SYNTHESIS OF NEW STEROID HAPTENS FOR RADIOIMMUNOASSAY. PART III. 15β-CARBOXYETHYLMERCAPTOSTEROID-BOVINE SERUM ALBUMIN CONJUGATES. SPECIFIC ANTISERA FOR RADIOIMMUNOASSAY OF 5α-DIHYDROTESTOSTERONE, 5α-ANDROSTANE-3β,17β-DIOL AND 5α-ANDROSTANE-3α,17β-DIOL.

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ABSTRACT

The syntheses of 15β-carboxyethylmercapto-5α-dihydrotestosterone, 15β-carboxyethylmercapto-5α-androstane-3β,17β-diol and 15β-carboxyethylmercapto-5α-androstane-3α,17β-diol and the preparation of their bovine serum albumin (BSA) conjugates are described. These conjugates were employed for the generation of specific antisera suitable for radioimmunoassay (RIA) of 5α-dihydrotestosterone (5α-DHT), 5α-androstane-3β,17β-diol (3β-diol) and 5α-androstane-3α,17β-diol (3α-diol).

INTRODUCTION

It is generally accepted that 5α-dihydrotestosterone (5α-DHT) is the major metabolite in human and animal prostate after testosterone administration. The presence of varying amounts of other 5α-reduced metabolites such as 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol) is also documented. In order to study the role of testosterone in the regulation of cellular function in male accessory organs of reproduction, it is essential to develop accurate and rapid assay procedures for measuring these metabolites. Radioimmunoassay (RIA) methods are far superior to other analytical techniques for micro-quantitation of steroid hormones, and we have therefore initiated a program to develop highly specific antisera for all the important androgen metabolites.

Recently, we have demonstrated (1,2) that highly specific antisera could be generated by coupling testosterone and dehydroepiandrosterone through the C-15 position from the β-side.

In this communication, we report the synthesis of three additional antigens obtained by coupling 5α-DHT, 3β-diol, and 3α-diol through C-15, and the generation of specific antisera useful for RIA.

MATERIALS AND METHODS

Solvents and reagents: The following chemicals were used as furnished by the suppliers: Tri-n-butylamine, isobutyl chloroformate, and Rivanol (6,9-diamino-2-ethoxyacridine lactate, K and K Laboratories, Inc.); Freund's complete adjuvant (Calbiochem); bovine serum albumin Fraction V (Sigma Chemical Co.); carbon decolorizing neutral Norit (Miles Laboratory, Inc.); dextran T-70 (Pharmacia Fine Chemicals); PPO and dimethyl-POPOP (Packard Instrument Co., Inc.); and Bio-Solv BBS-3 (Beckman Instruments, Inc.). Dioxane was purified by passing through a column of Woelm neutral alumina activity III. All solvents were reagent grade and were used without further purification.

All unlabeled steroids were purchased from Steraloids, Inc. Proof of chemical purity was established by infrared spectra, melting point determinations, and thin-layer chromatography. 5α-Dihydrotestosterone-[1,2,4,5,6,7-^{3}H], 107 Ci/mmole, 5α-androstane-3β,17β-diol-[1,2-^{3}H], 51 Ci/mmole and 5α-androstane-3α,17β-diol-[1,2-^{3}H], 44 Ci/mmole were purchased from Amersham Searle Corp. Melting point determinations were made on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were determined in a potassium bromide disc using a Perkin-Elmer Model 257 grating spectrophotometer. Ultraviolet spectra were recorded with a Cary Model 11 MS spectrometer. NMR spectra were obtained with a Varian A-60A spectrometer in deuterochloroform and are reported in ppm from the internal standard of TMS. Mass spectra were obtained with a Finnigan 1015F-L Quadrapole Mass Spectrometer. Dry column chromatography was performed on Woelm silica gel in a nylon column as described by Loev and Goodman (3). The microanalyses were performed by Micro-Tech Laboratories, Skokie, Illinois.

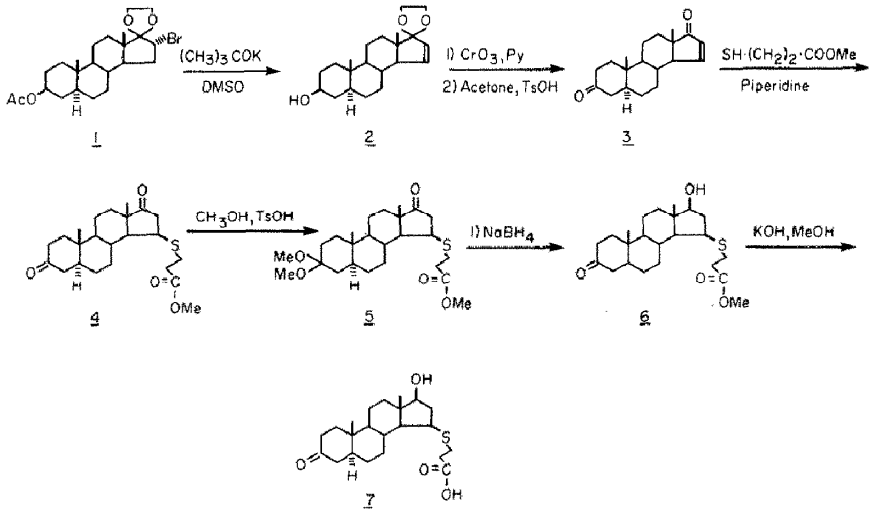
Preparation of immunogens

15β-Carboxyethylmercapto-5α-dihydrotestosterone 7 was prepared as indicated in scheme I. 17,17-Ethylenedioxy-5α-androst-15-en-3β-ol 2 was obtained using a modification of the method of Marquet *et al.* (4) in which 17,17-ethylenedioxy-16α-bromo-5α-androstan-3β-ol acetate 1 was subjected to dehydrobromination with potassium *t*-butoxide in dimethylsulfoxide at 40°C to give 2. The 3β-hydroxy group in 2 was oxidized to a 3-ketone with chromium trioxide-pyridine reagent (5) and then the 17-ethylene ketal group was removed by reaction with p-toluenesulfonic acid in aqueous acetone solution to give the Δ^{15}-3,17-dione 3. The conjugate ketone 3 readily reacted with methyl-3-mercaptopropionate in the presence of piperidine to yield 3,17-dioxo-5α-androstane-15β-yl 2′-methoxycarbonylethyl sulfide 4. The 3-oxo group in 4 was protected as dimethyl ketal by stirring in methanol in the presence of a trace of p-toluenesulfonic acid to give the 3,3′-dimethoxy derivative 5. Sodium borohydride reduction of compound 5 gave the 17β-hydroxy derivative 6 which on subsequent hydrolysis with potassium hydroxide led to the desired 15β-carboxyethylmercapto derivative 7.

15β-Carboxyethylmercapto-5α-androstane-3β,17β-diol 11 was obtained as outlined in scheme II. 3β-Hydroxy-5α-androst-15-en-17-one 8, described earlier by Sondheimer *et al.* (6), was obtained by hydrolysis of the ketal 2 with p-toluenesulfonic acid in aqueous acetone. Reacting 8 with methyl-3-mercaptopropionate in the presence of piperidine led to 3β-hydroxy-17-oxo-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide 9. The 17-oxo group in 9 was reduced with sodium borohydride to give the 3β,17β-diol 10 which was then hydrolyzed with potassium hydroxide in methanol to give 15β-carboxyethylmercapto derivative 11.

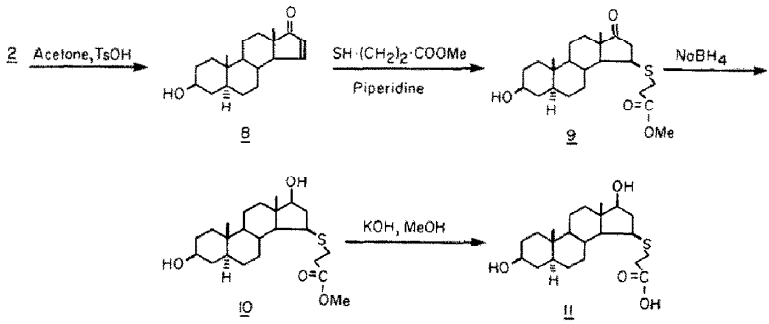
The synthesis of 15β-carboxyethylmercapto-5α-androstane-3α,17β-diol 19 was achieved as described in scheme III.

SCHEME I



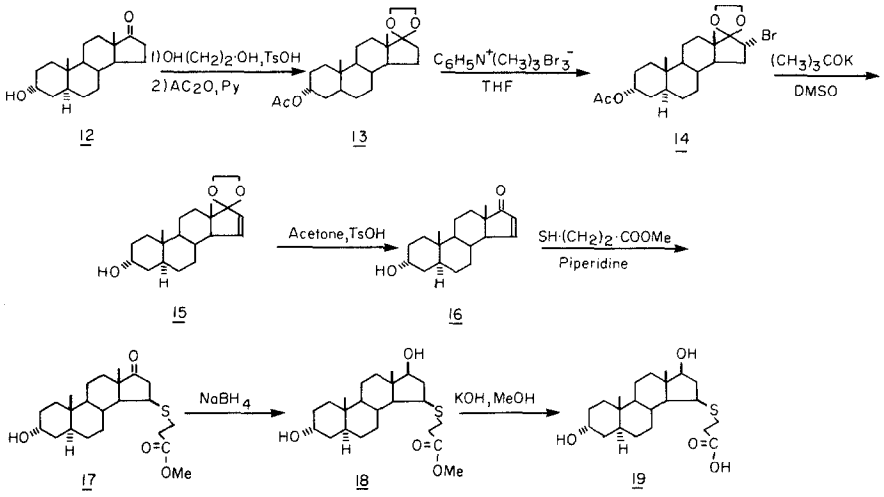
1 2 3 4 5 6 7

SCHEME II



8 9 10 11

SCHEME III



3α-Hydroxy-5α-androstan-17-one 12, obtained by alkaline hydrolysis of the corresponding 3α-formyloxy-5α-androstan-17-one prepared by Bose *et al.* (7), served as the starting material for the preparation of the desired steroid hapten 19. The 17-oxo group in 12 was ketalized by reacting with ethylene glycol and p-toluenesulfonic acid in benzene solution to give the 17-ethylene ketal which on subsequent treatment with acetic anhydride and pyridine led to the 3α-acetate derivative 13. The 17-ethylene ketal 13 was brominated according to the method of Marquet *et al.* (4) using phenyltrimethylammonium bromide perbromide in tetrahydrofuran to give the 16α-bromo derivative 14. The bromo-ketal 14 was subjected to dehydrobromination with potassium *t*-butoxide in dimethylsulfoxide at 40°C to give the 17,17-ethylenedioxy-5α-androst-15-en-3α-ol 15. Hydrolysis of the ketal with p-toluenesulfonic acid in aqueous acetone led to 3α-hydroxy-5α-androst-15-en-17-one 16. Conjugate addition of methyl-3-mercaptopropionate to the enone 16 gave the expected 3α-hydroxy-17-oxo-5α-androstane-15β-yl 2′-methoxycarboxylethyl sulfide 17 which was reduced with sodium borohydride to yield the 3α,17β-dihydroxy derivative 18. Finally, hydrolysis of the methyl ester 18 with methanolic potassium hydroxide led to the desired 15β-carboxyethylmercapto-5α-androstane-3α,17β-diol 19.

The following sequence describes the typical procedure employed in the workup and isolation of the reaction product. The reaction mixture was treated with ice-water and then extracted with a specified organic solvent. The organic extract was washed with brine, dried over anhydrous sodium sulfate, filtered and then 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.

17,17-Ethylenedioxy-5α-androst-15-en-3β-ol (2)

17,17-Ethylenedioxy-16α-bromo-5α-androstan-3β-ol acetate 1 (5.15 g) (4) was dissolved in hot dimethylsulfoxide (100 ml). After cooling the solution to 40°C, potassium *t*-butoxide (4.5 g) was added and the reaction mixture was stirred under nitrogen at 40°C overnight (18 hours). The cooled solution was diluted with water (250 ml) and the reaction product was isolated with ethyl acetate. The product crystallized from ether-hexane to give 2 (3.5 g, 95%) mp 146-148°C; ν_{max} 3420 cm^{-1}; δ 0.83 (s, C-18 Me), 0.90 (s, C-19 Me), 3.93 (s, C-17 ketal) 5.68 (d of d, J = 8Hz, C-15 H) 6.16 (d of d, J = 8Hz, C-16 H) ppm. Lit. (4) mp 152-153°C.

3,17-Dioxo-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide (4)

To a stirred solution of pyridine (19.3 ml) in dry methylene chloride (300 ml) chromium trioxide (12.0 g) was added portion wise under anhydrous conditions. After the addition was complete, the methylene chloride solution turned cherry red in color. To the above solution 17,17-ethylenedioxy-5α-androst-15-en-3β-ol 2 (7 g) was added in one portion. After stirring for an additional 1/2 hour, the solvent was decanted and the dark residue was washed with several portions of methylene chloride. The combined methylene chloride extracts were washed with a saturated solution of sodium bicarbonate and the reaction product isolated. 17,17-Ethylenedioxy-5α-androst-15-en-3-one (6.3 g, 90%) crystallized from ether-hexane mp 195-197°; MS, m/e = 330 (M^+).

To a solution of the above 17,17-ethylenedioxy-5α-androst-15-en-3-one (6.3 g) in acetone (300 ml) was added an aqueous solution of p-toluenesulfonic acid mono hydrate (500 mg in 50 ml water). The reaction mixture was stirred under nitrogen for 4 hours. Excess p-toluenesulfonic acid was neutralized with sodium bicarbonate solution and the reaction product was isolated with ethyl acetate. The residue crystallized from ether-petroleum ether to give 5α-androst-15-ene-3,17-dione 3 (4.3 g, 68%) mp 128-130°C. Without further purification, it was employed in the next step.

To a stirred solution of 5α-androst-15-ene-3,17-dione 3 (4.3 g) and methyl-3-mercaptopropionate (4.3 ml) in tetrahydrofuran (50 ml), piperidine (0.5 ml) was added. The solution was stirred for 1/2 hour at room temperature under anhydrous conditions. The solution was diluted with cold water (200 ml) and neutralized with hydrochloric acid and the product isolated with ethyl acetate. The residue was crystallized from acetone-hexane to give 3,17-dioxo-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide 4 (4.22 g, 69%) mp 158-159°C; ν_{max} 1735, 1720 and 1700 cm^{-1}; δ 0.85 (s, C-18 Me), 1.02 (s, C-19 Me), 2.8 (m, C-15-CH_2·CH_2) and 3.7 (s, ester Me) ppm;

Anal. Calcd. for $C_{23}H_{34}O_4S$: C, 67.96; H, 8.43
Found: C, 68.07; H, 8.50

3,3'-Dimethoxy-17-oxo-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide (5)

To a stirred solution of 4 (1.0 g) in dry methanol (55 ml) p-toluenesulfonic acid mono hydrate (10 mg) was added and the mixture stirred at room temperature overnight (18 hours). The solution was evaporated under a stream of nitrogen to give

5, which crystallized from ether-hexane (0.76 g, 69%) mp 127-128°C; ν_{max} 1735 cm^{-1}; δ 0.82 (s, C-18 Me), 0.95 (s, C-19 Me), 2.7 (m, C-15-CH_2·CH_2), 3.15 (s, C-3 -OCH_3), 3.2 (s, C-3'-OCH_3), 3.7 (s, ester Me) ppm; MS, m/e 453 (M^+).
Anal. Calcd. for $C_{25}H_{40}O_5S$: C, 66.35; H, 8.91
Found: C, 66.41; H, 9.00

17β-Hydroxy-3-oxo-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide (6)

To a stirred solution of sodium borohydride (0.4 g) in 95% ethanol (50 ml) was added dropwise a solution of 3,3'-dimethoxy-5α-androstane derivative 5 (2.0 g) in 95% ethanol (75 ml). After the addition was complete, stirring was continued for 2 1/2 hours at 0°C. Excess sodium borohydride was decomposed with glacial acetic acid. The resulting solution was evaporated under a stream of nitrogen. The residue was treated with water and the reaction product isolated with ethyl acetate. The residue was dissolved in dry THF and re-esterified by adding diazomethane. The residue was crystallized from acetone-hexane to give 6 (790 mg) mp 126-127°C; ν_{max} 3430, 1735, and 1700 cm^{-1}; δ 0.94 (s, C-18 Me), 1.01 (s, C-19 Me), 2.67 (m, C-15-CH_2·CH_2), 3.67 (s, ester Me) ppm; MS, m/e = 408 (M^+).
Anal. Calcd for $C_{23}H_{36}O_4S$: C, 67.62; H, 8.88
Found: C, 67.79; H, 9.03

3-Oxo-17β-hydroxy-5α-androstane-15β-yl 2'-carboxyethyl sulfide (7)

Compound 6 (0.59 g) was dissolved in methanolic potassium hydroxide (0.1 g in 10 ml absolute methanol). The solution was heated under reflux for 2 1/2 hours. The solution was cooled and diluted with water (75 ml) and acidified with conc. hydrochloric acid. The precipitated organic material was isolated with ethyl acetate. The residue was crystallized from ethyl acetate to give 7 (0.471 g, 82%), mp 198-199°C; ν_{max} 3300, and 1700 cm^{-1}; MS, m/e = 394 (M^+).
Anal Calcd. for $C_{22}H_{34}O_4S$: C, 66.98, H, 8.69
Found: C, 66.59; H, 8.81

3β-Hydroxy-5α-androst-15-en-17-one (8)

To a solution of 17,17-ethylenedioxy-5α-androst-15-en-3β-ol 2 (3.5 g) in acetone (200 ml), an aqueous solution of p-toluenesulfonic acid (200 mg in 15 ml water) was added. The reaction mixture was stirred under nitrogen for 4 hours. Excess p-toluenesulfonic acid was neutralized with sodium bicarbonate. The solution was diluted with water (500 ml) and the reaction product isolated with ethyl acetate. The residue was crystallized from ether-hexane to give 8 (1.8 g, 59%) mp 161-163°C; ν_{max} 3450, 1680 and 1630 cm^{-1}; MS m/e = 288 (M^+). Lit. (6) mp 161-163°C.

3β-Hydroxy-17-oxo-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide (9)

To a stirred solution of 3β-hydroxy-5α-androst-15-en-17-one 8 (1.8 g) and methyl-3-mercaptopropionate (1.36 ml) in tetrahydrofuran (30 ml), piperidine (0.2 ml) was added and the reaction mixture stirred for 1/2 hour under anhydrous conditions. After the reaction was complete, it was diluted with water (200 ml) and then neutralized with hydrochloric acid. The reaction product was isolated with ethyl acetate. The

residue crystallized from ether-petroleum ether to give 9 (1.82 g, 73%) mp 87-89°C; ν_{max} 3400 and 1725 cm^{-1}; MS, m/e = 408 (M^+).
Anal Calcd. for $C_{23}H_{36}O_4S$: C, 67.60; H, 8.86
Found: C, 67.60; H, 8.89

3β,17β-Dihydroxy-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide (10)

To a stirred solution of 9 (1.7 g) in absolute ethanol (40 ml) at 0°C was added dropwise a solution of sodium borohydride (0.36 g) in absolute ethanol (40 ml) and the solution stirred for 2 1/2 hours at 0°C. After the reaction was complete, excess sodium borohydride was decomposed with glacial acetic acid. The resulting solution was evaporated under vacuum and the residue was treated with ice water. The reaction product was isolated with ethyl acetate and crystallized from acetone-petroleum ether to give 10 (1.2 g) mp 118-121°C; ν_{max} 3370 and 1725 cm^{-1}; MS, m/e = 410 (M^+).
Anal. Calcd. for $C_{23}H_{38}O_4S$: C, 67.28; H, 9.33
Found: C, 67.16; H, 9.41

3β,17β-Dihydroxy-5α-androstane-15β-yl 2'-carboxyethyl sulfide (11)

Compound 10 (0.1 g) was dissolved in methanolic potassium hydroxide (0.1 g potassium hydroxide in 20 ml absolute methanol) and heated under reflux for 2 1/2 hours. The solution was cooled and diluted with water and acidified with concentrated hydrochloric acid. The solution was saturated with sodium chloride and the carboxylic acid isolated with ethyl acetate. The residue was crystallized from acetone to give 11 (0.065 g, 67%), mp 228-229°C; MS, m/e = 396 (M^+).
Anal. Calcd. for $C_{22}H_{36}O_4S$: C, 66.63; H, 9.15
Found: C, 66.55; H, 9.23

3α-Hydroxy-5α-androstan-17-one (12)

To a solution of 3α-formyloxy-5α-androstan-17-one (7) (0.302 g) in methanol (20 ml), a solution of potassium carbonate (0.124 g) in water (3 ml) was added and stirred at room temperature for 2 hours. The solvent was evaporated under a stream of nitrogen and the product isolated with ethyl acetate. The residue was crystallized from ether-hexane to give 3α-hydroxy-5α-androstan-17-one 12 (0.275 g, 91%), mp 183-184°C; δ 0.72 (s, C-18 Me), 0.77 (s, C-19 Me), 3.97 (m, W 1/2 = 8Hz, C-3β H) ppm.
Anal. Calcd. for $C_{19}H_{30}O_2$: C, 78.57, H, 10.41
Found: C, 78.94; H, 10.66

17,17-Ethylenedioxy-5α-androstan-3α-yl acetate (13)

3α-Hydroxy-5α-androstan-17-one 12 (6.95 g) was added to a solution of dry benzene (250 ml) and ethylene glycol (20 ml) containing p-toluenesulfonic acid (0.8 g) and the contents heated under azeotropic distillation for 4 hours. The solution was then cooled and neutralized with 10% methanolic potassium hydroxide and the product isolated with benzene. The residue so obtained was crystallized from ether-petroleum ether to give 17,17-ethylenedioxy-5α-androstan-3α-ol (7.1 g, 93%) mp

135-136°C; δ 0.76 (s, C-18 Me), 0.81 (s, C-19 Me), 3.86 (s, C-17 ketal), 4.01 (m, W 1/2 = 8 Hz, 3β-H) ppm.
Anal. Calcd. for $C_{21}H_{34}O_3$: C, 75.41; H, 10.25
Found: C, 74.96; H, 10.28

The above ketal (7 g) was dissolved in dry pyridine (70 ml) and treated with acetic anhydride (40 ml). The reaction mixture was set aside at room temperature overnight (18 hours), and then the solvent was evaporated under a stream of nitrogen. The residue was crystallized from ether-hexane to give 17,17-ethylenedioxy-5α-androstan-3α-yl acetate 13, (8.9 g, 83%) mp 125-126°C; δ 0.73 (s, C-18 Me), 0.78 (s, C-19 Me), 1.98 (s, C-3α acetate), 3.83 (s, C-17 ketal), 4.97 (m, W 1/2 = 9 Hz, 3β-H) ppm.
Anal. Calcd. for $C_{23}H_{36}O_4$: C, 73.37; H, 9.64
Found: C, 74.07; H, 9.87

17,17-Ethylenedioxy-16α-bromo-5α-androstan-3α-yl acetate (14)

To a cooled solution of 17,17-ethylenedioxy-5α-androstan-3α-yl acetate 13 (8.9 g) in tetrahydrofuran (50 ml) was added in one portion, a solution of phenyltrimethylammonium bromide perbromide (8.9 g) in dry tetrahydrofuran (50 ml). After mixing, molecular sieve (4 Å, four tablespoons) was added to the solution. A crystalline solid separated as the reaction progressed. The reaction mixture was kept in the refrigerator (4°C) overnight (18 hours) for completion of the reaction. The precipitated solid was separated by filtration. The filtered solution was evaporated under vacuum and the product isolated with methylene chloride. The residue was crystallized from methylene chloride-methanol to give 17,17-ethylenedioxy-16α-bromo-5α-androstan-3α-yl acetate 14 (8.5 g, 75%) mp 239-240°C; δ 0.75 (s, C-18 Me), 0.83 (s, C-19 Me), 2.0 (s, C-3α acetate), 4.25 (m, C-17 ketal), 5.0 (m, W 1/2 = 7 Hz, 3β-H) ppm.
Anal. Calcd. for $C_{23}H_{35}O_4$: C, 60.66; H, 7.75
Found: C, 60.65; H, 7.90

3α-Hydroxy-5α-androst-15-en-17-one (16)

17,17-Ethylenedioxy-16α-bromo-5α-androstan-3α-yl acetate 14 (5.0 g) was dissolved in hot dimethylsulfoxide (100 ml). After cooling the solution to 40°C, potassium *t*-butoxide (5.0 g) was added under anhydrous conditions. The reaction mixture was stirred under nitrogen at 40°C overnight (18 hours). The cooled solution was diluted with water (100 ml) and the product was isolated with ethyl acetate to give 17,17-ethylenedioxy-5α-androst-15-en-3α-ol 15 as a solid (3.33 g, 91%). No attempt was made to purify this compound. It gave a negative Beilstein test for halogen.

The above compound 15 (3.33 g) was dissolved in acetone (200 ml) and an aqueous solution of p-toluenesulfonic acid monohydrate (0.2 g in 15 ml water) was added. The reaction mixture was stirred under nitrogen for 4 hours. Excess p-toluenesulfonic acid was neutralized with sodium bicarbonate solution and then diluted with water (500 ml). The reaction product was isolated with ethyl acetate. The residue was crystallized from ether-petroleum ether to give 3α-hydroxy-5-androst-15-en-17-one 16 (2.5 g, 87%); mp 162-163°C; δ 0.85 (s, C-18 Me), 1.05 (s, C-19 Me), 4.06 (m, W 1/2 = 9 Hz, C-3β-H), 6.02 (d of d J = 8 Hz, C-15 H), 7.52 (d of d, J = 10 Hz, C-16 H) ppm; MS, m/e = 288 (M^+).

Anal. Calcd. for $C_{19}H_{28}O_2$: C, 79.12; H, 9.78
Found: C, 78.34; H, 9.93

3α-Hydroxy-17-oxo-5α-androstane-15β-yl 2'-methoxycarboxyethyl sulfide (17)

To a stirred solution of 16 (1.6 g) and methyl-3-mercaptopropionate (1.5 ml) in tetrahydrofuran (20 ml), piperidine (0.3 ml) was added and the solution stirred for 30 minutes under anhydrous conditions. After the reaction was complete, the reaction mixture was diluted with water (50 ml), neutralized with hydrochloric acid, and the product isolated with ethyl acetate. The residue was crystallized from ether-petroleum ether to give 17 (1.45 g, 64%) mp 109-110°C; δ 0.83 (s, C-18 Me), 1.07 (s, C-19 Me), 2.72 (m, C-15-CH_2·CH_2), 3.70 (s, ester Me), 4.06 (m, W 1/2 = 9 Hz, 3β-H) ppm; MS, m/e = 408 (M^+).
Anal. Calcd. for $C_{23}H_{36}O_4S$: C, 67.62; H, 8.82
Found: C, 68.07; H, 9.10

3α,17β-Dihydroxy-5α-androstane-15β-yl 2'-methoxycarbonylethyl sulfide (18)

To a stirred solution of sodium borohydride (0.14 g) in 95% ethanol (25 ml) a solution of compound 17 (1.3 g) in 95% ethanol (35 ml) was slowly added and stirred for 2 1/2 hours at 0°C. The excess sodium borohydride was decomposed with glacial acetic acid and the ethanol evaporated under a stream of nitrogen. The residue was treated with water and the product isolated with ethyl acetate. Crystallization of the residue from ether-petroleum ether gave 18 (0.16 g, 82%), mp 93-94°C; δ 0.82 (s, C-18 Me), 0.95 (s, C-19 Me), 2.70 (m, C-15 -CH_2·CH_2-), 3.7 (s, ester Me) ppm.
Anal. Calcd. for $C_{23}H_{38}O_4S$: C, 67.28; H, 9.33
Found: C, 67.36; H, 9.50

3α,17β-Dihydroxy-5α-androstane-15β-yl 2'-carboxyethyl sulfide (19)

The methyl ester 18 (1.2 g) was dissolved in methanolic potassium hydroxide (0.18 g in 50 ml absolute methanol) and the solution heated under reflux for 2 1/2 hours. It was then cooled and diluted with water and acidified with concentrated hydrochloric acid. The solution was saturated with sodium chloride and then extracted with ethyl acetate. The product was then crystallized from ethyl acetate to give 19 (0.87 g, 78%) mp 196-197°C; MS, m/e = 396 (M^+).
Anal. Calcd. for $C_{22}H_{36}O_4S$: C, 66.64; H, 9.15
Found: C, 66.45; H, 9.28

Preparation of the Steroid-Bovine Serum Albumin Conjugates and Determination of the Moles of Steroid Bound per Mole Protein: The three 15β-carboxyethylmercapto derivatives of 5α-dihydrotestosterone (7, 0.635 mmoles), 5α-androstane-3β,17β-diol (11, 0.126 mmoles), and 5α-androstane-3α,17βdiol (19, 0.505 mmoles) were coupled to bovine serum albumin (BSA, 0.0127, 0.0025, and 0.101 mmoles, respectively, based on the mol. wt. of 70,000) by the mixed anhydride procedure (8) with our modification described earlier (1).

Table I
Moles of Steroid per Mole of Conjugate as
Determined by Ninhydrin Procedure (9)

Conjugate	Moles of Steroid
15β-Carboxyethylmercapto-5α-DHT-BSA	18
15β-Carboxyethylmercapto-3β-diol-BSA	23
15β-Carboxyethylmercapto-3α-diol-BSA	30

Immunization Procedure and Collection of the Antibody: A group of five female New Zealand white rabbits were used for immunization with each of the three different conjugates. The primary injection (2 mg) of the conjugate in isotonic saline, mixed with Freund's complete adjuvant (1:1) for a final dilution of 1 mg/ml, was divided into four equal portions and injected intramuscularly into each thigh and below each shoulder blade. Intramuscular injections of 0.5 mg into each thigh were repeated 7, 14, and 21 days after the initial injections, and every 30 days thereafter. Plasma was collected 14 days after the third booster injection and every 30 days thereafter.

Standard Curve: A standard curve was established in each case by setting up duplicate centrifuge tubes (3 ml) containing 0, 50, 100, 250, 1000, and 2000 picograms of the steroid in a total volume of 0.5 ml of sodium phosphate assay buffer (0.1 M, pH 7, 0.9% NaCl, 0.1% sodium azide). The standards were prepared from a stock solution of unlabeled steroid in absolute ethanol (100 ng/ml). The labeled steroids were prepared in assay buffer at a concentration of 50 picograms/0.50 ml. The antiserum was prepared in BSA assay buffer (1 gm BSA/1,000 ml sodium phosphate buffer) at a concentration equal to five times the final working dilution. The antibody (0.25 ml) and the labeled steroid (0.50 ml) were added to all standard tubes containing 0.5 ml assay buffer; the tubes were mixed and allowed to incubate at 4°C overnight. 0.2 milliliters of cold gamma globulin dextran-coated charcoal (1 g charcoal, 0.1 g dextran, 0.2 g human gamma globulin, 200 ml deionized water), was added to each tube, mixed, and returned to the cold room for 20 minutes. After centrifugation at 2,500 rpm for 6 minutes, 0.5 ml of each supernatant was aliquoted into a counting vial. Then 15 ml of scintillation medium (4 g PPO, 50 mg dimethyl-POPOP, 100 ml Bio-Solv BBS-3, 1,000 ml toluene) was added to each vial. The samples were counted to a relative standard error of less than 2% in a Packard liquid scintillation counter Model 3320.

RESULTS AND DISCUSSION

All plasma was treated with Rivanol (10) prior to titer assessments. The titer was determined from the ability of the antibody to bind a constant amount (50 pg) of the labeled steroid as compared to plasma collected prior to the primary injection. In three to four months time all the 15 rabbits immunized with various steroid-BSA conjugates produced antisera with usable titers. However, the individual antiserum from each rabbit differed and exhibited varying degrees of sensitivity and specificity. As a first step, in each case we have evaluated the cross-reactivity of a structurally similar and

pertinent steroid. Accordingly, the cross-reactivity of testosterone with anti-5α-DHT serum (Table II), 5-androstene-3β,17β-diol with anti-3β-diol serum (Table III) and androsterone with anti-3α-diol serum (Table IV) was evaluated.

Table II
Testosterone Cross-Reactivity with Anti-5α-DHT Serum

Rabbit No.	Production Time	Titer	Percent Cross with Testosterone
X-137	3 months	1: 8,000	33.17
X-138	4 months	1: 1,500	28.75
X-139	6 months	1: 8,000	19.61
X-140	10 months	1:30,000	7.78
X-160	7 months	1: 5,000	8.11

Table III
5-Androstene-3β,17β-diol Cross-Reactivity with Anti-3β-diol Serum

Rabbit No.	Production Time	Titer	Percent Cross with 5-Androstene-3β,17β-diol
X-127	4 months	1:15,000	71.50
X-128	4 months	1: 9,000	74.24
X-129	6 months	1: 7,500	18.84
X-130	6 months	1: 3,000	28.00
X-131	7 months	1:25,000	15.27

Table IV
Androsterone Cross-Reactivity with Anti-3α-diol Serum

Rabbit No.	Production Time	Titer	Percent Cross with Androsterone
X-143	5 months	1:30,000	1.77
X-144	5 months	1:15,000	1.22
X-145	5 months	1:15,000	4.02
X-146	5 months	1:15,000	2.18
X-150	5 months	1: 4,000	2.23

Complete characterization of antiserum which exhibited lowest cross-reactivity with sufficiently high titer and sensitivity, was then carried out. Using this criteria, we have selected anti-5α-DHT serum from rabbit X-140, 3β-diol serum from rabbit X-131, and anti-3α-diol serum from rabbit X-144 for indepth characterization. The final

working dilution (titer), the antibody production time, and the binding affinity are presented in Table V.

Table V
Antibody Characterization

BSA Conjugate	Titer	Production Time	Binding Affinity
15β-Carboxyethylmercapto-5α-DHT	1:30,000	10 months	4.95×10^9 L/M
15β-Carboxyethylmercapto-3β-diol	1:25,000	7 months	1.00×10^9 L/M
15β-Carboxyethylmercapto-3α-diol	1:15,000	5 months	1.47×10^9 L/M

In establishing the binding affinity, varying nanomolar concentrations of the labeled steroid were incubated with a constant volume and dilution of antibody at 4°C for 18 hours. By varying only the labeled steroid, a relationship between the nanomolar concentration and the bound and free fractions was established and evaluated as a Scatchard plot (11). In Figure 1 we present the Scatchard plots for the three different antisera.

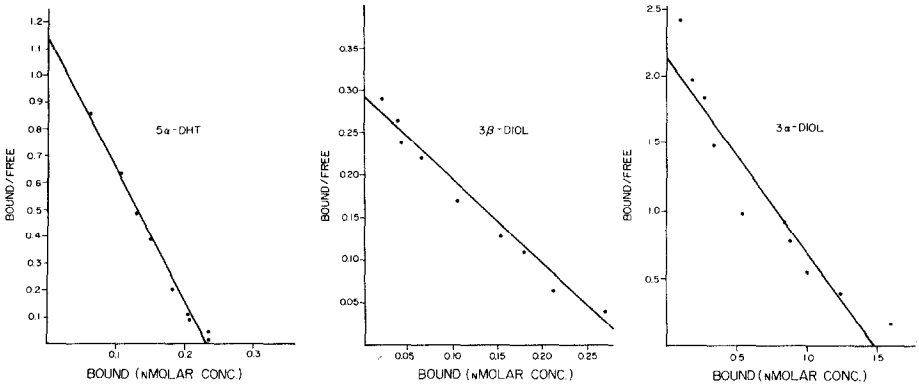


Figure 1. Binding Affinity Determination

The percent cross-reaction of each antibody was determined using the method of Abraham (10) and the data is presented in Table VI. Standard curves were established over the desired working range and compared to the inhibition curves of pertinent and structurally similar steroids.

Table VI
Percent Cross-Reactivity Data

Steroid	5α-DHT Antibody	3β-diol Antibody	3α-diol Antibody
1. 5α-Dihydrotestosterone	100.00	1.25	0.00
2. 5α-Androstane-3β,17β-diol	0.61	100.00	0.00
3. 5α-Androstane-3α,17β-diol	0.56	0.00	100.00
4. Testosterone	7.78	0.38	0.00
5. 5β-Dihydrotestosterone	1.45	0.00	0.00
6. 6β-Hydroxytestosterone	1.94	0.00	0.00
7. 11β-Hydroxytestosterone	0.00	0.00	0.00
8. Androstenedione	0.42	0.00	0.00
9. 11β-Hydroxyandrostenedione	0.00	0.00	0.00
10. 5α-Androstane-3,17-dione	2.92	0.00	0.00
11. 5β-Androstane-3,17-dione	0.00	0.00	0.00
12. Androsterone	0.00	0.00	1.22
13. Epiandrosterone	0.77	0.02	0.00
14. Dehydroepiandrosterone	0.00	0.00	0.00
15. 5-Androstene-3β,17β-diol	0.00	15.27	0.00
16. 3α-Hydroxy-5β-androstan-17-one	0.00	0.00	0.00
17. Progesterone	0.00	0.00	0.00
18. 17α-Hydroxyprogesterone	0.00	0.00	0.00
19. Deoxycorticosterone	0.00	0.00	0.00
20. Corticosterone	0.00	0.00	0.00
21. 3β-Hydroxy-5β-pregnan-20-one	0.00	0.00	0.00
22. Estrone	0.00	0.00	0.00
23. Estradiol-17β	0.00	1.35	0.00
24. Estriol	0.00	0.11	0.00

Anti-5α-DHT serum: The antiserum proved to be highly specific and exhibited less than 10% cross-reaction with testosterone. Other steroids that showed minor cross-reactions include 5α-androstane-3,17-dione (2.9%), 6β-hydroxytestosterone (1.9%), and 5β-dihydrotestosterone (1.5%).

Anti-3β-diol serum: The cross-reactivity data as presented indicates that the antiserum is sufficiently specific with the single exception of 5-androstene-3β,17β-diol which showed a 15% cross-reactivity.

Anti-3α-diol serum: The antiserum proved to be extremely specific for 5α-androstane-3α,17β-diol. Androsterone is the only steroid that exhibited minor cross-reaction (1.2%).

The particular clinical or experimental situation will have to determine whether the above cross-reaction will affect the measurement in a significant manner.

NOMENCLATURE

Trivial Name	
5α-Dihydrotestosterone	17β-Hydroxy-5α-androstan-3-one
Testosterone	17β-Hydroxyandrost-4-en-3-one
5β-Dihydrotestosterone	17β-Hydroxy-5β-androstan-3-one
6β-Hydroxytestosterone	6β,17β-Dihydroxyandrost-4-en-3-one
11β-Hydroxytestosterone	11β,17β-Dihydroxyandrost-4-en-3-one
Androstenedione	Androst-4-ene-3,17-dione
11β-Hydroxyandrostenedione	11β-Hydroxyandrost-4-ene-3,17-dione
Androsterone	3α-Hydroxy-5α-androstan-17-one
Epiandrosterone	3β-Hydroxy-5α-androstan-17-one
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one
Progesterone	Pregn-4-ene-3,20-dione
17α-Hydroxyprogesterone	17α-Hydroxypregn-4-ene-3,20-dione
Deoxycorticosterone	21-Hydroxypregn-4-ene-3,20-dione
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one
Estradiol-17β	1,3,5(10)-Estratriene-3,17β-diol
Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol

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