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PREPARATION AND ANTIGENIC PROPERTIES OF 5α-DIHYDROTESTOSTERONE-7-BOVINE SERUM ALBUMIN CONJUGATE

D. EXLEY and T. S. BAKER

Department of Biochemistry, Queen Elizabeth College, University of London, London W8, England

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SUMMARY

The C-7 (O-carboxymethyl) oxime derivative of 5α-dihydrotestosterone (5α-DHT) has been prepared from testosterone acetate. Synthetic steps involved the sequence, C-3 ketalation, introduction of a carbonyl at C-7, reduction of the bond at C-5, deacetylation prior to oximation and then finally deketalation. Condensation of this oxime to BSA afforded a conjugate which produced anti 5α-DHT antisera (5α-DHT) in inoculated rabbits. Apart from a 50% cross reaction with testosterone the antisera was reasonably specific for 5α-DHT.

INTRODUCTION

In 1970 Midgley and Niswender[1] reported that anti-progesterone sera produced by linking the antigenic carrier through C-11 hydroxy progesterone had greater specificity than antisera obtained by carrier linkage through C-3 or C-20 groups of progesterone. As a result these workers produced the hypothesis that conjugation of antigenic carrier through positions on steroids distal to their functional groups would elicit antibodies of greater specificity than those antibodies raised by conjugation through their functional groups. The first report indicating that this hypothesis could be correct was made a year later when in 1971, Exley, Johnson and Dean[2] reported on antisera raised by oestradiol-17β-6-(O-carboxymethyl) oxime-BSA conjugate [3] showing it to be highly specific for oestradiol-17β. Several workers have confirmed this finding for oestradiol-17β [4–7] and it has been shown to be true when similar C-6 or C-7 conjugation was used for oestrone and oestriol [8, 9].

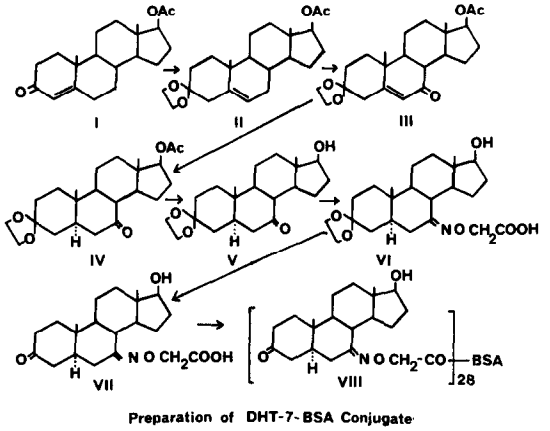

Fig. 1.

A specific interest in androgen action at the target site led to a demand for a specific radioimmunoassay for 17β-hydroxy-5α-androstan-3-one, 5α-dihydrotestosterone (5α-DHT). Unlike that for the oestrogens no highly specific antisera has been raised for this physiologically important androgen and as yet C-6 or C-7-(O-carboxymethyl)-oxime conjugation has not been attempted. Following the success of this type of conjugation for oestrogens we have therefore prepared the 5α-DHT C-7-(O-carboxymethyl) oxime-BSA conjugate.

This report is concerned with the preparation of the immunogen and the specificity of the antisera it elicited in rabbits.

METHODS AND MATERIALS

The preparation of the 5α-DHT conjugate is shown in Fig. 1. Synthesis steps I–VIII. The starting material for the synthesis was the readily available 17β-acetoxy-4-androsten-3-one (testosterone acetate) compound I. Testosterone acetate was first converted in good yield to 17β-acetoxy-5-androsten-3-on-3-ethylene ketal [10] by exchange dioxolanation [11]. Oxidation of this ketal with the tertiary butyl complex of Heusler and Wettstein [12] using the method of Rao and Kurath [13] afforded a 54·5% yield of 17β-acetoxy-5-androstene-3 7-dione-3-ethylene ketal (III). A catalytic method was then used to saturate the 5-ene bond of III. The method, which has not been reported to have been used for 7 keto steroids was that of Howarth and Liston [14] who utilized 10% palladium on charcoal. The reduction yielded 60% of the desired 5α compound (17β-acetoxy-5α-androstane-3, 7-dione-3-ethylene ketal) (IV). This 5α compound was separated from its 5β isomer by preparative t.l.c.

Deacetylation of IV by 1% K_2CO_3 in methanol–water afforded 17β-hydroxy-5α-androstane-3,7-dion-

3-ethylene ketal (V) which on oximation by carboxymethoxylamine hemihydrochloride gave 17β-hydroxy-5α-androstane-3,7-dion-3-ethylene ketal-7-(O-carboxymethyl) oxime (VI). Finally treatment of this oxime (VI) with acetic acid gave material which was deketalated but still retained its important oxime function. This was the desired hapten plus bridge i.e. 17β-hydroxy-5α-androstane-3, 7-dione-7-(O-carboxymethyl) oxime (VII). Compound VII was coupled to BSA to give compound VIII the desired conjugate by the mixed anhydride procedure of Erlanger, Borek, Bieser, and Liebermann[15]. Due to the lack of radioactive label and unsaturation of the A ring, calculation of the number of haptens bound to BSA was made by the method of Tamaoki *et al.*[16] utilizing nitrotropone, and checked by a modified Zimmermann [17] reaction.

EXPERIMENTAL

All melting points were determined on Reichert micro hot plate and were uncorrected.

Infrared spectra were obtained on a Perkin–Elmer 157 (NaCl spectrophotometer). Optical rotations were obtained from a Perkin–Elmer 141 polarimeter. Ultraviolet spectrophotometric determinations were made using Unicam SP 800 and SP 1800 instruments.

Nuclear magnetic resonance spectra were obtained from a Perkin–Elmer R12B spectrometer.

Gas–liquid chromatography was performed with a Perkin–Elmer F11 Gas Chromatograph fitted with flame ionisation.

Microanalyses were performed by the Isotope Unit, Queen Elizabeth College. Analyses for carbon, hydrogen and oxygen were made.

17β-acetoxy-5α-androstene-3-one-3-ethylene ketal [11]. This compound was prepared from testosterone acetate (I) by exchange dioxolanation using 2-methyl-2-ethyl-1,3-dioxolanone.

The 3 ethylene ketal (II) was then prepared using this reagent by mixing 10 g of testosterone acetate with 180 ml benzene, 200 ml of the dioxolanone and 200 mg of p-toluene sulphonic acid and gently distilling through a column of glass helices (20 × 2 cm.) for $5\frac{1}{2}$ h. About 200 ml of distillate was collected. The reaction mixture was washed successively with aqueous sodium bicarbonate solution and with water. After drying over anhydrous sodium sulphate the material was concentrated to dryness by rotary evaporation and recrystallized from methanol. Yield was 86% m.p. 197–199°. (Literature 202–203°). Thin-layer chromatography (t.l.c.) on silica gel using Benzene–ethyl acetate 4:1 (v/v) (R_F testosterone acetate = 0·39, R_F compound II = 0·56) revealed only a faint trace of starting material. Infrared (I.R.) (CCl_4-max):— 1732 cm.$^{-1}$ (—$OCOCH_3$), 1095 cm.$^{-1}$ and 1030 cm^{-1} (ketal).

17β-acetoxy-5-androstene-3,7-dion-3-ethylene ketal (III). 6·5 g of 17β-acetoxy-5-androsten-3-one-3-ethylene ketal (II) was dissolved in 60 ml carbon tetrachloride and warmed on an oil bath to 80°. A mixture of 80·2 ml carbon tetrachloride solution containing t-butyl chromate equivalent to 14·8 g CrO_3, 7·8 ml acetic acid and 20·8 ml acetic anhydride was added dropwise (over 15 min) and the mixture then stirred for 14 h. The solution was cooled on ice then 15·6 g oxalic acid in 121 ml water was slowly added with continual cooling and stirring. After the frothing subsided a further 6·5 g solid oxalic acid was added and stirring continued for 30 min. The aqueous phase was separated and extracted several times with carbon tetrachloride. The organic extractant was collected (900 ml), washed several times with distilled water until the yellow colour disappeared, dried over anhydrous sodium sulphate and taken to dryness by rotary evaporation. Successive recrystallizing from acetone afforded 2·81 g of pure 17β-acetoxy-5-androstene-3, 7-dion-3-ethylene ketal (III) and 0·86 g of a product containing traces of starting material. Yield = 54·5% m.p. = 255–257°. (Literature = 254–256°). (ref. 13). λ_{max} 241·5 nm (ethanol). $E = 1{\cdot}2 \times 10^4$ M^{-1}. I.R. (CCl_4) = 1732 cm.$^{-1}$ (—$OCOCH_3$), 1672 cm.$^{-1}$ (C=O), 1640 cm.$^{-1}$ (double bond).

17β-acetoxy-5α-androstane-3, 7-dion-3-ethylene ketal (IV). Catalytic hydrogenation was used to saturate the 5-ene bond present in (III). Palladium on charcoal was the method of choice because although its action on 7 keto steroids had not been reported, it has been shown to yield exclusively the required 5α isomer using 17β-acetoxy-5-androsten-3-ethylene ketal [13].

790 mg of 17β-acetoxy-5-androstene-3, 7-dion-3-ethylene ketal (III) and 400 mg 10% palladium on charcoal were placed in a 250 ml conical flask and 80 ml ethyl acetate added. The flask was flushed through with hydrogen for several min and then sealed. After shaking the flask mechanically for 2 h the contents were filtered through a layer of cellulose powder on a glass sinter. The sinter was washed with 25 ml ethyl acetate and the filtrate taken to dryness by rotary evaporation. Weight of oily residue = 780 mg. Thin-layer chromatography (t.l.c.) revealed absence of starting material and showed 2 spots. Gas–liquid chromatography (g.l.c.) also showed two peaks. Ratio of peaks 60:40. Major peak ran as 5α isomer.

The oily residue was taken up in a small amount of ethyl acetate and ran as bands on 8 preparative silica gel plates (PF_{254}). (20 × 20 × 0·075 cm.). The respective bands were developed 3 times in toluene–dioxane–ethanol (100:20:20 by vol.) viewed under U.V. light and the band containing the 5α isomer scraped off and extracted with ethyl acetate. After filtering through a layer of celite the filtrate was taken to dryness dissolved in petroleum ether (40–60) and recrystallized.

Weight of crystals = 475 mg. Yield 60% m.p. 176–177° $[\alpha]_D^{24} = -45{\cdot}5°$ (chloroform). λ_{max} (MeOH) = 287 nm. $E_{max} = 35 M^{-1}$ cm.$^{-1}$ (consistent with saturated ring ketone). I.R. = 1735 cm.$^{-1}$ (acetoxy), 1715 cm.$^{-1}$ (7-ketone), 1095 cm.$^{-1}$ and 1030 cm.$^{-1}$ (ketal). Disappearance of bands at 1672 cm.$^{-1}$ and

1640 cm.$^{-1}$ indicated double bond had been saturated. Molecular rotation $(M)_D^{24} = -177{\cdot}3°$. Calculated value for $5\alpha = -194°$ for $5\beta = -238°$ indicating 5α isomer. n.m.r. (C-18 proton), 1·05 (C-19), 2·00 (17β), 3·94 (ketal), 4·70 (17α). *Anal.* Calculated for $C_{23}H_{34}O_5$. C,70·73; H,8·77. Found: C,70·83, H,9·03.

Further evidence that compound IV was the 5α isomer (17β-acetoxy-5α-androstane-3,7-dion-3-ethylene ketal) was obtained by deketalation of this compound to give 17β-acetoxy-5α-androstane-3,7-dione.

100 mg of IV was dissolved in acetic acid with water (75:25 v/v) (7·5 ml) and heated on a steam bath for 30 min. Fifty ml ether was added, and the solution washed with sodium bicarbonate solution and then with sodium chloride before drying over anhydrous sodium sulphate and evaporating to dryness. Weight of crude material = 77 mg (85% yield). Recrystallization from ethanol gave 54 mg of material which ran as one spot on t.l.c. (toluene–dioxane–acetic acid 25:25:2 by vol.) R_f 0·58. I.R. = 1735 cm.$^{-1}$ (acetoxy), 1715 cm.$^{-1}$ = (7-ketone), 1705 cm.$^{-1}$ = (3-ketone). $M_D^{23} = -128{\cdot}5°$. Calculated value for $5\alpha = -137°$, for $5\beta = -166°$ confirming material was 5α.

17β-hydroxy-5α-androstane-3,7 dion-3-ethylene ketal (V). 280 mg of IV was dissolved in 100 ml of ether and 100 ml of 1% potassium carbonate in methanol–water (80:20 v/v) added. The mixture was stirred at room temperature for 2½ h then at 60° for 1 h. The mixture was then neutralized with 1N hydrochloric acid, 100 ml water added and the product extracted with 3 × 200 ml dichloromethane and solution then taken to dryness. Residue (206 mg) was dissolved in ethyl acetate and ran as bands on 4 preparative layer plates (20 × 20 × 0·075) cm. PF_{254} (Merck). 4 developments in toluene–ethyl acetate (3:1 v/v). Bands scraped off and extracted with methanol–ethyl acetate (1:1 v/v) then filtered through sinter which was washed with methanol.

The residue after evaporation to dryness was taken up in ethyl acetate. Recrystallization from ethyl acetate-pet ether gave a white gelatinous ppt = 190 mg. Yield 76% m.p. 195–196·5°. λ_{max} (MeOH) = 286 nm. E_{max} = 34 M^{-1} cm^{-1}. I.R. showed disappearance of bands at 1735 cm.$^{-1}$ and 1250 cm.$^{-1}$. (17β acetoxy). *Anal.* Calculated for $C_{21}H_{32}O_4$. C, 72·4; H, 9·2. Found C, 72·4. H. 9·6.

17β-hydroxy-5α-androstane-3,7-dion-3-ethylene ketal-7-(O-carboxymethyl) oxime (VI). A mixture of 180 mg of 17β-hydroxy-5α-androstane-3,7-dion-3-ethylene ketal (V) and 2 g of carboxymethoxylamine hemihydrochloride was dissolved in 150 ml plus 165 ml of 1M sodium acetate, and left overnight at room temperature.

The volume was first reduced by rotary film evaporation (40°) and water added to 600 ml. The solution was then adjusted to pH 9·0 with 1N NaOH and extracted with ethyl acetate (4 × 100 ml). The remaining aqueous layer was cooled on ice and adjusted to pH 2·0 by hydrochloric acid and extracted with ethyl acetate (6 × 100 ml) which was washed with water, dried over anhydrous sodium sulphate and taken to dryness by a rotary evaporator at 40°. Recrystallization from ethyl acetate–petroleum ether afforded 195 mg of crystals.

Yield = 89% m.p. 236–238°. I.R. showed presence of oxime peak (1690–1695 cm^{-1}). λ_{max} (Tris (0·05 M) pH 8·4) = 218 nm. $E_{max} = 4{\cdot}5 \times 10^3$ M^{-1} cm.$^{-1}$. T.l.c. using toluene–dioxane–acetic acid (25:25:2 by vol.) gave one spot R_F = 0·43 using toluene–dioxane–acetic acid (40:20:2 by vol.) gave one spot. R_f = 0·09.

17β-hydroxy-5α-androstane-3,7-dion-7-(O-carboxymethyl) oxime (VII). 130 mg of (VI) was dissolved in acetic acid–water (80:20 v/v) and heated on a boiling water bath for 20 min then taken to dryness by rotary evaporation (90°). The residue was purified by running as bands on 5 t.l.c. (20 × 20 × 0·025 cm.) Merck GF_{254} aluminium precoated plates. Development was made 4 times in toluene–dioxane–acetic acid (25:25:2 by vol.), bands located with Brady's reagent by spraying a strip cut off from the side of the plate. The material was eluted with methanol, and after taking to dryness recrystallized from ethyl acetate. Obtained 53 mg. Yield 36%. I.R. showed 1705 cm.$^{-1}$ (3-ketone) and 1690–1695 cm.$^{-1}$ (oxime). λ_{max} (Tris (0·05M) pH 8·4) = 218 nm. $E_{max} = 3{\cdot}6 \times 10^3$. M^{-1} cm^{-1}. Material ran as one spot on t.l.c.

17β-hydroxy-5α-androstane-3,7-dion-7-(O-carboxymethyl) oxime-BSA conjugate (VIII). A mixture of 41 mg of (VII), 27 μl of tri-n-butylamine and 1·4 ml of dry dioxane was cooled to 4°. 14·2 μl of isobutyl chloroformate was then added and the solution left for 30 min. 100 mg of BSA dissolved in 4·34 ml of dioxane–water (1:1 v/v) and 0·09 ml of 1N sodium hydroxide was then added and stirred for 1 h when a further 50 μl of 1N sodium hydroxide was added to maintain the pH at 8·0. Stirring was continued in the cold for 3 h. Low molecular weight materials were then removed from the reaction mixture using a Sephadex G-25 column (30 × 1 cm., equilibrated in 0·9% sodium chloride) by eluting with 0·9% sodium chloride solution. The material was then dialyzed against 20 l of distilled water overnight, and finally lyophylised. The conjugate was obtained as 65 mg of white fluffy powder. Estimation of the number of moles of steroid incorporated into a mole of BSA was made by the method of Tamaoki *et al.* [16] using nitrotropone was found to be 38 and be 28 when the conjugate was hydrolysed by hydrochloric acid and the resultant 17β-hydroxy-5α-androstane-3,7-dione estimated by the Zimmermann reaction [17] using a standard prepared by dekelation of (IV).

Preparation of antisera. Three male rabbits weighing 2–3 kg were immunized with 250 μg antigen, by the multiple site intradermal technique of Vaitukaitis *et al.*[18]. Each animal received 2 ml of a mixture consisting of the above amount of antigen in 1 ml saline and 1 ml Freund's incomplete adjuvant reinforced by 5 mg M. Butyricum. The backs of the animals were shaved and the injection made in 50–60

sites. Pertussis vaccine (0·5 ml) was also injected in one leg of the animals. Serum samples were obtained by puncture of an ear vein. A titre of 1:1500 was obtained after about six weeks.

Characterization of antisera. Assessment of the specificity of the antisera was made using the liquid phase radioimmunoassay method of Hotchkiss, Atkinson and Knobil[19]. Calculation of the cross reactions were made as indicated by Abrahams[20] at the 50% level.

RESULTS

Antisera from the rabbits did not differ significantly in specificity therefore detailed characterization of one antiserum only is presented. The results of the cross reactivity of various related steroids tested for their ability to compete with 9 pg radioactively labelled 5α-DHT for binding sites of the antiserum are shown in Table 1. The serum was reasonably specific with respect to substitution in rings A and D and it did not seriously cross react with steroids such as 17α-hydroxy-5α-androstan-3-one (epi-5α-DHT) (4·0%) or with the 3α or 3β, 17β diols (all less than 4%). The antiserum showed a strict requirement for the 5α configuration of the A/B ring junction in that the cross reaction with 5β-DHT was only 4%. The only major cross reactant was testosterone (51%) which has a similar overall skeletal shape to 5α-DHT.

DISCUSSION

This paper describes the preparation of an immunogenic conjugate of 5α-dihydrotestosterone (5α-DHT) in which the steroid was attached *via* a O-carboxymethyl oxime bridge to carbon 7. This type of conjugation is similar to that made by many investigators for oestrogens. Unfortunately the antisera to 5α-DHT is not as specific as those raised by the oestrogens since it seriously cross reacts with testosterone. However apart from this particular cross reaction the antisera to 5α-DHT is reasonably specific to all other androgens tested.

Table 1. Percentage cross reactions of the 5α-DHT antiserum

Steroid	Percentage cross reaction
5α-dihydrotestosterone	100
Testosterone	51
17β-hydroxy-5β-androstan-3-one	4.0
17α-hydroxy-5α-androstan-3-one	4.0
5α-androstane-3β, 17β-diol	2.5
17α-hydroxy-4-androsten-3-one	2.0
5α-androstane-3α,17β-diol	0.7
4-androstene-3, 17-dione	0.7
5α-androstan-17β-ol	< 0.5
5β-androstan-17β-ol	< 0.5
4-androsten-17β-ol	< 0.5
5α-androstan-3-one	< 0.5
5β-androstan-3-one	< 0.5
4-androsten-3-one	< 0.5
3β-hydroxy-5α-androstan-7-one	< 0.5
5α-pregnane-3, 20-dione	< 0.5
4-pregnane-3, 20-dione	< 0.1
17α-hydroxy-4-pregnane-3, 20-dione	< 0.1
3β-hydroxy-5-androstan-17-one	< 0.1
11β ,17, 21-trihydroxy-4-pregnane-3, 20-dione	< 0.001
11β, 21-dihydroxy-4-pregnane-3, 20-dione	< 0.001
17, 21-dihydroxy-4-pregnane-3, 20-dione	< 0.001
Cholesterol	< 0.001

Very recently (some time after this particular work was commenced), Bauminger, Kohen, Lidner and Weinstein [21] prepared 17β-hydroxy-5α-androstan-3-one-1α carboxymethyl thioether (5α-DHT-C_1 BSA). This antigen raised antisera to 5α DHT which possessed a 10% cross reaction with testosterone and a 16% cross reaction with 5α-androstone-3α-17β-diol. Thus this antisera had less cross reaction for testosterone than that reported in this paper, but the lack of specificity to the 5α diol which is known to exist in plasma in significant amounts [22] is in contrast to the present antisera which possesses reasonable specificity to all the androstanediols.

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