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New haptens for progesterone analogs: Synthesis and conformational analysis†

SHILPA S. KORDE AND GIRISH K. TRIVEDI*
Department of Chemistry, Indian Institute of Technology, Powai, Mumbai 400 076, India.
email: chgktia@ether.chem.iitb.ac.in; Phone: 91-22-5767173; Fax: 91-22-5767152.

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Abstract

Growing interest in fields like steroid–receptor interaction and membrane structure studies has promoted the synthesis of several steroidal haptens. The synthesis of four new analogous progesterone haptens, viz. 4-(carboxyethyl) thio-16α-methylprogesterone, 4-(carboxyethyl) thio-19-norethisterone-17-acetate, 20-(hemisuccinyloxymethyl)pregn-4-en-3-one and 6β-hemisuccinyloxy-16α-prop-2'-enylprogesterone is reported herein along with a fluorescent progesterone analog, (20*S*)-[3-[[[(2-oxo-2*H*-1-benzopyran-3-yl)amino]carbonyl]oxy]methyl]pregn-4-en-3-one. Molecular models obtained from MM2 calculations showed that the geometry of the A/B rings was unaltered, hence the new progesterone analogs show potential as haptens for progesterone immunoassay and membrane structure studies.

Keywords: Haptens, steroids, progesterone analogs, carboxyethylthio, hemisuccinyl, fluorescent progesterone analog, molecular modeling, A/B ring conformation.

1. Introduction

Immunoassays have revolutionized the field of medical diagnosis during the past few decades. Since Erlanger *et al.*[1] successfully produced antisteroid antisera, many radioimmunoassays for steroid hormones have been reported with high sensitivity, selectivity and specificity.[2–4] The specificity of an antibody increases when a bridge or a linking group is present between the steroid and the carrier protein. This was pointed out by Midgley and Niswender, and it is generally believed that increase in the number of spacer atoms of the linking group further enhances specificity of the corresponding antibody.[5–7] Although a number of haptens have been synthesized for progesterone,[8–11] little attention has been paid to the synthesis of analogous progesterone haptens.[12] The biological significance of 16-alkylated progesterone is obvious from the fact that 16α-methylprogesterone shows prolonged progestational activity while 9α-fluoro-16α-methylprednisolone enhances the glucocorticoid activity and completely counteracts the potent salt-retaining effect.[13] Similarly, norethisterone is a highly potent synthetic contraceptive and is used in the treatment of various menstrual disorders and fertility problems.[14]

Our group has been engaged in the synthesis of various steroidal labels for their application in immunoassays and membrane studies.[15–18] The present report describes the syntheses of carboxyethyl thioether derivatives of both 16α-methylprogesterone as well as norethisterone. The

†Dedicated to Prof. S. C. Bhattacharyya.
*Author for correspondence

syntheses of two hemisuccinyl haptens, viz. 20*S*-(hemisuccinyloxymethyl) progesterone and 6β-(hemisuccinyloxy-16α-prop-2′-enyl) progesterone have also been discussed.

The application of fluorescence properties to the study of biochemical phenomena has gained importance due to their distinct advantages over the radioanalytical techniques.[19] Synthesis of several steroidal fluorescent probes for their use in fluoroimmunoassays are known.[20–23] Bohme and Kempfle have synthesized 4,6,8(14)-trien-3-one steroids via 3,5,7-trien-3-ol ethers which functioned as probes in studying micellization.[24] We wish to report the synthesis of 20*S*-[3-[[(2-oxo-2H-1-benzopyran-3-yl)amino]carbonyloxy]methyl]pregn-4-en-3-one (**13**), a potential fluorescent probe for fluoroimmunoassay and membrane structure studies and its photophysical data.

In addition, the five haptens have also been modeled for minimised energy structures and their conformations have been analyzed herein.

2. Experimental

Norethisterone acetate (Aldrich) was used after recrystallization. 16α-Methylprogesterone, 6β-hydroxy-16α-prop-2′-enylprogesterone and 20*S*-(hydroxymethyl)pregn-4-en-3-one were synthesized from 16-dehydropregnenolone acetate and pregnenolone according to the reported procedure.[25,26]

Melting points are uncorrected. UV spectra were recorded on Shimadzu UV-260 spectrophotometer, while IR spectra were recorded on a Nicolet Impact-400 FT-IR spectrophotometer. Fluorescence studies were carried out on Spex Fluorolog-01681-0.22 m fluorescence spectrophotometer. A Hewlett-Packard MS Engine 5989-A spectrometer was used to record the mass spectra. ^{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 for recording ^{1}H NMR spectra, while about 15 mg was used in the case of ^{13}C NMR spectra.

The molecular modeling system used was Hyperchem Release 3 for Windows wherein energy minimizations were carried out using MM2 calculations.

2.1. *4,5-Epoxy-16α-methylpregn-3,20-dione (2)*

16α-Methylprogesterone (**1**; 1.5 g, 4.5 mmol) was added to 10% methanolic NaOH (5 mL) in a round bottom flask and cooled to 0°C. 30% H_2O_2 (0.5 ml, 4.5 mmol) was added and the reaction mixture was stirred for 2 h at 0°C. Neutralization was carried out with acetic acid and the product obtained was extracted with ethyl acetate, washed with water, brine and dried over anhydrous Na_2SO_4. Column chromatography of the crude afforded 83% epoxide (0.96 g). MS: (*m/z*) 344 (3%), 329 (3), 301 (10). IR (KBr): ν 3310, 3036, 2947, 2100, 1755, 1712, 1235 cm^{-1}. Analysis: Calc. for $C_{22}H_{32}O_3$: C, 76.70; H, 9.36%. Found: C, 76.73; H, 9.41%. ^{1}H NMR ($CDCl_3$): 4α,5α-isomer δ 2.99 (s, 1H, 4β-H), 2.67 (m, 1H, 16β-H), 2.12 (s, 3H, 21-H$_3$), 1.15 (s, 3H, 19-H$_3$), 0.94 (d, J = 6.9 Hz, 3H, 16-Me), 0.67(s, 3H, 18-H$_3$); 4β,5β-isomer δ 3.05 (s, 1H, 4α-H), 2.67 (m, 1H, 16β-H), 2.13 (s, 3H, 21-H$_3$), 1.15 (s, 3H, 19-H$_3$), 0.96 (d, J = 6.9 Hz, 3H, 16-Me), 0.68 (s, 3H, 18-H$_3$). ^{13}C NMR ($CDCl_3$): δ 214.5 (C-3), 209.2 (C-20), 20.3 (C-19), 14.04 (C-18).

2.2. *4-(Carboxyethyl)thio-16α-methylprogesterone (3)*

Compound **2** (0.75 g, 2.1 mmol) was dissolved in ethyl alcohol (5 ml) to which 10% NaOH (1 ml) was added. 3-mercaptopropanoic acid (0.22 g, 2.1 mmol) was added to the mixture and the reaction mixture stirred overnight. Another equivalent of the acid was added and the reaction mixture stirred for a further 36 h. It was then cooled to 0°C and acidified to pH 4 with dilute HCl. The mixture was then extracted with dichloromethane, washed with water, brine and dried over anhydrous Na_2SO_4. The solvent was removed and the column chromatographic purification of the crude using 5% methanol in benzene yielded 0.21 g (23%) of the pure product as a viscous semisolid. MS: (*m/z*) 432 (M^+, 15%), 387 (2), 327 (4). IR(KBr): ν 3440, 2930, 1720, 1700, 1685, 1464, 1383 cm^{-1}. Analysis: Calc. for $C_{25}H_{36}O_4S$: C, 69.41; S, 7.41; H, 8.39%. Found: C, 69.48, H, 8.30%. ^1H NMR ($CDCl_3$): δ 3.69 (dt, $J = 2.4$, 14.1, 1H, 6α-H), 2.93 (t, $J = 6.9$ Hz, 2H, SCH_2), 2.67 (m, 1H, 16β-H), 2.55 (t, $J = 6.9$ Hz, 2H, CH_2COO), 2.49–2.55 (m, 2H, 2α-H, 2β-H), 2.19 (m, 1H, 6β-H), 2.13 (s, 3H, 21-H_3), 1.22 (s, 3H, 19-H_3), 0.95 (d, $J = 7.0$ Hz, 3H, 16-Me), 0.70 (s, 3H, 18-H_3). ^{13}C NMR ($CDCl_3$): 209.2 (C-20), 195.0 (C-3), 176.7 (COOH), 175.8 (C-5), 128.3 (C-4), 76.6 (C-17), 18.0 (C-19), 14.0 (C-18).

2.3. *4, 5-Epoxy-19-norethisterone-17-acetate (5)*

Norethisterone acetate (**4**; 1 g, 2.94 mmol) was treated with H_2O_2 (0.33 ml, 2.94 mmol) following a procedure similar to that adopted for the synthesis of epoxy derivative of 16α-methylprogesterone. The reaction yielded 74% of 4,5-epoxy-19-norpregn-20-yn-3-one-17-acetate (**5**) as a colourless semisolid. MS: (*m/z*) 356 (M^+, 60%), 341 (65), 313 (20). IR(KBr): ν 3036, 2937, 1712, 1470, 1377 cm^{-1}. Analysis: Calc. for $C_{22}H_{28}O_4$: C, 74.55; H, 7.39%. Found: C, 74.61; H, 7.43%. ^1H NMR ($CDCl_3$): 4α, 5α-isomer δ 3.03 (s, 1H, 4β-H), 2.75 (m, 1H, 16β-H), 2.62 (s, 1H, 21-H), 2.05 (s, 3H, acetyl-H_3), 0.91 (s, 3H, 18-H_3). 4β, 5β-isomer δ 3.04 (s, 1H, 4α-H), 2.75 (m, 1H, 16β-H), 2.62 (s, 1H, 21-H), 2.05 (s, 3H, acetyl-H_3), 0.91 (s, 3H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 206.6 (C-3), 169.4 (acetyl-CO), 67.3 (C-5), 61.7 (C-4), 13.3 (C-18).

2.4. *4-(Carboxyethylthio)-19-norethisterone-17-acetate (6)*

An identical procedure as that used for the preparation of compound **3** was adopted and the mixture of α and β epoxides (0.5g, 1.40 mmol) was reacted with 3-mercaptopropanoic acid (0.15 ml, 1.40 mmol). The pure carboxyethylthio derivative (**6**) was obtained as a viscous solid in a yield of 40% (0.25 g). MS: (*m/z*) 444 (M^+, 15%), 387 (8), 327 (6). IR(KBr): ν 3440, 2930, 1716, 1700, 1685, 1464, 1384 cm^{-1}. Analysis: Calc. for $C_{25}H_{32}O_5S$: C, 67.54, S, 7.21, H, 7.25%. Found: C, 67.55, H, 7.20%. ^1H NMR ($CDCl_3$): δ 3.77 (dt, $J = 2.4$, 13.9 Hz, 1H, 6α-H), 2.94 (t, $J = 6.9$ Hz, 2H, SCH_2), 2.75 (m, 1H, 16β-H), 2.60 (s, 1H, 21-H), 2.56 (t, $J = 6.9$ Hz, 2H, CH_2COO), 2.05 (s, 3H, $COCH_3$), 2.02–2.07 (m, 1H, 6β-H), 0.93 (s, 3H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 195.7 (C-3), 176.2 (COO), 171.7 (COOH), 169.6 (C-5), 127.9 (C-4), 84.2 (C-20), 83.1 (C-17), 75.1 (C-21), 13.4 (C-18).

2.5. *6β-Hemisuccinoyl-16α-prop-2'-enylprogesterone (8)*

Compound **7** (0.25 g, 0.68 mmol) was dissolved in pyridine to which 4-dimethylaminopyridine (0.085 g, 0.68 mmol) and succinic anhydride (0.068 g, 0.68 mmol) were added. The reaction mixture was refluxed overnight, following which ethyl acetate was added. After filtering the

mixture through celite, the product was extracted with ethyl acetate. Column chromatography afforded 68% of the pure hemisuccinate (**8**) as a viscous semisolid. MS: (*m/z*) 470 (1%), 455 (5), 429 (8), 425 (15), 384 (20). IR(KBr): ν 3440, 3026, 2927, 2875 1740, 1720, 1703, 1683, 1660, 1466, 1380, 1245 cm^{-1}. Analysis: Calc. for $C_{28}H_{38}O_6$: C, 71.46; H, 8.14%. Found: C, 71.41; H, 8.19%. ^1H NMR ($CDCl_3$): δ 5.94 (bs, 1 H, 4-H), 5.65 (m, 1 H, 2′-H), 5.45 (bs, 1 H, 6α-H), 4.95 (m, 2 H, 3′-H_2), 2.67–2.74 (m, 5 H, 16β-H, succinyl H_4), 2.26 (m, 1 H, 17α-H), 2.11 (s, 3 H, 21-H_3), 1.29 (s, 3 H, 19-H_3), 0.72 (s, 3 H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 209.1 (C-20), 200.0 (C-3), 176.4 (COO), 170.8 (COOH), 161.9 (C-5), 137.3 (C-2′), 128.9 (C-4), 115.9 (C-3′), 74.4 (C-6), 70.3 (C-17), 29.3 (CH_2COO), 28.7 (CH_2COOH), 18.7 (C-19), 14.3 (C-18).

2.6. *20S-[(Hemisuccinyloxy)methyl]pregn-4-en-3-one (12)*

Compound **11** was dissolved in pyridine (0.06 ml) in a round bottom flask to which succinic anhydride (0.07 g, 0.7 mmol) was added and the mixture stirred for 5 h. Ethyl acetate was then added and the reaction mixture was filtered through celite. The mixture was extracted with ethyl acetate, washed with dilute HCl, water and brine. The organic extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Silica gel column chromatography afforded the pure hemisuccinate (**12**) in 33% yield (0.15 g). m.p. = 128°C. MS: (*m/z*) 430 (M^+, 80%), 312 (100), 297 (20). IR(KBr): ν 3421, 3036, 2918, 1737, 1703, 1681, 1656, 1464, 1383, 1210 cm^{-1}. Analysis: Calc. for $C_{26}H_{38}O_5$: C, 72.53; H, 8.90%. Found: C, 72.57; H, 8.94%. ^1H NMR ($CDCl_3$): δ 5.73 (bs, 1 H, 4-H), 4.12 (dd, J = 10.8, 3.6 Hz, 1 H, 22-H), 3.92 (dd, J = 10.8, 7.5 Hz, 1 H, 22-H), 2.66 (m, 4 H, succinyl H_4), 1.19 (s, 3 H, 19-H_3), 1.01 (d, J = 6.8 Hz, 3 H, 21-H_3), 0.79 (s, 3 H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 199.8 (C-3), 176.2 (COO), 172.3 (COOH), 171.6 (C-5), 123.8 (C-4), 69.8 (C-22), 29.0 (CH_2COO), 28.7 (CH_2COOH), 17.4 (C-19), 17.1 (C-21), 12.0 (C-18).

2.7. *20S-[3-[[[(2-oxo-2H-1-benzopyran-3-yl)amino]carbonyl]oxy]methyl]pregn-4-en-3-one (13)*

Coumarin-3-carboxylic acid (0.028 g, 0.15 mmol), triethylamine (0.03 ml, 0.03 mmol) and diphenylphosphoryl azide (0.08 ml, 0.03 mmol) dissolved in dry THF (5 ml) were taken in a three-necked flask fitted with a reflux condenser under nitrogen atmosphere and stirred for about 15 min. To this was added 20*S*-(hydroxymethyl)pregn-4-en-3-one (**7**; 0.05 g, 0.15 mmol) in dry THF (10 ml) and the reaction mixture refluxed for 48 h. It was then cooled and extracted with ether. The organic layer was washed with 5% $NaHCO_3$ solution followed by water and brine, and dried over Na_2SO_4. The crude product was subjected to silica gel column chromatography followed by recrystallization from methanol–chloroform to yield the pure fluorophore (**13**) (0.04 g). m.p. = 178°C. MS: (*m/z*) = 517 (M^+, 3%), 330 {M-[$C_{10}H_5O_3N$]$^+$, 2%}. IR(KBr): ν 3401, 3035, 2944, 1717, 1675, 1626, 1469, 1220, 1035 cm^{-1}. Analysis: Calc. for $C_{32}H_{39}O_5N$: C, 74.25; N, 2.71; H, 7.59%. Found: C, 74.31; N, 2.76; H, 7.64%. ^1H NMR ($CDCl_3$): δ 8.32 (s, 1 H, 4′-H), 7.56 (s, 1 H, NH), 7.42–7.48 (m, 2 H, 7′-H, 8′-H), 7.30–7.34 (m, 2 H, 5′-H, 6′-H), 5.73 (brs, 1 H, 4-H), 4.23 (dd, J = 10.8, 3.6 Hz, 1 H, 22-H) 3.93 (dd, J = 10.8, 7.5 Hz, 1 H, 22-H), 2.45 (m, 1 H, 2β-H), 2.35 (m, 1 H, 2α-H), 1.18 (s, 3 H, 19-H_3), 1.07 (d, J = 6.7 Hz, 3 H, 21-H_3), 0.78 (s, 3 H, 18-H_3). ^{13}C NMR ($CDCl_3$): δ 199.6 (C-3), 171.4 (C-5), 158.6 (C-2′), 153.6 (OCONH), 149.7 (C-3′), 123.9 (C-4), 71.0 (C-22), 17.5 (C-19), 17.2 (C-21), 12.1 (C-18). UV (Table I).

Table I
Photophysical data of 20*S*-[3-[[[(2-oxo-2H-1-benzopyran-3-yl)amino]carbonyl] oxy]methyl]pregn-4-en-3-one

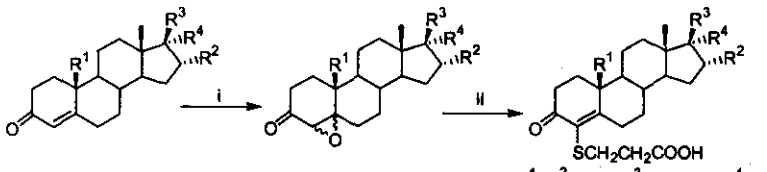
Sl no.	Solvent	λ_{ex}	ε	λ_{em}	ϕ_F
1.	Chloroform	320	4×10^5	383	0.13
2.	THF	318	4×10^5	380	0.132
3.	Acetonitrile	316	4×10^5	383	0.135
4.	Methanol	316	4×10^5	380	0.139

3. Results and discussion

A general procedure to yield carboxyethylthioethers from 4-en-3-one steroidal compound ensues that the enone is subjected to alkaline hydrogen peroxide reaction at 0°C to obtain the epoxide, which on treatment with mercaptopropanoic acid under alkaline conditions followed by dehydration gives the desired thioether derivative.

16α-Methylprogesterone (**1**) was epoxidised by alkaline hydrogen peroxide at 0°C to obtain the 4,5-epoxy derivative (**2**) (Scheme 1). Careful but fast column chromatography of the reaction mixture yielded an inseparable mixture of α and β epoxides (**2**). The mixture as such was subjected to various spectroscopic techniques for structure determination. The mass spectrum of compound **2** showed a weak M^+ peak at *m/z* 344 ($C_{22}H_{32}O_3$). A signal at 3036 cm^{-1} was observed in the IR spectrum which is characteristic of an epoxide, while the band at 1712 cm^{-1} indicated the absence of conjugated ketone. The ^1H NMR spectrum of the mixture of 4α, 5α- and 4β,5β-epoxides (**2**) showed a singlet for 4-H at 3.05 ppm along with another at 2.99 ppm, the integration values of which indicated that the α and β epoxides were formed in the ratio 2:1. Two singlets were observed for 21-H_3, at 2.13 ppm for β-epoxide and 2.12 ppm for α-epoxide while 18-H_3 resonance signal for the α-epoxide appeared at 0.67 ppm and that for β-epoxide at 0.68 ppm. The ^{13}C spectrum showed two peaks at 214.5 ppm for C-3 while C-20 signal was observed at 209.1 ppm. These data confirmed that the structure of compound **2** was 4,5-epoxy-16α-methylpregn-3, 20-dione.

This mixture was then subjected to reaction with 3-mercaptopropanoic acid in ethanolic KOH. Repeated additions of the mercaptopropanoic acid resulted in a carboxyethylthio derivative (**3**). The mass spectrum of compound **3** showed M^+ peak at *m/z* 432 ($C_{25}H_{36}O_4S$). The IR spectrum showed peaks at 3440, 1720, 1700 and 1685 cm^{-1} indicating that a carboxylic group



1 **2** (α:β 2:1) **3** R^1=R^2=CH_3; R^3=$COCH_3$; R^4=H
4 **5** (α:β 1:18) **6** R^1=R^2=H; R^3= C≡CH; R^4=OAc

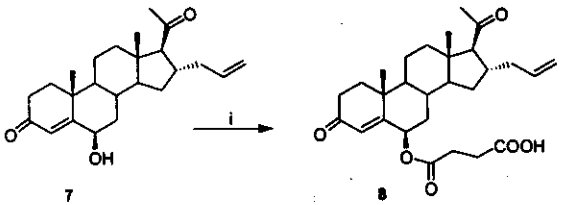
Scheme 1. Synthesis of 4-carboxyethylthio-16α-methylprogesterone (**3**). Conditions: (i) NaOH, H_2O_2/methanol; (ii) KOH, 3-thiopropanoic acid/ethanol.

might be present in addition to two carbonyl groups. The formation of thioether was further suggested by the ^{1}H NMR spectrum which showed a double triplet (J = 2.4, 14.1 Hz) at 3.69 ppm assigned to 6α-H. A triplet (J = 6.9 Hz) at 2.93 ppm was assigned to SCH_2 and another at 2.55 ppm to CH_2COO. The ^{13}C NMR spectrum of compound **3** showed the carboxyl signal at 176.7 ppm. Two additional methylene resonances were found at 34.8 and 28.6 ppm and were assigned to SCH_2 and CH_2COOH, respectively. This confirmed that compound **3** was 4-(carboxyethyl)thio-16α-methylprogesterone.

Similarly, the carboxyethylthio derivative (**6**) of 19-norethisterone-17-acetate (**4**) was prepared via 4,5-epoxy-19-norethisterone-17-acetate (**5**) (Scheme 1). The epoxidation of norethisterone acetate (**4**) with H_2O_2 in alkaline methanol led to compound **5** which showed M^+ peak at *m/z* 356 ($C_{22}H_{28}O_4$) in its mass spectrum. The IR spectrum showed a signal at 3036 cm^{-1} indicating the presence of an epoxide group while the signal at 1712 cm^{-1} indicated the presence of a nonconjugated ketone. The ^{1}H NMR spectrum of compound **5** indicated that it was a mixture of α and β epoxides, the ratio being 1:18. This is observed from the integral values of the singlets at 3.03 and 3.04 ppm for 4α-H and 4β-H, respectively. The ^{13}C NMR spectrum of compound **5** showed C-3 signal at 206.6 ppm, carbonyl signal of the acetyl group at 169.4 ppm while C-18 signal appeared at 13.3 ppm. These data authenticated compound **5** to be 17α-acetoxy-4,5-epoxy-19-norpregn-20-yn-3-one.

The M^+ peak at *m/z* 444 ($C_{25}H_{31}O_5S$) and IR peaks at 3440 and 1716 cm^{-1} of compound **6** obtained from the reaction of 3-mercaptopropanoicacid on the mixture of epoxides **5** indicated the presence of a carboxylic group. The ^{1}H NMR spectrum of compound **6** showed a double triplet (J = 2.4, 13.9 Hz) at 3.77 ppm while two triplets (J = 6.9 Hz) were seen at 2.94 and 2.56 ppm for SCH_2 and CH_2COO which established that the carboxyethylthio derivative (**6**) was indeed formed. The ^{13}C NMR spectrum of compound **6** showed carboxylic carbon resonating at 171.7 ppm, the S-C signal appeared at 34.8 ppm, while the methylene carbon adjacent to the carboxylic group resonated at 28.6 ppm. These data confirmed that the structure of compound **6** was 17α-acetoxy-4-(carboxyethyl)thio-19-norpregn-4-en-20-yn-3-one.

6β-Hydroxy-16α-prop-2′-enylprogesterone (**7**) was obtained during the reaction of PCC with 16α-prop-2′-enylpregnenolone.[25] To obtain its hemisuccinyl derivative **8**, compound **7** was treated with succinic anhydride, the reaction being catalysed by DMAP at reflux (Scheme 2). The IR spectrum indicated that at least three carbonyl groups are present in compound **8**. Its ^{1}H NMR spectrum showed that 6α-H signal was deshielded by ca. 0.9 ppm as compared to that in compound **7** indicating that the hemisuccinyl group is hooked onto the steroid. In the ^{13}C NMR spectrum of compound **8**, four carbonyl signals at 200.0, 209.1, 176.4 and 170.8 ppm

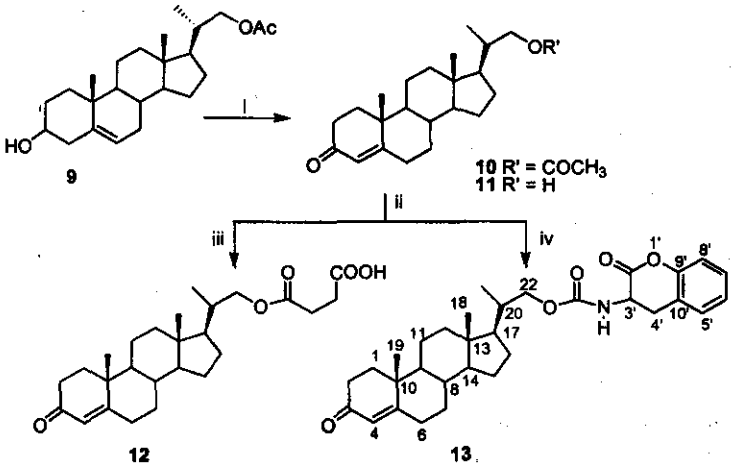


Scheme 2. Synthesis of 6β-hemisuccinoyl-16α-prop-2′-enylprogesterone. (i) Succinic anhydride, dimethylaminopyridine/pyridine.

were assigned to C-3, C-20, ester carbonyl and carboxylic carbon, respectively. Two methylene signals at 29.3 and 28.7 ppm were also seen and assigned to CH_2COO– and CH_2COOH, respectively. These data confirmed that compound **8** is 6β-hemisuccinoyl-16α-prop-2'-enylprogesterone.

To synthesize the second hemisuccinyl hapten, pregnenolone was converted into a tetrahydropyranyl ether and subjected to Wittig reaction. Hydroboration oxidation of the resultant alkene yielded a mono-protected diol which was then protected by acetic anhydride/pyridine and the 3β-hydroxy group was set free by acid hydrolysis to give a secondary alcohol (**9**). Compound **9** was then oxidized by pyridinium chlorochromate to its 4-en-3-one derivative (**10**), which on alkaline hydrolysis with 10% aqueous KOH in methanol gave a primary alcohol (**11**).[26] Compound **11** was then treated with succinic anhydride in pyridine to obtain the hemisuccinyl ester (**12**) in about three hours (Scheme 3). After purification by silica gel column chromatography, the hemisuccinyl derivative (**12**), in its mass spectrum, showed an 80% intense M^+ peak at *m/z* 430 ($C_{26}H_{38}O_5$). Its IR spectrum showed a signal at 3421 cm^{-1} and 1703 cm^{-1} which indicated that formation of the hemisuccinate had taken place. The ^1H NMR spectrum of compound **12** displayed a multiplet (4H) at 2.66 ppm assigned to the four protons of the two methylene groups of the succinyl moiety. The ester formation was also indicated by the deshielding of C-22 protons to 4.12 (J = 10.8, 3.6 Hz) and 3.82 ppm (J = 10.8, 7.5 Hz), which appeared as double doublets. The ^{13}C NMR spectrum of compound **12** showed the presence of three carbonyl signals, one at 199.8 ppm was assigned to C-3, the second at 176.2 ppm to the ester carbonyl while the third at 172.3 ppm was assigned to the carboxylic carbon. The two methylene signals at 29.0 and 28.7 ppm were attributed to the presence of succinyl group. Thus, compound **12** was confirmed to be 20*S*-(hemisuccinyloxymethyl)pregn-4-en-3-one.

Coumarin fluorescence in spirolactone derivatives of testosterone has been utilized to study their affinity for renal mineralocorticoid receptors of adrenalectomized rats. But it was found that long and bulky substitution at C-7 resulted in loss of affinity.[27] It was reasoned that if the



Scheme 3. Synthesis of 20*S*-(hemisuccinyloxymethyl)preg-4-en-3-one (**12**) and 20*S*-[3'-[[[(2'-oxo-2H-1'-benzopyran-3'-yl)amino]carbonyl]oxy]methyl]pregn-4-en-3-one (**13**), Conditions: (i) PCC/dichloromethane, (ii) KOH/aqueous methanol, (iii) Succinic anhydride/pyridine, (iv) Coumarin-3-carboxylic acid, DPPA, triethylamine/anhydrous THF.

coumarin species was introduced on the C-17 side chain, it would stay away from the A/B ring and may result in a fluorescent hapten with better activity. A terminal hydroxy group in the side chain of a steroid could well be utilized for this purpose. Coumarin-3-carboxylic acid was converted *in situ* to an isocyanate using diphenylphosphoryl azide (DPPA) and coupled to the alcohol (**11**) in the presence of triethylamine to give a fluorescent progesterone analog (**13**). The mass spectrum of compound **13** showed an M^+ peak at *m/z* 517 ($C_{32}H_{39}O_5N$) while the IR spectrum showed peaks at 3401, 2944, 1717, 1675, 1626, 1220 and 1035 cm^{-1} thus indicating the presence of an aminocarboxyl group. Aromatic signals were seen in the ^1H NMR spectrum of compound **13** which integrated to five protons. Apart from these, one singlet for NH was also observed. The olefinic signal at 5.73 ppm indicated the presence of 4-en-3-one moiety. The resonances of both the C-22 protons were deshielded by nearly 0.6 ppm as compared to those in compound **11**. The ^{13}C NMR spectrum also showed the required number of aromatic carbon signals in the region 116–130 ppm which indicated that the coumarin group had indeed been added to the steroid. These spectral data confirmed that compound **13** was 20*S*-[3'-[[(2'-oxo-2*H*-1'-benzopyran-3'-yl)amino]carbonyloxy]methyl]pregn-4-en-3-one.

The UV spectra of compound **13** were recorded in various solvents. The fluorescent steroid absorbed at a maximum wavelength of 320 nm and its extinction coefficient was calculated to be 4×10^5. Fluorescence studies were also carried out simultaneously and showed that compound **13** fluoresced with the wavelength maximum being 380 nm and the quantum efficiency of fluorescence being ca. 0.13 (Table I).

Finally, the five haptens, **3**, **6**, **8**, **12** and **13**, were subjected to energy minimizations using MM2 calculations.[28] It was observed that both the carboxyethyl thioethers as well as the hemisuccinyl esters showed similar conformations (Fig. 1). The 16α-methyl group in compound **3** and the 16α-allyl group in compound **8** occupied quasiaxial positions, thus maintaining the ring D conformation as that in the parent steroid hormone, progesterone. Ring A of all the four haptens was seen to exist in a normal 1α, 2β half chair conformation. The bulky carboxyethyl thioether substitutions in compounds affected ring A only by a fraction. Similarly, ring B was found to exist as a normal chair in all the five haptens indicating that even the 6β-hemisuccinyl group in compound **8** occupied an axial position. A slight distortion in ring B of these haptens was caused by Δ^4. In compound **12**, the hemisuccinyl group at C-22 extended away from ring D linearly. Despite the bulk, its side chain at C-17 retained its quasiaxial position and did not cause any distortion of conformation. The coumarin moiety was attached to C-22 via a urethane linkage and was away from the A/B ring system of the steroid skeleton. It was in the same plane as that of the steroid, being slightly tilted towards C-20 and it occupied a quasi-axial position. In the absence of any conformational transmission effects in the five haptens, the A and B rings were unaltered and hence similar in conformation to the parent steroid hormone, progesterone.

4. Conclusion

Potential haptens for analogous progesterone derivatives were synthesized with carboxyethylthio and hemisuccinyl linking groups. The progesterone side chain, elongated with one carbon, was thought to be an ideal position for the substitution of a coumarin moiety and the resultant urethane showed a blue fluorescence. Conformational analysis of these haptens showed that

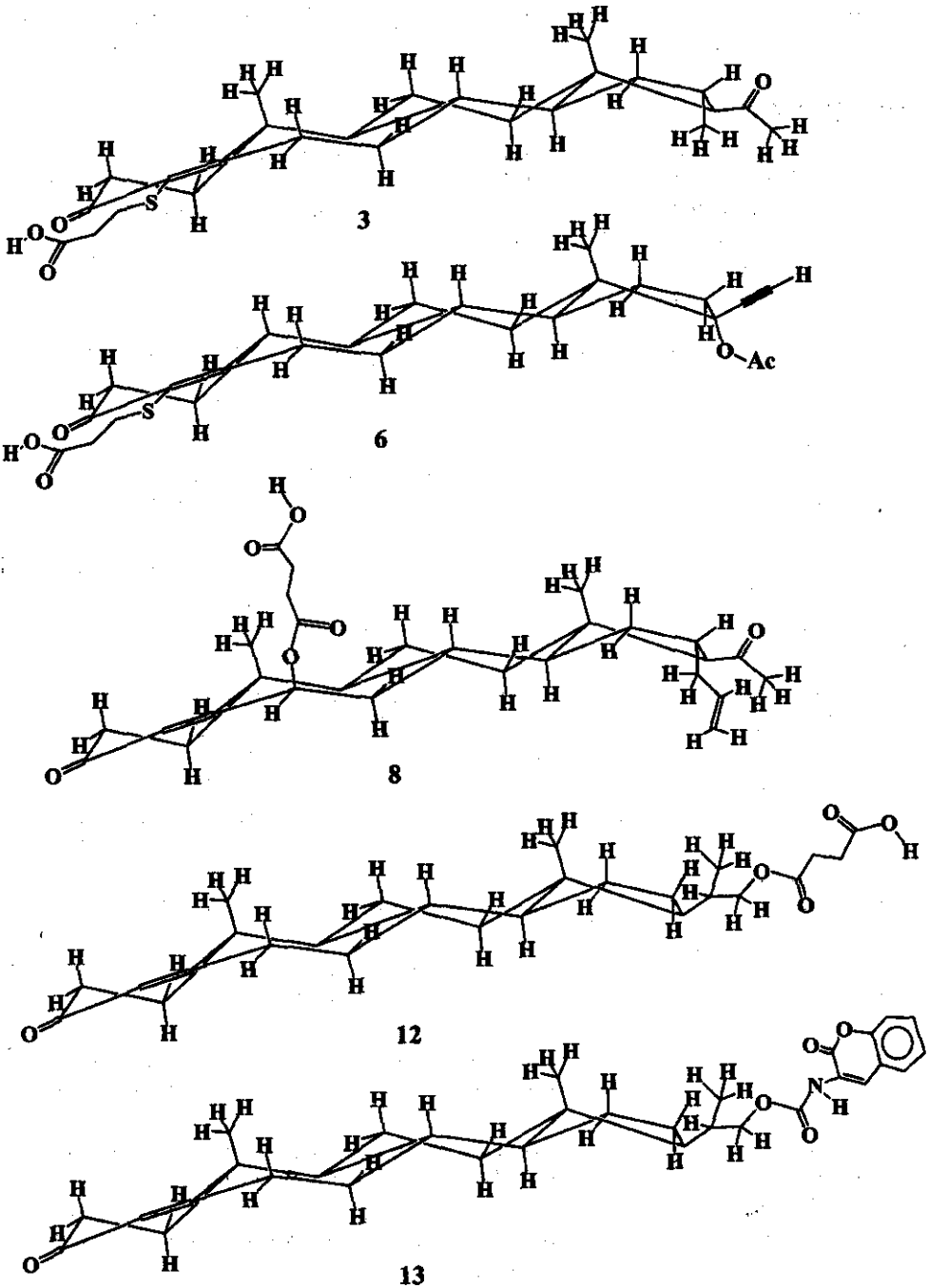


FIG. 1. Energy minimized structures of the five haptens **3**, **6**, **8**, **12** and **13**.

steroidal ring A exhibited a normal half chair while ring B exhibited a normal chair conformation.

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References

1. Erlanger, B. F., Borek, F., Beiser, S. M. and Liebermann, S. — Steroid–protein conjugates I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and cortisone, *J. Biol. Chem.*, 1957, **228**, 713–726.
2. Gupta, D. — *Radioimmunoassay of steroid hormones*, Verlag Chemie, 1980.
3. Lindner, H. R., Perel, E., Freidlander, A. and Zeitlin, A. — Specificity of antibodies to ovarian hormones in relation to the site of attachment of the steroid hapten to the peptide carrier, *Steroids*, 1972, **19**, 357–375.
4. Niswender, G. D. — Influence of the site of conjugation on the specificity of antibodies to progesterone, *Steroids*, 1973, **22**, 413–424.
5. Niswender, G. D. and Midgley, A. R. (jr) — *Immunological methods in steroid determination*, Meredith Corporation, 1970.
6. Cohen, F., Bauminger, S. and Lindner, H. R. — Preparation of antigenic steroid–protein conjugates, Steroid immunoassay, Tenovos Workshop, *5th Cardiff Wales Proc.*, 1974.
7. Pouzar, V. and Cerny, I. — Preparation and properties of 3-(*O*-(2-carboxyethyl))-oxime derivatives of steroid hormones, *Steroids*, 1996, **61**, 89–93.
8. Pratt, J. J. — Steroid immunoassay in clinical chemistry, *Clin. Chem.*, 1978, **24**, 1869–1890.
9. Hochberg, R. B., Hoyle, R. M. and Rosner, W. — *E*-17α-(2-[^{125}I]iodovinyl)-19-nortestosterone: the synthesis of a gamma-emitting ligand for the progesterone receptor, *Endocrinology*, 1985, **117**, 250–254.
10. Hoyte, R. M., Brown, T. J., MacLosky, N. J. and Hochberg, R. B. — 7α-Methyl-E-17α(2-[^{125}I]iodovinyl)-19-nortestosterone: A new radioligand for the detection of androgen receptor, *Steroids*, 1993, **58**, 13–23.
11. Kesner, J. S. *et al.* — Validations of time-resolved fluoroimmunoassays for urinary estrone 3-glucuronide and pregnanediol 3-glucuronide, *Steroids*, 1994, **59**, 205–211.
12. Bakos, T., Lokos, M., Vincze, I. and Volford, J. — Steroids 51. An improved synthesis of ORG2058 and synthesis of [^{3}H]ORG2058, *Steroids*, 1993, **58**, 115–118.
13. Gunthard, H. H., Beriger, E., Engel, C. R. and Heusser, H. — Eine neue vaiante der synthese der biologisch hoch alatiuen gestogens 17α-mehylprogesterone, *Helv. Chim. Acta*, 1952, **35**, 2437–2444.
14. Colton, F. B. — Steroids and "the pill": early research at Searle, *Steroids*, 1992, **57**, 624–630.

15. DESAI, U. R. AND TRIVEDI, G. K. New steroid haptens for radioimmunoassay; Synthesis of steroids with thioether or ester linkage at the 2α-position, *Steroids*, 1991, **56**, 185–188.

16. DESAI, U. R. AND TRIVEDI, G. K. Conformational analysis of ring A and total assignment of 19-functionalised 4-en-3-one steroids. Applicability of 2D NOE as a crucial technique, *J. Org. Chem.*, 1991, **56**, 4625–4629.

17. BANERJEE, S., DESAI, U. R. AND TRIVEDI, G. K. Synthesis of 2′-(3α-benzyloxy-24-norcholan-23-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazolidine-*N*-oxyl–A new potential spin probe for biomembranes, *Tetrahedron*, 1992, **48**, 133–148.

18. BANERJEE, S., TRIVEDI, G. K., SRIVASTAVA, S. AND PHADKE, R. S. 2′-(3α-Benzyloxy-24-norcholan-23-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazoline-N-Oxyl as a potential spin probe for model membranes, *Steroids*, 1994, **59**, 377–382.

19. MUNRO, C. J. AND LASELY, B. L. Nonradiometric immunoassays of steroid hormones, *Prog. Clin. Biol. Res.*, 1988, **285**, 289–329.

20. TEUSCH, G., KLICH, M., BOUCHOUX, F., CEREDE, E. AND PHILBERT, D. Synthesis of a fluorescent steroid derivative with high affinity for the glucocorticoid and progesterone receptors, *Steroids*, 1994, **59**, 22–29.

21. CHEN, H. AND BRUEGGEMEIER, R. W. Synthesis and biochemistry of fluorescent aromatase inhibitors, *Steroids*, 1990, **55**, 123–127.

22. MIKOLA, H., SUNDELL, A. AND HANNINEN, E. Labeling of estradiol and testosterone alkyloxime deivatives with a europium chelate for time resolved fluoroimmunoassays, *Steroids*, 1993, **58**, 330–334.

23. MIKOLA, H. AND HEDLOF, E. Synthesis of europium labeled digoxin derivatives and their time resolved fluoroimmunoassay, *Steroids*, 1994, **59**, 472–478.

24. BOHME, R. M. AND KEMPFLE, M. A. Synthesis of fluorescent 4,6,8(14)-trien-3-one steroids via 3,5,7-trien-3-ol ethers: important probes for steroid–protein interactions, *Steroids*, 1994, **59**, 265–269.

25. KORDE, S. S., UDASI, R. A. AND TRIVEDI, G. K. Oxidation of steroidal 5-en-3β-ol with pyridinium chlorochromate: Isolation of key intermediate, steroidal 6β-hydroxy-4-en-3-one, *Synth. Commun.*, 1997, **27**, 3419–3430.

26. KATOCH, R., KORDE, S. S., DEODHAR, K. D. AND TRIVEDI, G. K. Synthesis of potential pregnenolone and progesterone spin probes for biomembranes and immunoassays, *Tetrahedron*, 1999, **55**, 1741–1754.

27. AUZOU, G., NIQUE, F. AND NEDELAC, L. Synthesis of new fluorescent spirolactone derivatives: determination of their affinities for aldosterone receptors, *Steroids*, 1988, **51**, 465–469.

28 ALLINGER, N. L. Calculations of molecular structure and energy by force field methods, *Adv. Phys. Org. Chem.*, 1976, **13**, 1–82.