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PREPARATION OF SOME NOVEL STEROID HAPTENS FOR GENERATION OF ANTIBODIES TO TESTOSTERONE AND 5α-DIHYDROTESTOSTERONE

P. NARASIMHA RAO, S. A. SHAIN and L. R. AXELROD

Biological Growth and Development Group, Southwest Foundation for Research and Education, San Antonio, Texas 78284, U.S.A.

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SUMMARY

The syntheses of 6β-hydroxytestosterone 6-hemisuccinate, 17β-hydroxy-5α-androstane-3,6-dione 6-(O-carboxymethyl)oxime, 11β-hydroxy-19-nortestosterone 11-hemisuccinate, and 11β,17β-dihydroxy-5α-oestran-3-one 11-hemisuccinate are described. The compounds were conjugated with bovine serum albumin in aqueous dioxane by a mixed anhydride procedure and the number of moles of hapten incorporated per mole of albumin was determined.

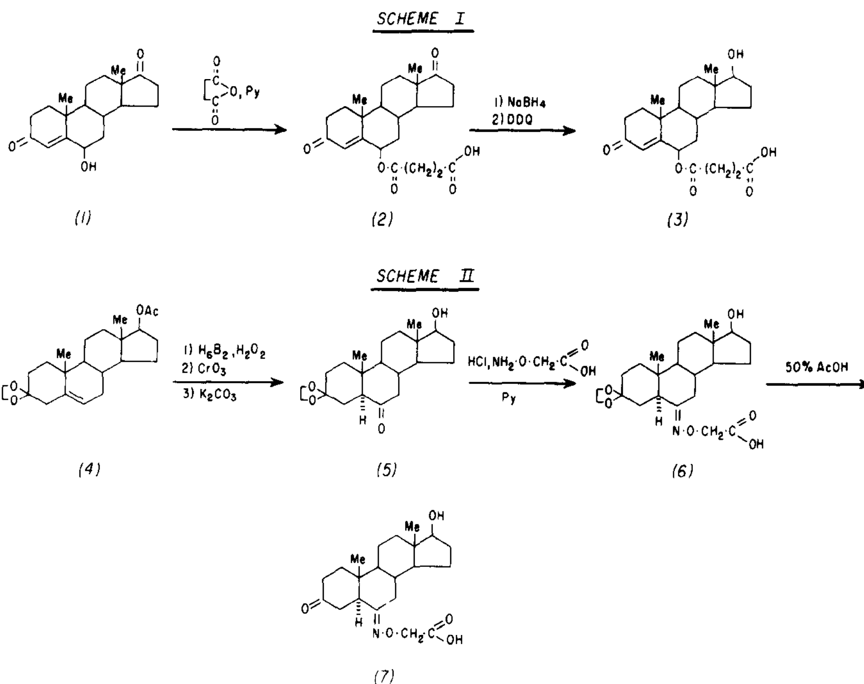
INTRODUCTION

The covalent linkage of steroids to macromolecules for the purpose of producing antibodies which demonstrate specificity towards the steroid hapten was first described by Erlanger *et al.* [1, 2]. The antibodies so generated were later successfully employed for radioimmunoassay of steroids in biological fluids. Initially, steroids were conjugated to the macromolecular carrier through functional groups located in the A and D rings of the steroid nucleus. These procedures resulted in the production of relatively nonspecific antibodies which demonstrated considerable cross-reactivity with structurally similar steroids [3, 4]. In an attempt to improve antibody specificity, steroid haptens were covalently linked to the macromolecular carrier through C-6, C-7 and C-11 positions [4–11], thus leaving both ends of the steroid molecule available for specific recognition by the antibody. These modifications resulted in the production of antibodies demonstrating markedly improved specificity for A and D ring substituents with oestrogens, progesterone, testosterone and androstenedione [4, 5, 8–11]. Furthermore, these investigations demonstrated that moderate specificity with respect to the A–B ring junction could be achieved when the steroid was joined through α-linkage at the C-11 position [4, 8, 11].

Because of our continuing interest in the role of testosterone and 5α-dihydrotestosterone in the regulation of cellular function in male accessory organs of reproduction, we have initiated a program to produce highly specific antibodies for these two androgens. Our approach has been to prepare steroid haptens which could be covalently attached to the carrier protein from the β-side of the steroid nucleus. We reasoned that the preparation of steroid haptens derivatized from the β-side as opposed to the α-side of the steroid nucleus might lead to more specific recognition by the antibody of the conformationally unique 5α configuration of A–B ring junction as opposed to the C_4–C_5-double bond. The preparation of C-6 β-derivatives was achieved with relative ease. However the requirement for β-attachment of the steroid haptens through the C-11 position necessitated selection of 19-nor derivatives of testosterone and dihydrotestosterone for the following reasons: (a) numerous attempts to derivatize the 11β-hydroxyl function in a steroid nucleus containing both C-18 and C-19 methyl groups were unsuccessful; (b) acylation of the 11β-hydroxyl function in the absence of C-19 methyl group was readily accomplished; (c) examination of molecular models suggested that the absence of the C-19 methyl group should not significantly affect the ability of the antibody to recognize the α-side of the steroid nucleus.

In this communication we report the preparation of four antigens which we are presently employing in our attempt to generate antibodies of high specificity.

6β-Hydroxy-4-androstene-3,17-dione(1) was reacted with succinic anhydride in pyridine solution (scheme I) to give the 6β-hemisuccinate derivative (2). Sodium borohydride reduction of (2) followed by oxidation of the allylic hydroxyl function at C-3 with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in *t*-butanol



readily yielded 6β-hydroxytestosterone 6-hemisuccinate (3) in good yield.

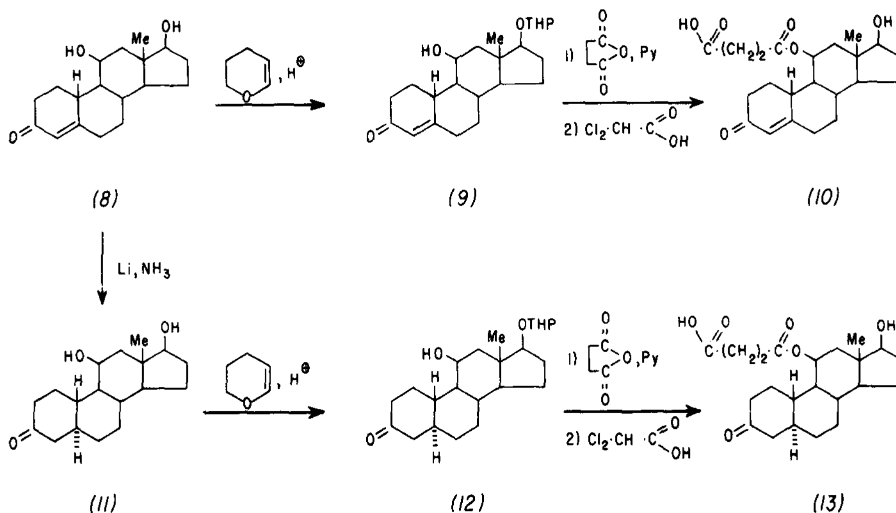
Three,3-ethylenedioxy-5-androsten-17β-ol 17-acetate (4) [12] was subjected to hydroboration [13] (scheme II) and the C-6 hydroxy intermediate so obtained was oxidized with Jones' reagent [14] at 0°C to yield the 6-oxo-17β-acetoxy product. Refluxing the 6-oxo product with potassium carbonate in methanol, resulted in simultaneous hydrolysis of the 17-acetate and isomerization of the 6-ketone to the stable 5α-configuration to yield 3,3-ethylenedioxy-17β-hydroxy-5α-androstan-6-one (5) [15]. Compound (5) was then condensed with carboxymethoxylamine hemihydrochloride in pyridine solution to give the 6-(O-carboxymethyl)oxime derivative (6) which was subsequently reacted with 50% acetic acid to remove the protective 3-ketal group and ultimately yield 17β-hydroxy-5α-androstane-3,6-dione 6-(O-carboxymethyl)oxime (7).

The 17β-hydroxyl function present in 11β-hydroxy-19-nortestosterone (8) [16, 17] was selectively protected by reacting with a limited amount of dihydropyran and p-toluenesulfonic acid in methylene chloride solution (scheme III), to give the 17-tetrahydropyranyl ether (9). The absence of the C-19 methyl group in (9) allowed the succinylation of the 11β-hydroxyl function in (9). The intermediate was subjected to mild acid hydrolysis with 60% dichloroacetic acid to remove the 17-THP ether and yield the 11β-hydroxy-19-nortestosterone 11-hemisuccinate (10).

The conjugated double bond in 11β-hydroxy-19-nortestosterone (8) was stereospecifically reduced with lithium in ammonia to give the 5α intermediate (11). The 17β-hydroxyl group was selectively protected as described above to give the tetrahydropyranyl ether (12). Succinylation of (12) and subsequent hydrolysis with 60% dichloroacetic acid yielded 11β,17β-dihydroxy-5α-oestran-3-one 11-hemisuccinate (13).

EXPERIMENTAL

Melting point determinations were made on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were determined in a potassium bromide pellet on a Perkin–Elmer Model 257 grating spectrometer. Ultraviolet spectra were recorded on a Cary Model 11MS spectrophotometer. n.m.r. spectra were obtained with a Varian A-60A spectrometer in deuterochloroform and are reported in ppm from the internal standard of TMS. Dry column chromatography was performed on Woelm silica gel in nylon columns as described by Loev and Goodman [18]. Woelm neutral aluminum oxide activity III was employed for column chromatography and purification of dioxane. Reagent grade silica gel G used for thin layer chromatography was obtained from E. Merck. Petroleum ether was Mallinckrodt reagent grade and had a boiling range of 30–60°C. The microanalyses were performed by Micro-Tech Laboratories, Skokie, Ill.



The following sequence describes the typical procedure employed in the work-up and isolation of the reaction product. The reaction mixture was treated with ice water and subsequently extracted with the specified organic solvent. The solvent extract was washed with brine, dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated under reduced pressure on a rotary evaporator at 60–65°C. The residue remaining in the flask was then purified as described.

6β-Hydroxy-4-androstene-3,17-dione 6-hemisuccinate (2)

Succinic anhydride (2 g) was added to a solution of 6β-hydroxy-4-androstene-3,17-dione (1, 1 g) in anhydrous pyridine (10 ml) and heated on a steam bath for 5 h. The majority of the pyridine was evaporated under a stream of nitrogen and the product was isolated with chloroform. The crude product was decolorized with charcoal and crystallized from ether to yield 6β-hydroxy-4-androstene-3,17-dione 6-hemisuccinate (2, 0·96 g), m.p. 177–178°C, ν_{max} 3420 (broad), 1735, 1710, 1680, 1655 and 1230 cm^{-1}, λ_{max}^{MeOH} 235 nm (ϵ = 13,855), δ 0·95 (s, 18-Me), 1·3 (s, 19-Me), 2·65 (s, succinate), 5·51 (t, $W_{1/2}$ = 7Hz, 6α-H), 5·96 (s, 4-vinyl H), 9·45 (s, —COOH), (Found: C, 68·51; H, 7·51. $C_{23}H_{30}O_6$ requires C, 68·64; H, 7·51%).

6β,17β-Dihydroxy-4-androsten-3-one 6-hemisuccinate (3)

A solution of 2 (1·0 g) in ethanol (30 ml) was cooled to −20°C and sodium borohydride (0·28 g) in ethanol (30 ml) was added and the mixture was stored at −20°C overnight. Excess sodium borohydride was then decomposed by the addition of glacial acetic acid (0·5 ml) and the reaction product was isolated with chloroform. The infrared spectrum of the crude product revealed that both the C-3 and C-17 keto functions had been reduced. The crude product (0·67 g) was dried by azeotropic distillation with benzene and then dissolved in *t*-butanol (22 ml). A solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1 g) in *t*-butanol was added and the mixture was stored overnight in the dark. The reaction mixture was then applied to a dry column (50 × 720 mm) of silica gel and the column was developed with a solvent mixture consisting of chloroform–ethanol–acetic acid (90:10:0·25). The band between 300–412 mm from the origin was removed and the product was isolated with ethyl acetate. Crystallization from acetone–ether gave 6β,17β-dihydroxy-4-androsten-3-one 6-hemisuccinate (3, 0·19 g), m.p. 157–158°C, ν_{max} 3420, 1745, 1710 (shoulder), 1680, 1610 and 1170 cm^{-1}, λ_{max}^{MeOH} 236 nm (ϵ 13,760), δ 0·83 (s, 18-Me), 1·31 (s, 19-Me), 2·65 (s, succinate), 5·48 (t, $W_{1/2}$ = 7Hz, 6α-H), 5·68 (m, $W_{1/2}$ = 18Hz, 17α-H) and 5·95 (s, 4-H). (Found: C, 68·57; H, 7·81. $C_{23}H_{32}O_6$ requires C, 68·29; H, 7·97%).

3,3-Ethylenedioxy-17β-hydroxy-5α-androstan-6-one (5)

(i) *Hydroboration*. Compound (4) was prepared by the procedure of Liston and Howarth [12]. A solution of 4 (2·5 g) in tetrahydrofuran (50 ml) was cooled to 0°C and maintained under a nitrogen atmosphere. A solution of diborane in tetrahydrofuran (15 ml, 1 M BH_3–THF complex) was added and the mixture was

stirred at room temperature for 1 h. A 10% solution of sodium hydroxide (15 ml) was then added dropwise and the alkaline reaction mixture was brought to 0°C. A solution of 30% hydrogen peroxide (10 ml) was slowly added over a period of 15 min and the resultant solution was stirred for an additional hour after which the reaction product was isolated with ethyl acetate. Infrared analysis of the crude product demonstrated the presence of the C-6 hydroxyl function. Thin-layer chromatography and n.m.r. spectrometry revealed that the crude product consisted of a mixture (*Ca* 1:2) of 17β-acetoxy-3,3-ethylenedioxy-5α-androstan-6α-ol and 17β-acetoxy-3,3-ethylenedioxy-5α-androstan-6β-ol. No attempt was made at this stage to separate the isomers.

(ii) *Oxidation with 8 N chromic acid.* The crude hydroboration product (1·05 g) was dissolved in acetone (25 ml) and the contents were cooled to −5°C. A solution of 8 N chromic acid [14] was added with stirring until the orange color persisted. Excess reagent was destroyed by the addition of a few drops of methanol and solid sodium bicarbonate (3 g) was added to neutralize excess acid. The reaction mixture was filtered through celite and the gummy reaction solids were triturated with ethyl acetate. The acetone and ethyl acetate solutions were combined and concentrated under reduced pressure to yield the oxidation product (1 g) after isolation with ether.

(iii) *Hydrolysis and equilibration with potassium carbonate.* The oxidation product (1 g) was dissolved in methanol (24 ml) and a solution of potassium carbonate (1·13 g) in water (6 ml) was added and the mixture was refluxed for 1·5 h. After removal of the methanol under reduced pressure the crude product (0.9 g) was isolated with ethyl acetate. Crystallization from benzene–petroleum ether yielded 3,3-ethylenedioxy-17β-hydroxy-5α-androstan-6-one (5, 0·64 g), m.p. 187–188°C, ν_{max} 3460, 1710, 1100 cm^{-1}. (Lit. [15] m.p. 188·2–188·7°C.)

3,3-Ethylenedioxy-17β-hydroxy-5α-androstan-6-one 6-O-carboxymethyl)oxime (6)

To a solution of compound 5 (0·53 g) in pyridine (20 ml) was added an aqueous solution (5 ml) of carboxymethoxylamine hemihydrochloride (0·7 g) and the solution was refluxed for 18 h. The reaction product was isolated with ethyl acetate to yield compound 6 (0·5 g) which was further purified by repeated crystallization from ethyl acetate to yield 3,3-ethylenedioxy-17β-hydroxy-5α-androstan-6-one 6-(O-carboxymethyl)oxime, m.p. 214–215°C (dec.), ν_{max} 3340, 1770 (shoulder), 1705, 1620, 1100 cm^{-1}. (Found: C, 66·12; H, 8·43. $C_{23}H_{35}NO_6$ requires C, 65·54; H, 8·37%).

17β-hydroxy-5α-androstane-3,6-dione 6-(O-carboxymethyl)oxime (7)

A solution of 6 (0·64 g) in 80% acetic acid (15 ml) was heated on a steam bath for 1 h after which the solvent was evaporated to dryness under vacuum. Crystallization from ether–petroleum ether yielded 17β-hydroxy-5α-androstane-3,6-dione 6-(O-carboxymethyl)oxime (7, 0·1 g), m.p. 152–155°C, ν_{max} 3420, 1745 (shoulder), 1710, 1640, 760 cm^{-1}. (Found: C, 67·05; H, 8·36. $C_{21}H_{31}NO_5$ requires C, 66·82; H, 8·28%).

11β,17β-Dihydroxy-4-oestren-3-one 11-hemisuccinate (10)

11β,17β-Dihydroxy-4-oestren-3-one (8) was prepared by established procedures [16, 17]. To a solution of 8 (0·38 g) in anhydrous dichloromethane (100 ml) was added dihydropyran (0·25 g) and *p*-toluenesulfonic acid (25 mg) and the solution was stirred at room temperature. The progress of the reaction was monitored by t.l.c. which indicated that the reaction was completed within 1 h. The reaction mixture was washed with sodium bicarbonate solution and the product was isolated with dichloromethane. The material was further purified by chromatography on a column of alumina (30 g) and the fractions eluted with ether and ether–ethyl acetate (1:1) were combined to yield 17β-tetrahydropyranyloxy-11β-hydroxy-4-oestren-3-one (9, 0·29 g) as a gum, ν_{max} 3445, 1670, 1610, 1120 cm^{-1}, δ 0·933 (s, 18-Me), 4·65 (t, $W_{1/2}$ = 9 Hz, 11α-H), 5·85 (s, 4-H). A comparison of the C-18 methyl resonances in the n.m.r. spectrum of the starting material 8, (δ 1·06 ppm) with that of the THP-ether, 9, indicated that the C-17 THP–ether function caused a pronounced shielding effect of about 8 Hz and lent additional evidence for the structure of compound (9).

To a solution of 9 (0·32 g) in pyridine (3 ml) succinic anhydride (0·32 g) was added and the mixture was heated at 100°C for 5 h under a nitrogen atmosphere. The reaction mixture was then evaporated to dryness and the product (0·44 g) isolated with ethyl acetate. The product was dissolved in 60% dichloroacetic acid (10 ml) and subsequently stirred for 1 h at room temperature. Most of the dichloroacetic acid was then evaporated under vacuum and the residue was dissolved in acetone and decolorized with charcoal. Trituration of the residue with ether and crystallization yielded 11β,17β-dihydroxy-4-oestren-3-one 11-hemisuccinate (10, 0·2 g), m.p. 221–223°C, ν_{max} 3500, 1740, 1710, 1650, 1610, 1310 cm^{-1}, λ_{max}^{MeOH} 242 nm (ε 16,090), δ 1·06 (s, 18-Me), 2·66 (s, succinate), 5·36 (t, $W_{1/2}$ = 7 Hz, 11α-H), 5·9 (s, 4-H). (Found: C, 67·34; H, 7·80. $C_{22}H_{30}O_6$ requires C, 67·67; H, 7·74%).

11β,17β-Dihydroxy-5α-oestran-3-one (11)

To a solution of 8 (0·7 g) in tetrahydrofuran (40 ml) and liquid ammonia (200 ml) was added lithium (0·12 g) and the mixture was stirred for 20 min. Sufficient ammonium chloride was then added to discharge the blue color and the reaction product was isolated with ethyl acetate and subjected to chromatography on a column of alumina (21 g). The fractions eluted with benzene were combined to give 11β,17β-dihydroxy-5α-oestran-3-one (11, 0·28 g). Crystallization from acetone yielded compound (11), m.p. 235–236°C, ν_{max} 3440, 1700, 1130 cm^{-1}, δ 1·01 (s, 18-Me), 4·68 (m, $W_{1/2}$ = 18 Hz, 11α-H). (Found: C, 73·90; H, 9·64. $C_{18}H_{28}O_3$ requires C, 73·93; H, 9·56%).

11β,17β-Dihydroxy-5α-oestran-3-one 11-hemisuccinate (13)

To a solution of 11 (0·15 g) in dichloromethane (40 ml) was added dihydropyran (0·2 g) and *p*-toluenesulfonic acid (20 mg) and the reaction was performed as described for the preparation of compound (9). Purification of the reaction product on a column of alumina (20 g) yielded 17β-tetrahydropyranyloxy-11β-hydroxy-5α-oestran-3-one (12, 0·11 g). The I.R: ν_{max} 3445, 1710, 1030 cm^{-1} and n.m.r. δ 0·93 (s, 18-Me) spectra are consistent with the structure assignment. A solution of 12 (0·11 g) in pyridine (2 ml) was reacted with succinic anhydride (0·11 g) as described for the synthesis of compound (10). Isolation of the reaction product and removal of the 17-THP ether with 60% dichloroacetic acid yielded 11β,17β-dihydroxy-5α-oestran-3-one 11-hemisuccinate (13, 80 mg) which was crystallized from ether–petroleum ether to yield (13), m.p. 188–190°C, ν_{max} 3450, 1740, 1710 cm^{-1}, (Found: C, 67·50; H, 8·43. $C_{22}H_{32}O_6$ requires C, 67·32; H, 8·22%).

Preparation of the steroid—Bovine serum albumin conjugate and determination of the mol of steroid bound per mol protein

The steroid–protein conjugates were prepared by a modification of the procedure of Erlanger *et al.* [1]. In a typical preparation 0·5 mmol of the steroid hapten was dissolved in 5 ml anhydrous dioxane and 1·5 mmol of tri-*n*-butylamine was added with mixing. The solution was cooled to 11°C and 1·5 mmol of isobutyl chlorocarbonate was added with vigorous mixing and the solution was maintained at 11°C for 60 min. A weight of bovine serum albumin (BSA), equivalent to 0·6 mmol of available amino groups, was dissolved in 18 ml water and the pH was adjusted to 8·5 at 0°C. To this solution was added an equal volume of dioxane and the apparent pH was maintained at 8·5. The mixed anhydride prepared above was slowly added to the well stirred aqueous dioxane solution of BSA at 0°C. During the addition procedure the apparent pH of the reaction mixture was maintained between 8·3 and 8·5 by the simultaneous addition of 0·3 N NaOH. After the first 30 min the major portion of the reaction was completed as judged by stabilization of the apparent pH. The reaction mixture was stirred at 0°C for an additional 3½ h, the apparent pH was re-adjusted to 7·0 and dialyzed overnight at 2°C against running distilled water.

The dialysate was then made 25% in acetone and the protein conjugate was precipitated by adjusting the apparent pH to 5·0 at 0°C. The precipitate was collected by centrifugation for 10 min at 10,000 *g* at 2°C. The pellet was resuspended in distilled water and the protein was solubilized by adjusting the pH to 7·0 at 0°C. The acetone precipitation procedure was repeated a total of three times. The final precipitate was solubilized as described, dialyzed overnight at 2°C, and lyophilized. The final protein recovery in this procedure was usually 90%. Quantitation of the moles of steroid bound per mole protein was obtained either by ultraviolet absorption spectrometry [1] or by determination of free amino groups in the conjugate by a minor modification of the quantitative ninhydrin procedure [19].

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Table 1. Moles of steroid per mole of conjugate

Steroid	U.V. method [1]	Ninhydrin method [19]
6β,17β-Dihydroxy-4-androsten-3-one 6-hemisuccinate	27	27
17β-Hydroxy-5α-androstane-3,6-dione 6-(O-carboxymethyl)oxime	—	33
11β,17β-Dihydroxy-4-oestren-3-one 11-hemisuccinate	33	36
11β,17β-Dihydroxy-5α-oestran-3-one 11-hemisuccinate	—	27

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