PREPARATION AND ANTIGENIC PROPERTIES OF ANDROSTERONE-7-BSA CONJUGATE

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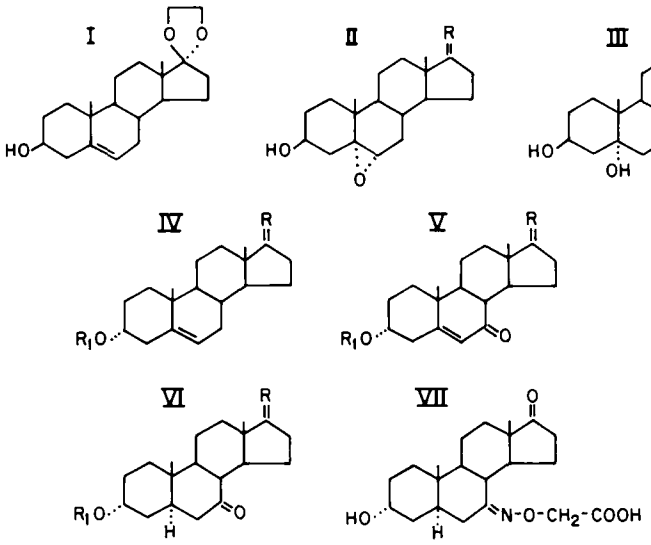
ABSTRACT

The 7-carboxymethoximino derivative of androsterone (1) has been prepared from dehydroisoandrosterone-17-ethyleneketal by a sequence involving inversion at C-3, introduction of a carbonyl at C-7, and reduction of the double bond at C-5. The substance was condensed with BSA by the carbodiimide procedure to afford a conjugate which produced anti-androsterone antiserum in innoculated rabbits. The antiserum is sufficiently active to be useful in radioimmunoassay procedures.

The measurements of free dehydroisoandrosterone (DHA) and androsterone in the plasma by RIA have recently been reported (2-4). In these assays, a separation step prior to binding is usually included because the antibody, generally produced against the 17-carboxymethoxime-BSA conjugate, displays significant cross reactivity with the corresponding 17-hydroxy compounds, i.e., 5-androstene-3β,17β-diol and 5α-androstane-3α,17β-diol, which are present in plasma in significant amounts (5-7). With the purpose of obviating this difficulty, a 17-ketosteroid-protein conjugate, 3β-hydroxy-5-androstene-7,17-dione-7-(O-carboxymethyl) oxime BSA, was prepared in which both the Δ^5-3β-hydroxy- and 17-ketone functions are available for eliciting a more specific antibody (8). This report is concerned with the preparation of an androsterone-BSA conjugate, 3α-hydroxy-5α-androstane-7,17-dione 7 (O-carboxymethyl) oxime BSA, which also possesses antigenic determinants in both the A and D rings. C-7 substituted derivatives of testosterone and 4-androstene-3, 17-dione for preparation of antigens have recently been described (9).



DHA-7α-^{3}H,4-^{14}C was converted to the ethyleneketal (I) which was reacted with m-chloroperbenzoic acid to yield 17,17-ethylenedioxy-5,6α-oxido-androstan-3β-ol (IIa) in essentially quantitative yield. Treatment of IIa with lithium aluminum hydride in refluxing tetrahydrofuran (10) afforded the 3β,5α-diol (IIIa) in over 90% yield. The 3-mesylate of IIIa underwent inversion at C-3 with re-formation of the C-5 double bond after refluxing in N,N-diethylaniline and acetyl chloride (10,11) to give 17,17-ethylenedioxy-3α-acetoxy-5-androstene (IVac) in about 45% yield after chromatographic separation of this substance from the 17-ketone (IVbc) and two other minor unidentified components shown to be present by GLC.

The ethyleneketal IVac was oxidized with chromium trioxide-pyridine complex in methylene chloride (8,12) to give a mixture which, by ultraviolet spectrum,

contained about 60% Δ^5-7-ketone. After chromatography on silica gel, the expected amount of crystalline 17,17-ethylenedioxy-3a-acetoxy-5-androsten-7-one (Vac) was obtained along with lesser amounts of the starting material and its corresponding 17-ketone, IVbc.

A portion of the Δ^5-7-ketone (Vac) was reduced with lithium-ammonia according to the method of Barton and Robinson (13) which yielded a mixture showing only traces of unsaturated ketone. The mixture was deparated (by silica gel chromatography) into 5 components most of which were formed through reductive cleavage or hydrolysis at C-3 and C-17 during the reaction or in the subsequent work-up; the desired product, 17,17-ethylenedioxy-3a-hydroxy-5a-androstan-7-one (Vlad), was obtained in greatest amount making up more than 35% of the product.

Conversion of Vlad to the 7-carboxymethoximino derivative by reaction with carboxymethoxylamine hemihydrochloride was accompanied by extensive deketalization at C-17 since estimation of the composition of the acidic product by infrared spectrum analysis showed a mixture of 3a-hydroxy-5a-androstane-7,17-dione-7-(O-carboxymethyl)oxime (VII) and its corresponding 17-ketal in approximately a 3:1 ratio. Apparently derivatization at C-7 occurred before hydrolysis of the 17-ethyleneketal since there was no evidence of oxime formation at C-17; in all likelihood, deketalization took place during isolation of the product. Deketalization was completed in acidified ethanol and after purification by TLC, VII was obtained in good yield.

Since the carboxymethoxime VII contained water of crystallization, the carbodiimide technique (14) was chosen for covalently binding the steroid to BSA. Assuming a molecular weight of 70,000 for the protein, the product 3a-hydroxy-

5α-androstane-7,17-dione-7-(O-carboxymethyl) oxime-BSA (androsterone-7-BSA conjugate) contained about 13 steroid residues per protein molecule.

Three rabbits were immunized against androsterone-7-BSA conjugate and were tested for the presence of antibodies at weekly intervals. Antibodies appeared by the fourth week and by the eighth week, the best animal afforded antiserum which at 1:10000 dilution bound 70% of 40 pg of androsterone-1,2-^{3}H; antisera from the other rabbits bound 50% and 27% under identical conditions. The two most active antisera were diluted 1:14000 and 1:10000 respectively and androsterone-^{3}H binding was measured over the range from 5-250 pg. The dose response curves are shown in Figure 1 and indicate the potential usefulness of the antisera in radioimmunoassays for circulating androsterone.

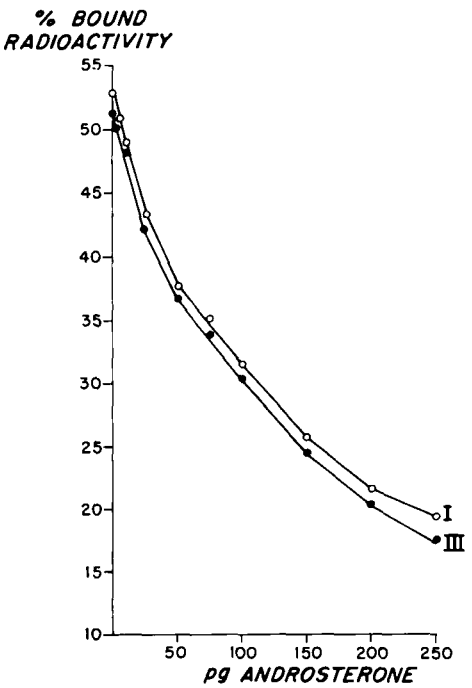


Figure 1. Dose response curve of androsterone-1,2-^{3}H to antisera from androsterone-7-BSA injected rabbits. O, Rabbit I, 8 weeks after immunization, 1:14000 dilution; ●, Rabbit III, 11 weeks after immunization, 1:10000 dilution.

EXPERIMENTAL

Melting points were taken on a micro hot stage and are corrected. Optical rotations were measured in chloroform. Infrared spectra were determined in potassium bromide dispersions. NMR spectra were obtained on a Varian EM-360 60 mHz spectrometer. Simultaneous assays for ^{3}H and ^{14}C were carried out in a Packard-Tricarb liquid scintillation spectrometer, model 3375 according to methods in routine use in this laboratory (15).

17,17-Ethylenedioxy-5,6α-oxido-5α-androstan-3β-ol(IIa)(16). DHA-7α-^{3}H and DHA-4-^{14}C both obtained from New England Nuclear Corporation, Boston, Mass, 02118, were mixed in tracer amounts with non-radioactive DHA to afford the starting material. DHA-7α-^{3}H,4-^{14}C, specific activity 66 cpm per mg ^{3}H and 232 cpm per mg ^{14}C, was converted to the 17-ethyleneketal I as described (8) and was obtained in about 73% yield after separation from unreacted DHA by chromatography on neutral alumina (Woelm, Waters Associates, Framingham, Mass.) deactivated with 10% water; I was eluted in benzene while starting material was recovered in benzene containing 0.5% ethanol.

I, 6.82 g, was dissolved in 30 ml chloroform and to the stirred solution, 4.4 g m-chloroperbenzoic acid in 48 ml chloroform was added dropwise during 10 minutes at room temperature; the solution was stirred for an additional 20 minutes. Excess reagent was decomposed with 40 ml of 10% sodium sulfite and the product was diluted with chloroform which was washed 3 times with 10% sodium bicarbonate, 3 times with saturated sodium chloride and dried over anhydrous sodium sulfate. Removal of the solvent in vacuo yielded about 7 g of product which by GLC consisted of 17,17-ethylenedioxy-5,6α-oxido-5α-andorstan-3β-ol (IIa) with trace amounts of the 5β,6β-epimer.

17,17-Ethylenedioxy-androstane-3β,5α-diol (IIIa). Without further purification, 3.12 g of IIa, dissolved in 125 ml of tetrahydrofuran (freshly distilled from lithium aluminum hydride) was added dropwise into a stirred suspension of 1.68 g of lithium aluminum hydride in tetrahydrofuran. After refluxing for 1.5 hr and cooling, the suspension was mixed with 10 ml of ethyl acetate, and 75 ml of 10 percent sodium hydroxide was slowly introduced. The product was extracted with ethyl acetate, washed three times with saturated sodium chloride and the solvent removed in vacuo to yield about 3.0 g of IIIa, m.p. 216° after crystallization from methanol which is in agreement with published data (17). GLC showed a single peak (Table I) and the NMR spectrum was consistent with the structure IIIa: δ (in ppm) = 0.80 (18 methyl), 0.93 (19 methyl), 3.83 (ethyleneketal).

TABLE I. Relative Retention Times of Androsterone Derivatives

Substance	RRT*
5α-cholestane	1.00
DHA-17-ketal (I)	1.58
17,17 ethylenedioxy-5,6α-oxido-5α-androstan-3β-ol (IIa)	2.58
17,17 ethylenedioxy-androstane-3β,5α-diol (IIIa)	3.04
17,17 ethylenedioxy-3α-acetoxy-5-androstene (IVac)	1.75
17,17 ethylenedioxy-3α-acetoxy-5-androsten-7-one (Vac)	4.43
17,17 ethylenedioxy-3α-hydroxy-5α-androstan-7-one (VIad)	3.66
3α-hydroxy-5α-androstane-7,17-dione (VIbd)	3.90
3α-hydroxy-5α-androstane-7,17-dione-7-(O-carboxymethyl) oxime (VII)	3.20+

*RRT, relative retention time to 5α-cholestane (6.9 minutes)
GLC on 3 percent OV-22 on 80/100 supelcoport (Supelco, Bellefonte, Pa), T,268°.
+Chromatographed as the trimethylsilyl derivative.

17,17-Ethylenedioxy-3α-acetoxy-5-androstene (IVac) (18). Approximately 6.2 g of IIIa was dissolved in 140 ml of pyridine containing 7.0 ml of methanesulfonyl chloride. After 2.5 hr at room temperature, ice and water were added and 30 minutes later the 3α-mesylate was extracted into ethyl acetate. The organic layer was washed with 5 percent sulfuric acid, water, 5 percent sodium hydroxide and water, then dried over sodium sulfate. Removal of the solvent afforded a crystalline residue which showed a single rapidly eluted peak in GLC (RRT, 1.17) consistent with loss of methanesulfonic acid on passing through the column.

The mesylate was dissolved in 40 ml of chloroform to which were added 40 ml N,N-diethylaniline and 40 ml of acetyl chloride (10, 11); the mixture was refluxed for 5 hours, cooled, transferred to a large round bottom flask with ethyl acetate and evaporated to an oil. After extraction into ethyl acetate which was washed with water, 5 percent hydrochloric acid, dilute alkali, and water, removal of the solvent afforded 6.3 g of oil. GLC showed two major peaks corresponding to 17, 17-ethylenedioxy-3α-acetoxy-5-androstene (IVac) and the 17-ketosteroid IVbc in a ratio of 7:3 in addition to two other unidentified minor components. The ketal IVac was separated from the ketone IVbc by chromatography on a large alumina column; about 4.3 g of the ketal (RRT, 1.75, Table I) was eluted in benzene:petroleum ether (1:1) and oxidized without further purification.

17,17-Ethylenedioxy-3α-acetoxy-5-androsten-7-one (Vac). Oxidation of 3.5 g of IVac with the chromic oxide-pyridine complex (12) was carried out as recently described (8). Briefly, 21 g of the dry complex suspended in 45 ml of methylene chloride was rapidly added to a stirred solution of IVac in 180 ml of the same solvent under nitrogen. After stirring overnight at room temperature, an additional 7.5 g of the oxidizing agent slurried in 15 ml of methylene chloride was introduced and the reaction allowed to continue for 6 more hours. The methylene

chloride solution was diluted with ether, decanted from the reaction flask and the tarry residue was washed with ether. The combined ether solution was repeatedly washed with saturated sodium bicarbonate until most of the yellow color was removed, then with water. Removal of the ether in vacuo resulted in 3.22 g of an oily residue:ultraviolet spectrum, maximum at λ_{max} = 236 nm (ethanol) corresponding to about 60 percent Δ^5-7 ketone.

In order to separate the major components of the product mixture, about 3 g were chromatographed on 450 g of silica gel. Column development was begun with 500 ml fractions of diethyl ether:petroleum ether (1:19) and continued with increasing amounts of diethyl ether. Two main fractions were eluted:17,17-ethylenedioxy-3a-acetoxy-5-androstene (IVac) and small amounts of its corresponding 17-ketone, IVbc, in diethyl ether:petroleum ether (1:9-2:8) and 1.6 g of oily crystals in the solvent mixtures from 4:6 to 1:1. This latter material showed a uv maximum at 236 nm corresponding to 90 percent Δ^5-7 ketone; GLC gave a major peak, RRT=4.44 (Table I). The infrared spectrum showed bands at 1110, 1370 and 1380 cm^{-1} associated with the ethyleneketal structure, 1675 cm^{-1} (conjugated carbonyl), and 1740 cm^{-1} (3a-acetoxy). NMR spectrum displayed signals at δ = 0.88 (18-methyl), 1.2 (19-methyl), 2.0 (3a-acetoxy), 3.84 (ethyleneketal), broad low band, centered at 4.0 (3β-H), and 5.08 (vinyl H at C-4). These data were in accord with the structure Vac,17,17-ethylenedioxy-3a-acetoxy-5-androsten-7-one. After two recrystallizations from methanol, the substance melted at 155-157°; $[\alpha]_D^{24.5}$ -112.1° (chloroform). Analysis: calculated for $C_{23}H_{32}O_5$ (MW, 388.3); C, 71.05, H, 8.30 Found: C,71.07, H, 8.38.

17,17-Ethylenedioxy-3a-hydroxy-5a-androstan-7-one (VIad). By means of a syringe, a solution of 535 mg of 17,17-ethylenedioxy-3a-acetoxy-5-androstene-7-one (Vac) in 20 ml of dry ether was rapidly added to 35 ml of a dark blue solution of lithium in ammonia cooled in a dry-ice acetone bath and equipped with a cold-finger condenser charged with dry-ice and acetone. The bath was removed from the flask and the reaction mixture was allowed to reflux for one hour with stirring. The blue color disappeared about mid-way but was restored by the addition of a small piece of lithium to the vessel. Acetone was introduced to destroy the lithium-ammonia complex and the solution rapidly decolorized. The condenser was removed to permit the ammonia to evaporate and the mixture was transferred to separatory funnels with ether and water and extracted into ether. After washing the ether solution with water, the solvent was evaporated and 465 mg of yellow oil was recovered which showed only traces of unsaturated ketone at 236 mμ. TLC indicated the presence of at least 5 substances; therefore the mixture was chromatographed. An 80 g column of silica gel was prepared which was developed with 200 ml portions of diethyl ether-petroleum ether starting with a 1:19 ratio and increasing the proportions of ether stepwise; 111 fractions were thus obtained with preliminary identification by GLC and infrared spectroscopy. The following compounds were tentatively identified: Fractions 20-22, ether-petroleum ether (1:19), 10.5 mg, m.p. 122° after crystallization from methanol, RRT, 1.47 (Table I) 17,17-ethylenedioxy-5a-androstene-7-one (the double bond is probably in ring A). Fractions 40-47 (4:6), 19.2 mg, oil, RRT, 3.74, 17,17-ethylenedioxy-3a-acetoxy-5a-

androstan-7-one (VIac). Fractions 60-75 (6:4), 115 mg, consisted mainly of a mixture of 3α-hydroxy-5α-androstane-7,17-dione (VIbd), RRT, 3.94, and 17,17-ethylenedioxy-3α-hydroxy-5α-androstan-7-one (VIad), RRT, 3.72 in about a 6:4 ratio. These components were separated by rechromatography on an alumina column deactivated with 10 percent water and eluted with benzene containing 0.5 percent ethanol where the diketone, VIbd, emerged first. After crystallization from benzene-isooctane it melted at 155-158°; $[\alpha]_D^{25}$, -43.0° (chloroform). The 17-ketal, VIad, was combined with the last major fraction from the silica gel column. Fraction 76-91 (7:3), 181 mg, the major and desired product, VIad, was crystallized twice from benzene-isooctane m.p. 199-202°, $[\alpha]_D^{25}$, -72.4° (chloroform). The NMR signal for the ethyleneketal (δ, 3.82 ppm) was still evident. Analysis: calculated for $C_{21}H_{32}O_4$ (MW, 348.3); C, 72.36, H, 9.26. Found: C, 72.19, H, 9.09. Specific activity, 179 cpm per mg ^{14}C, no ^{3}H present.

3α-Hydroxy-5α-androstane-7,17-dione-7-(O-carboxymethyl) oxime (VII). 17,17-Ethylenedioxy-3α-hydroxy-5α-androstan-7-one (VIad) (172 mg) was converted to the 7-carboxymethoximino derivative exactly as described (8). Briefly, the compound was refluxed for 3 hours in 10 ml of ethanol containing 143 mg of carboxymethoxylamine hemihydrochloride and 0.6 ml of 2 N sodium hydroxide. Upon cooling the suspension was diluted with water and adjusted to pH 10-11 with dilute alkali. After extraction of neutral material with ether, the alkaline solution was acidified to pH 1 with 6N hydrochloric acid and the precipitate was immediately extracted with ethyl acetate which was washed with water, dried over sodium sulfate and concentrated to afford 184 mg of a non-crystallizable oil. Since the infrared spectrum showed bands associated with the 17-ketone and the 17-ethyleneketal function, the mixture was dissolved in 25 ml ethanol and treated with one milliliter of concentrated hydrochloric acid for one hour at 60° to complete the deketalization. After diluting with water and extracting the product with ethyl acetate, 167 mg of oil (VII) was obtained after removal of the solvent. The NMR spectrum displayed signals at δ = 4.1 and 4.51 ppm characteristic of the 3β-H and the methylene hydrogens of the carboxymethoximino group. Final purification was achieved by chromatographing approximately 20 mg portions on 20 x 20 plates coated with a 250 μ layer of silica gel G in the system benzene-dioxane-acetic acid (75:20:2) (19). After eluting the major band (R_f, 0.45) and combining, 160 mg of crystals were obtained. The substance was recrystallized from methanol-water and yielded small needles which melted to an oil on drying at 100° in vacuo and cooled to a hard glass. Analysis: calculated for $C_{21}H_{31}O_5N$ (MW, 377.3); C, 66.80, H, 8.28, N, 3.71. Found: C, 67.45, H, 8.52, N, 3.56.

The glass was recrystallized again from methanol-water and the needles were air dried, then dried in vacuo at room temperature. Analysis: calculated for $C_{21}H_{31}O_5N \cdot H_2O$ (MW, 395.3), C, 63.76, H, 8.41, N, 3.54. Found: C, 63.88 H, 8.51, N, 3.36. Specific activity 162 cpm per mg ^{14}C.

Conjugation of 3α-hydroxy-5α-androstane-7,17-dione-7-(O-carboxymethyl) oxime (VII) to BSA. In a 5 ml volumetric flask, 40.3 mg of VII was dissolved in 0.25 ml pyridine; 0.25 ml of water was introduced and the mixture was stirred with

a magnetic spin bar. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (Aldrich Chemical Co.) (98.5 mg) was dissolved in 0.25 ml pyridine and 0.25 ml water and slowly dropped into the stirring steroid solution; stirring was continued for 35 minutes. BSA (21.4 mg) was dissolved in 0.5 ml water and introduced dropwise to the aqueous pyridine solution. After 2.5 hrs of stirring, the turbid solution was dialyzed against 3 changes of 500 ml of 0.9 percent sodium chloride over 3 days at 4°. The solution was analyzed for protein (20) and a portion was counted, 10.8 cpm ^{14}C per mg of conjugate. Assuming a molecular weight of 70,000 for BSA, about 13 steroid residues were attached to the protein molecule in the androsterone-7-BSA conjugate.

Production of Antisera Against Androsterone-7-BSA Conjugate. Three white New Zealand mature male rabbits were each innoculated intradermally with a homogenate of 1.5 mg (0.5 ml) of conjugate in 1 ml of Freund's adjuvant at multiple sites on the back at weekly intervals for 4 weeks. Four weeks later and again, six weeks after this (14 weeks after the initial immunization) each animal received "booster" innoculations of 0.50 mg and 0.90 mg of conjugate respectively. Weekly blood samples were withdrawn and the serum was tested for antibody formation against 40 pg of androsterone-1,2-^{3}H (6000 cpm) at a 1:200 dilution in 0.05 M borate buffer (pH, 8.0) containing 0.5% BSA and 0.05% human gamma globulin; bound androsterone was separated from the free steroid by ammonium sulfate precipitation (2). Before the start of the immunization procedure sera from the rabbits bound from 1.6 - 2.4% of the labeled androsterone and by the 4th week two of the rabbits produced sera which bound 90.6% and 76.3% of the steroid at the 1:200 dilution. Antisera selected from two of the rabbits 8 weeks and 11 weeks after the start of immunization proved to be potentially useful for radioimmunoassay at dilutions of 1:14,000 and 1:10,000 respectively (Fig. 1).

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1. Abbreviations: Androsterone, 3α-hydroxy-5α-androstan-17-one; DHA, dehydroisoandrosterone, 3β-hydroxy-5-androsten-17-one; BSA, bovine serum albumin; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; RRT, relative retention time.
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