to that in compound **12** and appeared as a multiplet at 3.58 ppm. The ^{13}C NMR spectrum of compound **15** confirmed that it possessed only one carbonyl group resonating at 209.4 ppm. The C-3 signal appeared at 71.5 ppm while three methyl signals appeared at 36.0, 19.3 and 14.1 ppm for C-21, C-19 and C-18, respectively. These data confirmed the structure of compound **15** to be 16α-prop-2'-enylpregnenolone. The major oxidation product from the reaction of the allyl derivative **15** and PCC was compound **16** which, with the 4-H resonance at 5.74 ppm and ^{13}C-3 signal at 199.4 ppm, was characterized as 16α-prop-2'-enylpregn-4-en-3,20-dione.

In a similar manner to compound **12**, the allyl enone **16** was converted to the diol **17** by treatment with AD-mix-β. The M^+ peak at m/z 388 in the mass spectrum and band at 3427 cm^{-1} in the IR spectrum indicated diol formation. The ^{1}H NMR of the diol **17** showed resonances for 4-H at 5.72 ppm indicating that the enone was intact. Additional downfield signals 3.60 and 3.50 ppm were assigned to 2'-H and 3'-H_2 which confirmed that compound **17** was indeed 16α-prop-2',3'-diolylpregn-4-en-3,20-dione. Esterification of diol **17** with 3-carboxyproxyl was carried out in the presence of DCC and DMAP to give the nitroxide **18**. Compound **18** possessed a molecular weight of 557 ($C_{33}H_{50}O_6N$) with the IR spectrum showing the presence of an ester carbonyl, a conjugated carbonyl and a nonconjugated carbonyl group. Deshielding of the 3'-H_2 resonances to 4.22 and 4.11 ppm in the ^{1}H NMR spectrum indicated the formation of the product **18**. Four singlets at 1.64, 1.58, 1.32 and 1.25 were assigned to the gem-methyl groups of the proxyl moiety. These data proved that compound **18** was 3"-[(pregn-5-en-3,20-dione-16α-yl)prop-2'ξ-ol-3'ξ-oate]-2",2",5",5"-tetramethylpyrrolidine-1"-oxyl.

To conclude, spin probes being convenient tools in the study of biomembrane dynamics and spin immunoassays, our efforts have resulted in the synthesis of four new spin labels of progesterone and pregnenolone. Ring D, being at the other terminal of the active site, *i.e.* ring A, was functionalised at C-20 by selective hydroboration with 9-BBN to obtain a terminal alcohol. The other functionalisation was succesfully attempted via Sakurai reaction to yield a stereoselective 16α-allylated 1,4-addition product, which was then hydroxylated by AD-mix-β to obtain a diol. In both the cases, the primary hydroxy group was then converted to the corresponding proxyl esters which have been characterised by NMR and ESR spectroscopy. The applications of these spin labelled compounds in probing certain properties like fluidity, phase transition temperature etc. of membranes are in progress.

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. Pregnenolone and 16-DPA were purchased from Sigma-Aldrich Chemicals and were used after recrystallization. 3-Carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl was purchased from Fluka and was used as such. A Hewlett Packard MS Engine 5989-A spectrometer was used to record the mass spectra. IR spectra were recorded on a Nicolet Impact 400 FT IR spectrophotometer. Elemental analysis was done on a CEST 1106. ^{1}H and ^{13}C 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 while recording ^{1}H NMR spectra while about 15-20 mg of the sample was used for recording ^{13}C NMR spectra. 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 phenylhydrazine. Only relevant NMR assignments have been reported. ESR spectra were recorded at ambient temperature on a 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, the concentrations of the nitroxides being ca. 10^{-5} M.

Tetrahydropyranyl ether of pregnenolone (2)

Pregnenolone (10 g, 31.6 mmol) was dissolved in dichloromethane (150 mL) and dihydropyran (2.65 g, 31.6 mmol) was added along with a catalytic amount of *p*-TSA (0.54 g, 3.2 mmol). The reaction mixture was stirred for 3 h after which the product was extracted with ethyl acetate, washed with 2% NaOH, water, brine and dried (anhydrous Na_2SO_4). Silica gel column chromatography with ethyl acetate/ pet. ether yielded the pure protected pregnenolone **2** as a white solid (12.1 g, 96 %).

m.p.= 118 ^{o}C; MS: m/z 400 (M^+, 1 %), 316 (20), 298 (100), 283 (15); IR(KBr): ν 3050, 3027, 2925, 2750, 1720, 1461, 1379 cm^{-1}; Anal.: Calcd for $C_{26}H_{40}O_3$: C, 77.95; H, 10.06. Found: C, 77.85; H, 10.12; ^{1}H NMR ($CDCl_3$): δ 5.35(bs, 1H, 6-H), 4.72(bs, 1H, 2'-H), 3.92(m, 1H, 6'-H), 3.53(m, 1H, 3α-H), 3.51(m, 1H, 6'-H), 2.12(s, 3H, 21-H_3), 1.01(s, 3H, 19-H_3), 0.63(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 209.4(C-20), 141.1(C-5), 121.2(C-4), 96.9(C-1'), 75.9(C-3), 31.8(C-21), 19.3(C-19), 13.2(C-18).

3β-(Tetrahydropyran-2'-yl)oxypregna-5,20(22)-diene (3)

Dry t-butanol (20 mL) was taken in a dry three necked flask to which potassium (5.85 g, 150 mmol) was added under nitrogen atmosphere. After complete dissolution of potassium, methyl triphenyl phosphonium iodide (53.55 g, 150 mmol) was added along with dry tetrahydrofuran (150 mL). The reaction mixture was allowed to stir for 2 h after which the tetrahydropyranyl ether **2** of pregnenolone (10 g, 25 mmol) was added. The reaction was stirred for 14 h after which ether (450 mL) was added and the precipitate filtered. The ether extract was washed with water, brine and dried over anhydrous Na_2SO_4. After concentrating *in vacuo*, the crude product was subjected to column chromatography using ethyl acetate/ pet. ether as the eluent which afforded the pure olefin **3** as a white solid in 82 % yield (8.2 g).

m.p.= 104 ^{o}C; MS: m/z 399 (M+1, 1 %), 296 (80), 281 (10); IR(KBr): ν 3050, 3027, 2918, 2750, 1640, 1460, 1379 cm^{-1}; Anal.: Calcd for $C_{27}H_{42}O_2$: C, 81.35; H, 10.62. Found: C, 81.32; H, 10.70; ^{1}H NMR ($CDCl_3$): δ 5.36(bs, 1H, 6-H), 4.85(bs, 1H, 22-H), 4.71(bs, 2H, 22-H, 2'-H), 3.91(m, 1H, 6'-H), 3.54(m, 1H, 3α-H), 3.50(m, 1H, 6'-H), 1.76(s, 3H, 21-H_3), 1.01(s, 3H, 19-H_3), 0.58(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 145.7(C-20), 140.1(C-5), 121.4(C-6), 112.7(C-22), 96.9(C-1'), 76.0(C-3), 24.7(C-21), 19.4(C-19), 12.7(C-18).

20S-Hydroxymethyl-3β-(tetrahydropyran-2'-yl)oxypregn-5-ene (4)

Olefin **3** (8 g, 20 mmol) dissolved in dry THF (120 mL) was taken in a three necked flask to which 9-BBN (2.42 g, 20 mmol) in tetrahydrofuran (30 mL) was added under nitrogen. The mixture was stirred for 15 h followed by the addition of ethanol (12 mL), 5N NaOH (10 mL) and 30 % v/v H_2O_2 (20 mL). The reaction mixture was refluxed for 1 h and the mixture was saturated with potassium carbonate and extracted with ethyl acetate. After the usual workup and silica gel column chromatography with ethyl acetate/ pet. ether as the eluent, the primary alcohol **4** was obtained as a white solid in 78 % yield (6.5 g).

m.p.= 140 ^{o}C; MS: m/z 398 (M-18, 10 %), 314 (100), 299 (10), 255 (8); IR(KBr): ν 3530, 3050, 3027, 2918, 2750, 1460, 1380, 1100 cm^{-1}; Anal.:Calcd for $C_{27}H_{44}O_3$. C, 77.84; H, 10.64. Found: C, 77.79; H, 10.72; ^{1}H NMR ($CDCl_3$): δ 5.35(m, 1H, 6-H), 4.72(bs, 1H, 2'-H), 3.92(m, 1H, 6'-H), 3.64(dd, 1H, J=3.3, 10.8 Hz, 22-

H), 3.53(m, 1H, 3α-H), 3.50(m, 1H, 6'-H), 3.37(dd, 1H, J=7.5, 10.8 Hz, 22-H), 1.05(d, 3H, J=5.7 Hz, 21-H_3), 1.01(s, 3H, 19-H_3), 0.70(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 140.9(C-5), 121.3(C-6), 96.8(C-1'), 75.9(C-3), 71.2(C-22), 19.3(C-19), 11.8(C-18).

3'-[[[(3β-tetrahydropyranyloxy)pregn-5-en-20S-yl]methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (5)

Alcohol **4** (1 g, 2.40 mmol) was dissolved in dry ether (20 mL) and to this solution, DMAP (0.293 g, 2.40 mmol), DCC (0.312 g, 2.40 mmol) and 3-carboxyproxyl (0.446 g, 2.4 mmol) were added. The reaction mixture was stirred for 30 h at room temperature, filtered and the filtrate concentrated to give yellow a viscous product. The crude product was then purified over silica gel column using ethyl acetate in pet. ether as eluent to give the desired product **5** as a viscous yellow oil in 30% yield (0.42 g).

MS: m/z 585 ($M^{\cdot}$,2%), 555 (M-30, 10); IR(KBr): ν 3050, 3027, 2925, 2750, 1736, 1461, 1379 cm^{-1}; Anal.: Calcd for $C_{36}H_{58}O_5N$: C, 73.93; H, 10.00; N, 2.39. Found: C, 73.70; H, 10.45; N, 2.67; ^1H NMR ($CDCl_3$): δ 5.35(m, 1H, 6-H), 4.72(bs, 1H, 2"-H), 4.11(dd, J=10.7, 7.8Hz, 22-H), 3.91(m, 1H, 6"-H), 3.81(dd, 1H, J=10.7, 7.8 Hz, 22-H), 3.50 (m, 2H, 3-H, 6"-H), 1.38 (3H, s, gem-methyl), 1.26 (3H, s, gem-methyl), 1.20 (3H, s, gem-methyl), 1.07 (3H, s, gem-methyl), 1.04(d, 3H, J=6.6 Hz, 21-H_3), 1.01(s, 3H, 19-H_3), 0.70(s, 3H, 18-H_3); ESR (10^{-5} M in $CHCl_3$); symmetrical triplet with g_o = 2.0055 and A_o = 14.5 G

3'-[[(pregn-5-en-3β-ol-20S-yl)methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (6)

THP protected proxyl ester **5** (0.2 g, 0.342 mmol) was suspended in methanol (10 mL) followed by the addition of *p*-toluene sulphonic acid (0.002 g, 0.01 mmol). The reaction mixture was stirred at room temperature for 3 h while monitoring the reaction progress by TLC. It was then concentrated in *vacuo* and the residue was extracted with dichloromethane, washed with 5% sodium bicarbonate solution, water and brine and dried over anhydrous sodium sulphate. After solvent removal, the crude product was purified over silica gel using ethyl acetate in pet. ether to elute 90% (0.15 g) of the desired alcohol **6** as a yellow viscous oil.

MS: m/z 501 ($M^{\cdot}$, 8%), 483 (M-18, 15), 471 (25); IR(KBr): ν 3427, 3026, 2973, 2933, 2868, 1730, 1532, 1472, 1380, 1216, 1064 cm^{-1}; Anal.: Calcd for $C_{31}H_{50}O_4N$: C, 74.36; H, 10.06; N, 2.80. Found: C, 74.79; H, 10.32; N, 2.54; ^1H NMR ($CDCl_3$): δ 5.38(m, 1H, 6-H), 4.15(m, 1H, 22-H), 3.86(m, 1H, 22-H), 3.55(m, 1H, 3 α-H), 1.65(s, 3H, gem-methyl), 1.57(s, 3H, gem-methyl), 1.48(s, 3H, gem-methyl), 1.25(s, 3H, gem-methyl), 1.03(d, 3H, J=6.6 Hz, 21-H_3), 1.01(s, 3H, 19-H_3), 0.70(s, 3H, 18-H_3); ESR (10^{-5} M in $CHCl_3$); symmetrical triplet with g_o = 2.0056 and A_o = 14.7 G

20S-(Acetoxymethyl)pregn-5-en-3β-ol (7)

Alcohol **4** (5 g, 12.02 mmol) was stirred with acetic anhydride (1.2 mL) and catalytic amount of pyridine at room temperature for two hours. The product was extracted with ethyl acetate, washed with sodium bicarbonate solution, water and brine and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the crude product obtained was dissolved in methanol (15 mL) and *p*-TSA (0.132 g, 0.75 mmol) was added to it. The reaction mixture was stirred for 2 hours. Methanol was evaporated *in vacuo* and the residue was extracted with ethyl acetate, washed with 5 % sodium bicarbonate solution, water and brine and dried over anhydrous Na_2SO_4. The pure colourless crystalline product **7** was obtained after column chromatography using ethyl acetate and pet.ether as the eluent and was found to be in a yield of 84% (3.77 g).

m.p.= 128 °C; MS: m/z 374 (M^+, 90 %), 356 (75), 341 (50); IR(KBr): ν 3520, 3020, 2927, 2865, 1735, 1675, 1460, 1380, 1230, 1100 cm^{-1}; Anal. Calcd for $C_{24}H_{38}O_3$: C, 76.96; H, 10.23. Found: C, 76.91; H, 10.31; ^1H NMR ($CDCl_3$): δ 5.35(m, 1H, 6-H), 4.07(dd, 1H, J=3.6, 10.8 Hz, 22-H), 3.77(dd, 1H, J=7.5, 10.8 Hz, 22-H), 3.53(m, 1H, 3α-H), 3.50(m, 1H, 6'-H), 2.06(s, 3H, acetyl-H_3), 1.02(d, 3H, J=5.7 Hz, 21-H_3), 1.01(s, 3H, 19-H_3), 0.71(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 171.5(acetyl-CO), 140.8(C-5), 121.2(C-6), 71.8(C-3), 69.6(C-22), 21.0(acetyl-CH_3), 19.4(C-19), 11.9(C-18).

20S-(Acetoxymethyl)pregn-4-en-3-one (8)

Compound 7 (2.4 g, 6.42 mmol) was oxidized using PCC(4.12 g, 19.26 mmol).[15] After purification by silica gel column chromatography using ethyl acetate and pet.ether as the eluent, the 4-en-3-one derivative **8** was obtained as a white solid in a yield of 89 % (2.12 g).

m.p.= 112 °C; MS: m/z 372 (M^+, 10 %), 329 (8), 312 (12), 270 (40); IR(KBr): ν 3039, 2920, 2875, 1736, 1696, 1460, 1383, 1096 cm^{-1}; Anal. Calcd for $C_{24}H_{36}O_3$: C, 77.38; H, 9.74. Found: C, 77.32; H, 9.71; ^1H NMR ($CDCl_3$): δ 5.73(bs, 1H, 4-H), 4.08(dd, J=3.6, 10.8 Hz, 1H, 22H), 3.77(dd, J=7.5, 10.8 Hz, 1H, 22-H), 2.43(ddd, J=5.1, 14.7, 16.8 Hz, 1H, 2β-H), 2.27(ddd, J=3.3, 4.2, 14.7 Hz, 1H, 6α-H), 2.06(s, 3H, acetyl-H_3), 1.19(s, 3H, 19-H_3), 1.02(d, J=6.6 Hz, 3H, 21-H_3), 0.74(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 199.6(C-3), 171.5(C-5, acetyl-CO), 123.8(C-4), 69.4(C-22), 21.0(C-11, acetyl-CH_3), 17.4(C-19), 17.1(C-21), 12.0(C-18).

20S-(Hydroxymethyl)pregn-4-en-3-one (9)

The acetate **8** (1 g, 2.69 mmol) was dissolved in methanol (10 mL) and a 10% solution of KOH in methanol (2 mL) was added. The reaction mixture was stirred for 8 h at room temperature and the solvent was evaporated under reduced pressure. The residue was extracted with ethyl acetate, washed with dilute HCl, water, brine and dried over anhydrous sodium sulfate. The colourless solid primary alcohol **9** obtained after column chromatography using ethyl acetate and pet.ether as the eluent was found to be 98% in yield (0.87 g).

m.p.= 126 °C. MS: m/z 330 (M^+. 80 %), 315 (15). 271 (10); IR(KBr): ν 3447, 3036. 2925, 2875, 1687, 1458, 1383 cm^{-1}; Anal. Calcd for $C_{22}H_{34}O_2$: C, 79.88; H, 10.43. Found: C, 79.95; H, 10.37; ^1H NMR ($CDCl_3$): δ 5.73(bs, 1H, 4-H), 3.63(dd, J=3.3 Hz, 10.8 Hz 22-H), 3.37(dd, 1H, J=6.6 Hz, 10.8 Hz, 22-H), 2.27(ddd, 1H, J= 2.7, 4.5, 14.7 Hz, 6α-H), 1.19(s, 3H, 19-H_3), 1.05(d, 3H, J=6.6 Hz, 21-H_3), 0.74(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 199.7(C-3), 171.6(C-5), 123.8(C-4), 67.9(C-22), 17.4(C-19), 16.7(C-21), 12.0(C-18).

3'-[[(Pregn-4-en-3-one-20S-yl)methyl]oate]-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (10)

Alcohol 9 (0.5 g, 1.515 mmol) was dissolved in dry ether (10 mL) and DMAP (0.185 g, 1.515 mmol), DCC (0.312 g, 1.515 mmol) and 3-carboxyproxyl (0.282 g, 1.515 mmol) were added. The reaction mixture was stirred for 30 h at room temperature, filtered and the filtrate concentrated to give a yellow viscous product. The crude product was then purified over silica gel column using ethyl acetate in pet. ether as eluent to get the desired product as a viscous yellow oil in 35% yield (0.27 g).

MS: m/z 499 (M^+, 4%), 484 (M-15, 12); IR(KBr): ν 3027, 2925, 2875, 1887, 1736, 1680, 1458, 1383 cm^{-1}; Anal.: Calcd for $C_{31}H_{48}O_4N$: C, 74.66; H, 9.71; N, 2.81. Found: C, 75.06; H, 9.28, N, 2.93; ^1H NMR ($CDCl_3$): δ 5.72(bs, 1H, 4-H), 4.07(dd, 1H, J=3.5, 10.7 Hz, 22-H), 3.77(dd, 1H, J=7.5, 10.7 Hz, 22-H), 1.25(s, 6H, gem-methyls), 1.16(s, 3H, 19-H_3), 1.12(s, 3H, gem-methyl), 1.06(s, 6H, gem-methyls), 1.01(d, J=6.6 Hz, 3H, 21- H_3), 0.72(s, 3H, 18-H_3); ESR (10^{-5} M in $CHCl_3$); symmetrical triplet with g_o = 2.0069 and A_o = 14.625 G

3β-Acetoxy-16α-prop-2'-enylpregn-5-en-20-one (12)

$TiCl_4$ (0.13 g, 0.7 mmol) was added dropwise to a stirred solution of 16-DPA (**11**; 2.5 g, 7.0 mmol) in dry dichloromethane (25 mL) at room temperature under nitrogen atmosphere. Allyl trimethyl silane (1.2 g, 10.5 mmol), in dichloromethane (0.5 mL) was then added slowly and the reaction mixture was stirred for 5 h. The reaction was quenched by addition of water (15 mL), extracted with chloroform, washed with water, brine, filtered through celite, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Medium pressure liquid chromatographic separation of the crude with 5 % ethyl acetate in pet. ether as eluent afforded 90% of the 1,4-adduct **12** (2.51 g) as white crystals.

m.p.= 105 °C; MS: m/z 398 (M^+, 3 %), 357 (2), 339 (40); IR(KBr): ν 3026, 2927, 1736, 1709, 1660, 1466, 1387, 1249 cm^{-1}; Anal.: Calcd for $C_{26}H_{38}O_3$: C, 78.35; H, 9.61. Found: C, 78.43; H, 9.67; ^1H NMR ($CDCl_3$): δ 5.67(m, 1H, 2'-H), 5.37(bs, 1H, 6-H), 4.90-5.01(m, 2H, 3'-H_2), 4.61(m, 1H, 3α-H), 2.34(m, 1H, 16β-H), 2.24(d, J=9.0 Hz, 1H, 17α-H), 2.10(s, 3H, 21-H_3), 2.04(s, 3H, acetyl-H_3), 1.01(s, 3H, 19-H_3), 0.65(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 208.9(C-20), 170.3(acetyl-CO), 139.6(C-5), 137.4(C-2'), 122.2(C-6), 115.5(C-3'), 73.7(C-3), 70.4(C-17), 32.0(C-21), 21.3(acetyl-CH_3), 19.2(C-19), 14.0(C-18).

3β-Acetoxy-16α-prop-2',3'-diol-1'ylpregn-5-en-20-one (13)

A 25 mL round bottom flask, equipped with a stirrer was charged with t-butanol (5 mL), water (5 mL) and AD-mix-β (1.4 g). Stirring at room temperature produced two clear phases, the lower aqueous phase was bright yellow. The mixture was cooled to 0°C whereupon some of the dissolved salts precipitated. To this mixture, olefin **12** (0.398 g, 1mmol) was added in one portion and the heterogenous slurry stirred vigorously at 0°C for 1 h followed by stirring at room temperature for 48 h. (The reaction progress was monitored by TLC) The mixture was cooled to 0°C, solid sodium sulfide (1.5 g) was added and the mixture was allowed to warm to room temperature and stirred for an additional 1 h. This was followed by addition of ethyl acetate (10 mL) and after separation of the layers, the aqueous phase was further extracted with ethyl acetate, the combined organic layers dried over anhydrous sodium sulfate and concentrated. The crude product thus obtained was purified over silica gel column using ethyl acetate in pet. ether to obtain 1,2-diol **13** as a white solid in 70% yield (0.3 g).

m.p.=146-147°C; MS: m/z: 432 (M^+,6%), 414 (28), 389 (56); IR(KBr): ν 3440, 3026, 2940, 2861, 1729, 1690, 1249, 1080 cm^{-1}; Anal.: Calcd for $C_{26}H_{40}O_5$: C, 72.17; H, 9.33. Found: C, 71.98; H, 9.67; ^1H NMR ($CDCl_3$): δ 5.37(bs, 1H, 6-H), 4.59(m, 1-H, 3-H), 3.60(m, 2H, 2'-H, 3'-H), 3.37(dd, 1H, J= 11.7, 8.05 Hz, 3'-H), 2.72(m, 1H, 16β-H), 2.04(s, 3H, acetyl H_3), 1.01(s, 3H, 19-H_3), 0.65(s, 3H, 18-H_3); $\delta^{13}C$ NMR ($CDCl_3$) δ 209.0(C-20), 170.6(acetyl CO), 139.7(C-5), 122.2(C-6), 73.8(C-3), 71.59(C-17), 71.49(C-2'), 67.1(C-3'), 31.9(C-21), 21.3(acetyl H_3), 19.1(C-19), 13.9(C-18).

3"-[[(3β-Acetoxypregn-5-en-20-one-16α-yl)prop-2'ξ-ol-3'ξ-oate]-2",2",5",5"-tetramethylpyrrolidine-1"-oxyl (14)

Compound **13** (1 g, 2.3 mmol) was dissolved in dry ether (20 mL) and to this solution, DMAP (0.28 g, 2.3 mmol), DCC (0.48 g, 2.3 mmol) and 3-carboxyproxyl (0.43 g, 2.3 mmol) were added. The reaction mixture was stirred for 28 h at room temperature, filtered and the filtrate concentrated to give a yellow viscous product. The desired product **4** was obtained as a viscous yellow oil (0.55 g, 40%) after purification over silica gel column using ethyl acetate in petroleum ether as eluent.

MS: m/z 601 (M^+, 8%); IR(KBr): ν 3442, 3026, 2940, 1732, 1700, 1564, 1499, 1459, 1367 cm^{-1}; Anal. Calcd for $C_{26}H_{38}O_3$: C, 69.95; H, 9.06; N, 2.33. Found: C, 69.43; H, 9.02; N, 2.36; ^1H NMR ($CDCl_3$): δ 4.20(m, 1H, 3'H), 4.20(m, 1H, 3'H), 3.65(m, 1H, 2'H), 3.51(m, 1H, 3α-H), 3.05(t, 1H, 3"-H), 2.05(s, 3H, acetyl-H_3), 1.75(s, 3H, gem-methyl), 1.65(s, 3H, gem-methyl), 1.35(s, 3H, gem-methyl), 1.25(s, 3H, gem-methyl), 1.16(s, 3H, 19-H_3), 0.65(s, 3H, 18-H_3); ESR (10^{-5} M in $CHCl_3$); symmetrical triplet with g_o = 2.0057 and A_o = 14.75 G

16α-Prop-2'-enylpregnenolone (15)

The acetate **12** (2.5 g, 6.28 mmol) was dissolved in methanol (10 mL) and a 10% solution of KOH in methanol (2 mL) was added. The reaction mixture was stirred for 8 h at room temperature and the solvent was evaporated under reduced pressure. The residue was extracted with ethyl acetate, washed with dilute HCl, water, brine and dried over anhydrous sodium sulfate. The pure primary alcohol **15** was obtained as a white solid (2.19 g, 98%) after column chromatography using ethyl acetate/ pet. ether as the eluent.

m.p.= 112 °C; MS: m/z 356 (20 %), 341(15), 338 (5), 323 (8); IR(KBr): ν 3414, 3026, 2940, 1709, 1660, 1459, 1387 cm^{-1}; Anal. Calcd for $C_{24}H_{36}O_2$: C, 80.85; H, 10.18. Found: C, 80.81; H, 10.11; ^{1}H NMR ($CDCl_3$): δ 5.71(m, 1H, 2'-H), 5.39(bs, 1H, 6-H), 4.96-5.07(m, 2H, 3'-H_2), 3.58(m, 1H, 3α-H), 2.34(m, 1H, 16β-H), 2.24(d, J=9.0 Hz, 1H, 17α-H), 2.10(s, 3H, 21-H_3), 2.04(s, 3H, acetyl-H_3), 1.01(s, 3H, 19-H_3), 0.65(s, 3H, 18-H_3); ^{13}C NMR ($CDCl_3$): δ 209.4(C-20), 140.7(C-5), 137.4(C-2'), 121.2(C-6), 115.5(C-3'), 71.5(C-3), 70.4(C-17), 36.0(C-21), 19.3(C-19), 14.1(C-18).

16α-Prop-2'-enylpregnen-4-en-3,20-dione(16)

Compound **15** (1 g, 2.8 mmol) was dissolved in dry dichloromethane (100 mL). To this, pyridinium chlorochromate (1.8 g, 8.4 mmol) was added and the solution stirred for 1 h. The solvent was then evaporated to half its volume and the slurry was poured onto a silica gel column and eluted with 50% ethyl acetate in petroleum ether. Evaporation of the solvent gave a mixture which on further purification over a silica gel column using ethyl acetate in pet. ether as the eluent gave the 4-en-3-one derivative **16** (0.89 g, 89%).

m.p. = 145 °C; MS: m/z = 354 (M^+, 4%), 313 (80)., IR($CHCl_3$): ν 3032, 2940, 1709, 1681, 1660, 1459, 1387 cm^{-1}; Anal. Calcd for $C_{24}H_{34}O_2$: C, 81.31; H, 9.67. Found: C, 81.40; H, 9.54; ^{1}H NMR ($CDCl_3$): δ 5.74 (bs, 1H, 4-H), 5.66 (m, 1H, 2'-H), 4.95 (m, 2H, 3'-H_2), 2.72 (m, 1H, 16βH), 2.10 (s, 3H, 21-Me), 1.18 (s, 3H, 19-Me), 0.69 (s, 3H, 18-Me); ^{13}C NMR ($CDCl_3$): δ 209.0 (C-20), 199.4 (C-3), 170.9 (C-5), 137.4 (C-2'), 123.9 (C-4), 115.8 (C-3'), 36.1 (C-21), 17.4 (C-19), 14.3 (C-18).

16α-Prop-2',3'-diol-1'-ylpregn-4-en-3,20-dione (17)

Compound **16** (0.354 g, 1 mmol) was treated with AD-mix-β (0.4 g) following the same procedure as that for compound **12** to obtain the desired product **17** as a colourless viscous oil (0.25 g, 65%).

MS: m/z 388 (M^+, 26%), 370 (M-18, 12), 345 (65); IR(KBr): ν 3427, 3026, 1683, 1616, 1429, 1380, 1246, 1080 cm^{-1}; Anal. Calcd for $C_{24}H_{36}O_4$: C, 74.19; H, 9.3. Found: C, 74.86; H, 9.45; ^{1}H NMR ($CDCl_3$): δ 5.72 (bs, 1H, 4-H), 3.60 (m, 1H, 2'-H, 3'-H), 3.40 (m, 1H, 3'-H), 2.83 (m, 1H, 16-H), 2.20 (s, 3H, 21-H_3), 1.22 (s, 3H, 19-H_3), 0.69 (s, 3H, 18-H_3).

3"-[(Pregn-4-en-3,20-dione-16α-yl)prop-2'ξ-ol-3'ξ-oate]-2",2",5",5"-tetramethylpyrrolidine-1"-oxyl (18)

Compound **17** (0.5 g, 1.37 mmol) was condensed with 3-carboxyproxyl (0.26 g, 1.37 mmol) in the presence of DCC (0.28 g, 1.37 mmol) and DMAP (0.17 g, 1.37 mmol) following the same procedure as that for compound **13** to obtain the desired product **18** as a yellow viscous oil (0.29 g, 38%).

MS: m/z 557 (M^+, 4%), 542 (4), 527 (8); IR(KBr): ν 3448, 3023, 2940, 2854, 1736, 1703, 1668, 1525, 1466, 1420, 1367, 1216, 1025 cm^{-1}; Anal. Calcd for $C_{33}H_{50}O_6N$: C, 71.19; H, 9.06; N, 2.52. Found: C, 70.99; H, 9.35; N, 2.84; ^{1}H NMR ($CDCl_3$): δ 5.66(bs, 1H, 4-H), 4.22(m, 1H, 3'-H), 4.11(m, 1H, 3'-H), 3.61(m, 1H, 2'-H), 2.82(m, 1H, 3''-H), 2.16(s, 3H, 21-H_3), 1.64(s, 3H, gem-methyl), 1.58(s, 3H, gem-methyl), 1.32(s, 3H, gem-methyl), 1.25(s, 3H, gem-methyl), 1.18(s, 3H, 19-H_3), 0.69(s, 3H, 18-H_3); ESR (10^{-5} M in $CHCl_3$); symmetrical triplet with g_o = 2.0059 and A_o = 14.75 G

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