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SYNTHESIS OF A PROTEIN CONJUGATE OF 16α-HYDROXYPREGNENOLONE

B. K. PARK and P. D. G. DEAN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, England

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SUMMARY

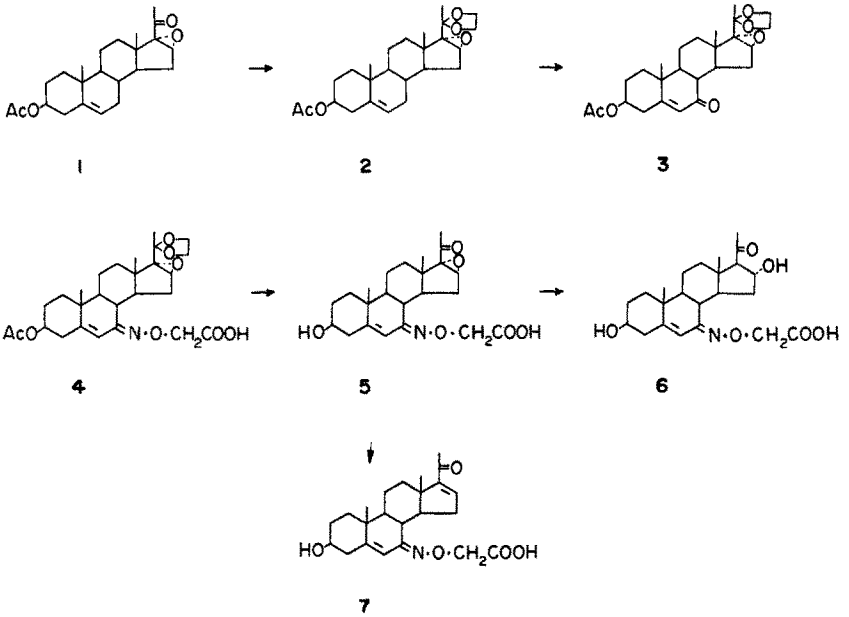
The synthesis of 3β, 16α-dihydroxy-5-pregnene-7,20)-dione 7-(O-carboxymethyl)oxime from 3β-acetoxy-20,20-ethylenedioxy-16α, 17α-oxido-5-pregnene is described. The derivatised steroid was conjugated to bovine serum albumin using the mixed anhydride technique.

DISCUSSION

We are currently developing a series of steroid hormone immunoassays which may be useful for monitoring foetal well-being during late pregnancy [1, 2]. Since, ideally, such assays should reflect the immediate health of the foetus, steroids of foetal origin may be particularly important. One such steroid is 16α-hydroxypregnenolone which is produced in relatively large quantities by the foetal liver [3], but to date it has not been measured in peripheral plasma. In this report we describe the synthesis of an antigen for the steroid as the first part of the development of the immunoassay.

The site of covalent linkage on the steroid nucleus of the immunogenic carrier plays a crucial role in determining the specificity of the antibodies raised against it [4] and highly specific antisera are normally only produced with antigens in which the unique functional groups of the steroid are accessible [5]. We therefore chose C-7 as the point of attachment as models indicated that this should leave both the structurally unique A and D rings free to act as immunogenic determinants. Furthermore, it has been demonstrated that conjugation through this position does not obscure a C_5-C_6 double bond [6].

The synthesis of the antigen is outlined in scheme 1. Epoxypregnenolone acetate (**1**) was chosen as starting material because it contained a functional group (the 16α,17α-epoxide) which could be easily transformed at a later stage into the chemically labile 16α-hydroxyl function. This compound was ketalised using a previously reported procedure [7] and the product (**2**) characterised by spectroscopy. Selective oxidation of the C-7 allylic methylene group in compound (**2**) with chromium trioxidepyridine complex in methylene chloride [7] gave the 7-ketone (**3**). The



Scheme 1.

chemical shift of the C-6 proton in the n.m.r. spectrum was consistent with the position of the ketone [8]. We found that higher yields of the ketone were obtained by addition of the oxidising agent in small portions. Condensation of this compound with carboxymethoxylamine hemihydrochloride in pyridine, gave the 7-(O-carboxymethyl)oxime (**4**) in quantitative yield. The n.m.r. spectrum of this compound was consistent with it being a single isomer since neither of the signals for the C-6 proton nor the methylene protons adjacent to the carboxyl residue showed any unexpected splitting. The ketal and acetoxy protecting groups were then removed by acid and base hydrolysis respectively to give the O-(carboxymethyl)oxime (**5**). The epoxide ring in (**5**) was reduced to a 16α-hydroxyl function with chromous acetate [9] which has been used previously to effect similar stereospecific conversions [10, 11]; the stereochemistry was confirmed by the coupling constant of the 17α-proton in the n.m.r. spectrum. Also formed in the reaction was the 16-dehydropregnenolone derivative (**7**), satisfactory separation of the two products was achieved by fractional crystallisation.

Finally, the 16α-hydroxypregnenolone derivative (**6**) was coupled to bovine serum albumin using the mixed anhydride technique [2].

EXPERIMENTAL

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. Infra-red spectra were determined from Mulls in Nujol on a Unicam SP 200 spectrophotometer. Ultraviolet spectra were determined for solutions in methanol on a Unicam SP 800 spectrophotometer. Nuclear magnetic resonance proton spectra were recorded on a Varian HA 100 spectrometer. Epoxypregnenolone acetate was purchased from Steraloids Inc., General reagents were of Analar grade and obtained from B.D.H. and all solvents were redistilled before use.

3β-Acetoxy-20,20-ethylenedioxy-16α,17α-oxido-5-pregnene (**2**). A mixture of 16α,17α-epoxypregnenolone acetate (10.00 g), redistilled ethylene glycol (10 ml) and benzene (500 ml) was refluxed in a Dean–Stark apparatus for 3 h to remove traces of moisture. After the addition of 4-methylphenylsulphonic acid monohydrate (0.650 g) the solution was refluxed for a further 5 h during which time it darkened considerably. The reaction was then quenched by the addition of solid sodium bicarbonate (10 g). After dilution with ether (500 ml) the reaction mixture was washed with saturated brine (100 ml × 4) and dried (Na_2SO_4). Pyridine was added to the solution which was then concentrated to an oil, which, on trituration with methanol, afforded a white solid which was recrystallised from cyclohexane–pyridine to give 3β-acetoxy-20,20-ethylenedioxy-16α,17α-oxido-5-pregnene as white needles m.p. 195°C (lit. m.p. 195–197°C).

i.r., ν_{max} 1705 cm^{-1} (acetate).

n.m.r. δ, ($CDCl_3$) 1.00 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.42 (3H, s, 21-CH_3) 2.04 (3H, s, $OCOCH_3$), 3.38 (1H, s, 16β-H), 3.94 (4H, s, OCH_2CH_2O) and 5.34 (1H, m, 6-H).

3β-Acetoxy-20,20-ethylenedioxy-16α,17α-oxido-5-pregnen-7-one (**3**). To a well stirred solution of the above ketal (4.2 g) in dichloromethane (distilled from calcium hydride: 250 ml), under an atmosphere of dry nitrogen, was added chromium trioxide–pyridine complex (42 g) in five portions over two days. The reaction mixture was then poured onto ether (100 ml) and the tarry residues washed with ether (100 ml × 7). The combined ether extracts were washed with saturated aqueous sodium bicarbonate (100 ml × 12), dried (Na_2SO_4) and concentrated. Recrystallisation of the residue from ethyl acetate gave 3β-acetoxy-20,20-ethylenedioxy-16α,17α-oxido-5-pregnen-7-one [3] as white prisms (3.52 g) m.p. 222–224°C.

i.r., ν_{max} 1710 (acetate), 1670 (7-ketone) and 1630 cm^{-1} (5-ene-alkene).

u.v., λ_{max} 236 nm (ε 12,500).

n.m.r., δ ($CDCl_3$) 1.00 (3H, s, 18-CH_3), 1.20 (3H, s, 19-CH_3), 1.41 (3H, s, 21-CH_3), 2.05 (3H, s, $OCOCH_3$), 3.39 (1H, s, 16β-H) 3.94 (4H, s, OCH_2CH_2O) and 5.70 (1H, m, 6-H).

Analysis Calculated for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96

Found: C, 69.76; H, 7.78

3β-Acetoxy-20,20-ethylenedioxy-17α-oxido-5-pregnen-7-one 7(O-carboxymethyl)oxime (**4**). A solution of the above ketone (2.50 g) and O-(carboxymethyl)hydroxylamine hemihydrochloride (2.50 g) in anhydrous pyridine (65 ml) was stirred at 40°C for 24 h.

Removal of the pyridine, in a stream of dry nitrogen, left an oil which was partitioned between ether (500 ml) and M hydrochloric acid (100 ml). The ether phase was washed with brine (100 ml), dried (Na_2SO_4) and concentrated. Recrystallisation of the residue from acetone gave 3β-acetoxy-20,20-ethylenedioxy-16α,17α-oxido-5-pregnen-7-one 7(O-carboxymethyl)oxime (3.02 g) as rhombohedra m.p. 214°C.

i.r., ν_{max} 1720 (carboxyl), 1710 (acetate) and 1638 cm^{-1} (C_5-C_6 alkene)

u.v., λ_{max} 242 nm (ε 13,100)

n.m.r., δ (d_6-DMSO) 0.95 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 1.27 (3H, s, 21-CH_3), 2.00 (3H, s, $OCOCH_3$), 3.29 (1H, s, 16β-H), 3.84 (4H, s, OCH_2CH_2O), 4.44 (2H, s, OCH_2CO) and 6.40 (1H, m, 6-H).

Analysis calculated for $C_{27}H_{37}NO_8$: C, 64.39; H, 7.41; N, 2.78

Found: C, 64.19; H, 7.36; N, 2.93

3β-Hydroxy-16α,17α-oxido-5-pregnene-7,20-dione 7-(O-carboxymethyl)oxime (**5**). A solution of the above ketal (2.50 g) and *p*-toluenesulphonic acid monohydrate (0.125 g) in acetone (250 ml) and water (25 ml) was refluxed for 3 h. After removal of the solvent *in vacuo* the crude reaction mixture was dissolved in *t*-butanol (250 ml) and 8M sodium hydroxide (50 ml) and stored, under nitrogen at 30° for 24 h. The *t*-butanol was then removed by rotary evaporation and

the residue diluted with water (500 ml). When the resulting solution was acidified (pH 5) with formic acid it gave a precipitate which was extracted with ether (200 ml × 3). The combined extracts were dried (Na_2SO_4) and concentrated to a solid which on recrystallisation from ethyl acetate gave 3β-hydroxy-16α,17α-oxido-5-pregnene-7,20-dione 7-(O-carboxymethyl)oxime as white needles (1.10 g) m.p. 218°C.

i.r., ν_{max} 3400 (hydroxyl), 1710 (carboxyl), 1700 (20-ketone) and 1640 cm^{-1} (5-ene-alkene)

u.v., λ_{max} 242 nm (ε 13,600)

n.m.r., δ (d_6-DMSO) 1.02 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 2.00 (3H, s, 21-CH_3), 3.59 (1H, s, 16β-H), 4.60 (2H, s, OCH_3CO) and 6.54 (1H, m, 4-H).

Analysis calculated for $C_{23}H_{31}NO_6$: C, 66.16; H, 7.48; N, 3.36

Found: C, 66.19; H, 7.27; N, 3.42.

3β,16α-Dihydroxy-5-pregnene-7,20-dione 7-(O-carboxymethyl)oxime (**6**). To a stirred solution of the foregoing oxime (1.0 g) in acetic acid (55 ml) and water (20 ml), under nitrogen, was added chromous acetate (1.8 g). The resulting deep red solution was stirred at 20°C for 4 h and then poured onto brine (200 ml). The resulting emulsion was extracted with ethyl acetate (200 ml × 3). The combined extracts were washed with M hydrochloric acid (100 ml × 2) and brine (100 ml) then dried (Na_2SO_4) and concentrated. Recrystallisation of the residue from aqueous methanol gave 3β-16α-dihydroxy-5-pregnene-7,20-dione 7-(O-carboxymethyl)oxime (0.41 g) as colourless needles m.p. 225°C.

i.r., ν_{max} 3450 (broad, hydroxyls), 1735 (carboxyl), 1700 (20-ketone) and 1640 $cm.^{-1}$ (5-ene-alkene)

u.v., λ_{max} 241 (ε 13,400)

n.m.r., δ d_6-DMSO) 0.58 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.09 (3H, s, 21-CH_3), 2.32 (1H, d, J = 7 Hz, 17-H), 4.46 (2H, s, OCH_2CO), 4.64 (1H, m, 16-H), and 6.38 (1H, s, 6-H).

Analysis calculated for $C_{23}H_{33}NO_6H_2O$: C, 63.14; H, 8.06; N, 3.20

Found: C, 63.33; H, 8.06; N, 3.36

Slow recrystallisation of the mother liquor gave 3β-hydroxy-pregna-5,16-diene-7,20-dione 7-(O-carboxymethyl)oxime as white prisms (0.15 g) m.p. 217°C.

i.r., ν_{max} 3350 (hydroxyl), 1700 (carboxyl), 1660 (20-ketone) and 1640 $cm.^{-1}$ (alkene).

u.v., λ_{max} 235 and 245 (sh) nm (ε 2240, 21,600)

n.m.r., δ (d_6-DMSO) 0.88 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 8.20 (3H, s, 21-CH_3), 4.49 (2H, s, OCH_2CO), 6.38 (1H, m, 6-H) and 6.85 (1H, m, 16-H).

BSA conjugate of 16α-hydroxypregnenolone. The 16α-hydroxypregnenolone derivative (**6**) was coupled to BSA as described previously [2], using 3 molar equivalents of tri-*n*-butylamine and 2 molar equivalents of isobutylchlorocarbonate. The molar steroid: protein ratio was determined spectrally to be 26.

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