Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1085–1090. Pergamon Press. Printed in Great Britain

THE PREPARATION OF THREE 5α-DIHYDROTESTOSTERONE–BSA CONJUGATES: A COMPARISON OF THE ANTIGENIC PROPERTIES

R. CONDOM* and B. DESFOSSES†
*Laboratoire de Chimie Structurale Organique, Université de Nice, Parc Valrose, 06034 Nice Cédex, and
†Laboratoire de Chimie Biologique, U.E.R. Biomédicale des Saints Pères, 45, rue des Saints Pères, 75270 Paris, France

(*Received* 12 *January* 1977)

Abstract—Antisera against dihydrotestosterone (DHT) were raised in rabbits with conjugates obtained by coupling 5α-DHT-1α-carboxymethyl,5α-DHT-6-(*O*-carboxymethyl) oxime, 5α-DHT-7-(*O*-carboxymethyl) oxime with bovine serum albumin. The preparation of the haptens has been described. The comparison of cross reactions of the antisera induced by these immunogens confirmed the importance of the site of linkage on the steroid. All antisera showed high cross-reaction with testosterone. The most specific antiserum was obtained with 5α-DHT-7-(*O*-carboxymethyl) oxime:BSA.

INTRODUCTION

After the results obtained with progesterone 11α-hemi-succinate:BSA, Midgley and Niswender [1] put forward the hypothesis that an antigen where the steroid has free functional groups must raise more specific antibodies than antigens where the steroid functional groups are used to bind the hormone to the protein. This hypothesis proved particularly accurate in the case of estrogenic [2] and androgenic [3] hormones.

We have studied several androgenic steroids which we have used in the preparation of haptens and antigens where the steroid is bound to bovine serum albumin (BSA) by a linkage (*O*-carboxymethyl) oxime at C-6 or C-7 or a linkage carboxymethyl at C-1 or C-15 [4] of the steroid.

This report relates to the preparation of the following antigens 5α-dihydrotestosterone-6-CMO:BSA and 5α-dihydrotestosterone-7-CMO:BSA. The synthesis of the antigen 5α-dihydrotestosterone-1α-carboxymethyl:BSA has already been described [4]. The specificity of the antibodies which these three antigens elicited in rabbits has been compared.

METHODS AND MATERIALS

1. *Preparation of the 5α-DHT-6-(O-carboxymethyl) oxime:BSA conjugate is outlined in Fig. 1.* 17β-Acetoxy-5-androsten-3-one-3-ethylene ketal was prepared and this compound was submitted to the action of perbenzoic acid. The resulting epoxide ring was opened and the diol formed when refluxed in a solution of formic acid in methanol gave the di-ketone I. Ketalisation of the ketone at C-3 was achieved by exchange with 3,3-ethylenedioxy-butane. Thus II was obtained. Condensation of II with carboxymethoxylamine hemi-hydrochloride followed by a deketalisation gave the hapten IV. The condensation of IV to bovine serum albumin (BSA) according to the method

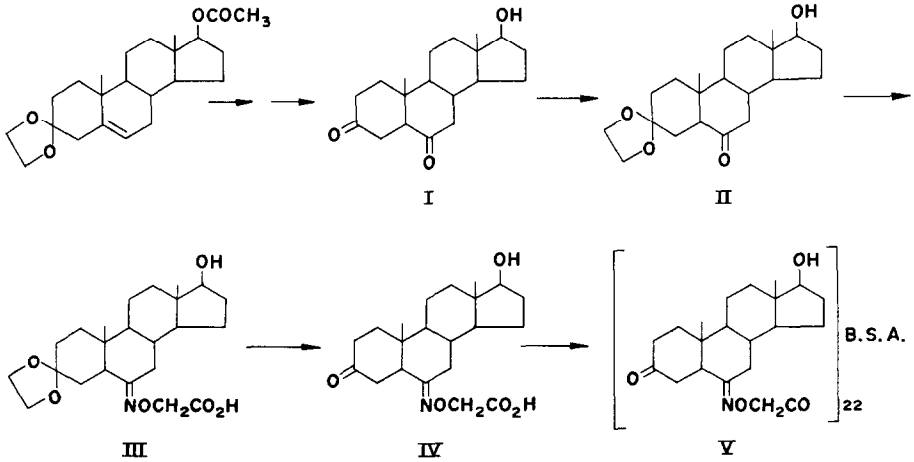

Fig. 1. Preparation of 5α-DHT-6-CMO:BSA conjugate

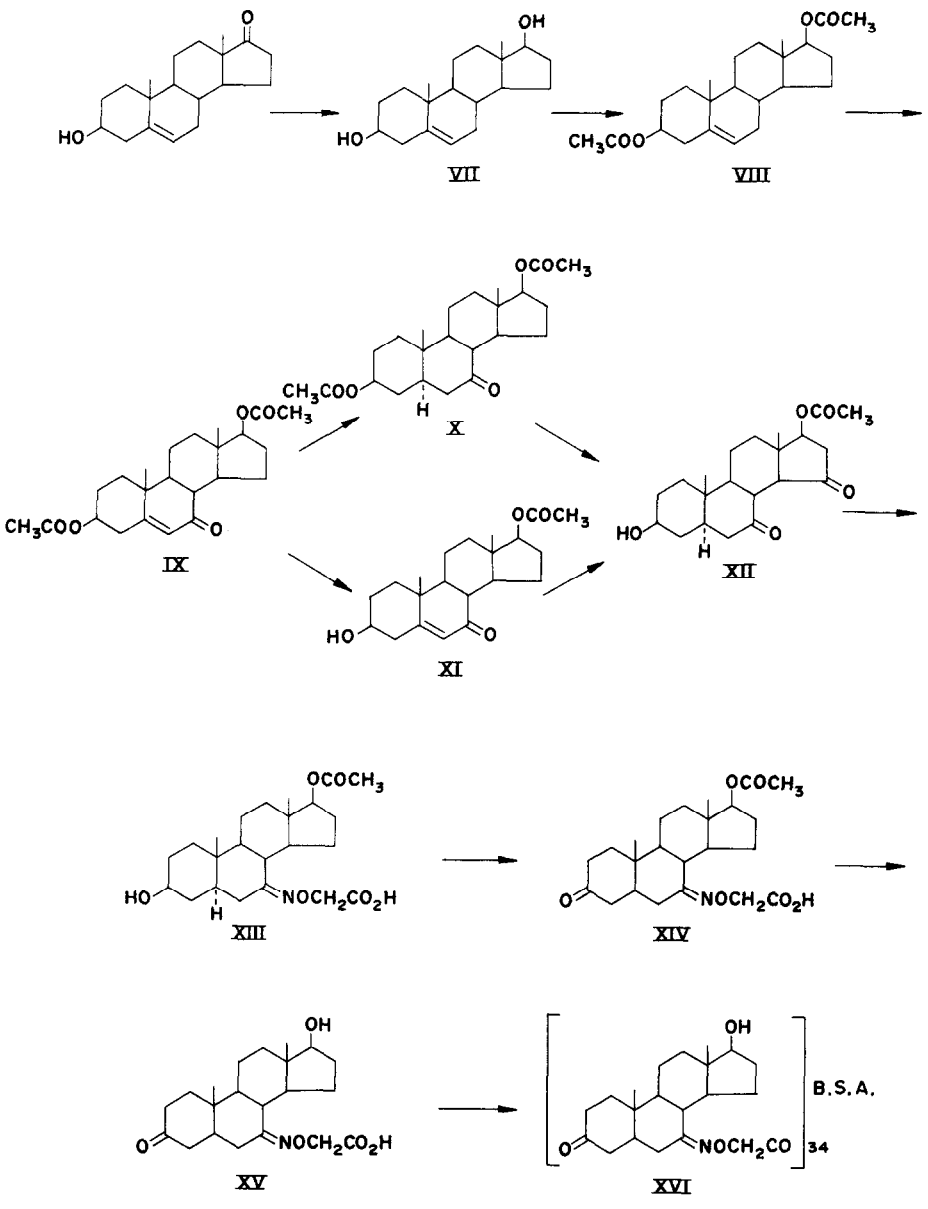

Fig. 2. Preparation of 5α-DHT-7-CMO:BSA conjugate.

of Vaughan[5] gave the antigen V. The number of steroid molecules incorporated per mol of BSA was measured according to Tamaoki *et al.* [6] with nitrotropone. Optical density was measured taking as reference the extinction coefficient of nitropone-BSA as 5 $10^3 M^{-1}cm^{-1}$ at 420 nm, 22 mol of haptens were linked to one mol of BSA.

2. *Preparation of 5α-DHT-7-(O-carboxymethyl) oxime:BSA conjugate is outlined in Fig. 2.* Dehydroepiandrosterone was reduced with potassium borohydride then acetylated giving compound VIII. Oxidation of the diacetoxy VIII with Na_2CrO_4 in a mixture of acetic anhydride–acetic acid gave after precipitation in $NaHCO_3$-saturated aqueous solution the 3β,17β-diacetoxy-5-androsten-7-one (IX). 17β-Acetoxy-3β-hydroxy-5α-androstan-7-one (XII) can be obtained either by the reduction of the double bond of IX on 10% palladium on charcoal in chloroform followed by a saponification of C-3-acetoxy, or by monosaponification of 3β,17β-diacetoxy-5-androsten-7-one (IX) yielding the 3β-hydroxy compound reduced in the same way as X and giving XII. The monosaponification was carried out with KOH in methanol at 0° and was followed by t.l.c. Condensation of XII in pyridine with carboxymethoxylamine hemi-hydrochloride gave the acid XIII which was submitted to oxidation with Jones reagent in acetone at 0°. The C-3-one compound XIV was obtained. Saponification of 17β-acetoxy of XIV gave the hapten XV. Condensation with BSA, as described for 5α-DHT-6-CMO : BSA, gave an antigen XVI in which 34 mol of hapten XV were linked to one mol of BSA.

EXPERIMENTAL

All melting points were determined on a Tottoli apparatus and were uncorrected. Infrared spectra were obtained on a Perkin Elmer 254 (KBr pellets). Nuclear Magnetic Resonance spectra were obtained from a Varian EM 360 with tetramethylsilane as the internal standard. Microanalyses were done by Service de Microanalyse, Centre National de la Recherche Scientifique, Thiais, France.

17β-Hydroxy-5α-androstane-3,6-dione (*I*). 17β-Acetoxy-5-androsten-3-one-3-ethyleneketal (9 g) was dissolved in 200 ml of perbenzoic acid (53 mEq) in benzene. The mixture was kept in the dark at room temperature for 2 days. This mixture was washed with a solution of $NaHCO_3$, dried and evaporated to dryness. The residue was dissolved in a mixture of methanol (100 ml) and formic acid (150 ml). After 5 h, the solution was poured into cold water and extracted with $CHCl_3$ (3 × 75 ml). The organic layer was washed with a solution of $NaHCO_3$ (5%), then water, and dried. The residue was dissolved in methanol (300 ml) to which a solution (65 ml) of KOH (13 g) was added. The mixture was refluxed for 1 h under N_2 then neutralized with acetic acid and distilled off under reduced pressure. The residue was dissolved with chloroform, washed with water, and evaporated to dryness.

The product was crystallized from methanol, 2.5 g, m.p. 220–228°. An analytical sample was recrystallized from aqueous methanol, m.p. 228–229°. (lit. [7] m.p. 234–236°C, $CHCl_3$–Hexane). Anal. Calc. for $C_{19}H_{28}O_3$: C, 75.00; H, 9.21. Found: C, 75.16; H, 9.43. n.m.r. ($CDCl_3$) δ ppm 0.80 (C_{18} methyl, 3H, *S*) 0.97 (C_{19} methyl, 3H, *S*). 3.70 (H_{17},1 H, m). I.R. $\bar{\nu}$ max. 3520; 2900; 1700 cm^{-1}.

17β-Hydroxy-5α-androstan-3-one-3-ethylene ketal (*II*). The di-ketone (I) (400 mg) was dissolved in 3,3-ethylenedioxy butane (10 ml) and refluxed. *P*-toluene sulphonic acid (10 mg) in 3,3-ethylene dioxybutane (1 ml) was added and the mixture was refluxed 6.5 min. The solution was cooled and a few milliliters of an aqueous solution of $NaHCO_3$ were added. After separation, the organic layer was collected, and evaporated to dryness.

The residue was crystallized from ethyl acetate giving II (370 mg), m.p. 184–187°. One recrystallization from the same solvent gave the analytical sample, m.p. 188–190° (lit. [8] m.p. 188.2–188.7°). Anal. Calc. for $C_{21}H_{32}O_4$: C, 72.41; H, 9.19. Found: C, 72.16; H, 9.46. I.R. $\bar{\nu}$ max. 3480; 2900; 1720 cm^{-1}.

17β-Hydroxy-5α-androstane-3,6-dione-3-ethylene-ketal-6-(O-carboxymethyl)oxime (*III*). The ketone (II) (184 mg) was dissolved in a mixture of carboxy methoxylamine hemihydrochloride (184 mg) in pyridine (20 ml). This mixture was stirred, at 40°, for 48 h. Pyridine was distilled off under reduced pressure and the residue dissolved in $CHCl_3$ and an aqueous basic solution. The aqueous layer was collected, acidified and extracted with $CHCl_3$. This organic solution was washed and evaporated to dryness. The residue was crystallized from methanol to give the analytical product III (121 mg), m.p. 214–217°.

After addition of water, the mother liquors gave a second crop (65 mg), m.p. 210–213°. Anal. Calc. for $C_{23}H_{35}O_6N$: C, 65.53; H, 8.37; O, 22.77. Found: C, 65.32; H, 8.23; O, 22.49. I.R. $\bar{\nu}$ max. 3350; 2900; 1720 cm^{-1}.

17β-Hydroxy-5α-androstane-3,6-dione-6-(O-carboxymethyl) oxime (*IV*). The ketal (III) (700 mg) was dissolved in acetone (50 ml). *P*-toluene sulfonic acid (50 mg) was added; the solution was stored at room temperature for 72 h and then evaporated to dryness. The residue was dissolved in $CHCl_3$. The organic solution was washed with water, dried over Na_2SO_4 and evaporated.

Crystallization from diethyl ether gave IV (452 mg), m.p. 125–128°.

An analytical sample was recrystallized from a mixture of diethyl ether–hexane, m.p. 145–146°. Anal. Calc. for $C_{21}H_{31}O_5N$: C, 66.82; H, 8.27; O, 21.19; N, 3.71. Found: C, 66.60; H, 8.17; O, 21.19; N, 3.82. n.m.r. (DMSOd6) δ ppm 0.65 (C_{18}methyl, 3H, *S*) 0.92 (C_{19} methyl, 3H, *S*); 4.50 (CH_2 oximino, 2H, *S*). I.R. $\bar{\nu}$ max. 3350; 2900; 1700 cm^{-1}.

17β-Hydroxy-5α-androstane-3,6-dione-6-(O-carboxymethyl)oximino BSA–conjugate (*V*). A mixture of 187.5 mg of IV, 98.5 mg of *n*-tributylamine and 4 ml of dioxane was cooled to 4°. Isobutyl chloroformate (74 mg) was added and the mixture stored for 1 h.

A mixture of 690 mg of BSA (Armour Fraction V) in 18 ml of distilled water 0.69 ml of 1M NaOH and 18 ml of dioxane was prepared and stored for 0.5 h at 4°.

The first solution was slowly added to the second mixture. After 2h, 0.33 ml of 1M NaOH was added and the solution was stirred for 2 h at 4°. The solution was dialyzed against cold water for 12 h. The antigen was precipitated at pH 4.5 with dilute HCl. After two days, centrifugation gave the antigen which was dissolved by addition of a solution of $NaHCO_3$. This solution was then dialyzed against distilled water and lyophilized.

3β,17β-Acetoxy-5-androsten-7-one (*IX*). 5-Androsten-3β,17β-diol-3β,17β-diacetate (12 g) was poured into a mixture of acetic anhydride (70 ml) and acetic acid (140 ml). The mixture was stirred, cooled at 20°, and 18.5 g of sodium chromate added in small amounts. The mixture was stirred for 48 h at room temperature, then poured into a saturated aqueous solution of $NaHCO_3$. The precipitate was filtered and dissolved in chloroform. This organic solution was washed with distilled water, dried and taken to dryness. The residue which was crystallized from methanol gave 5.0 g of (IX), m.p. 215–217° (lit. [9] m.p. 219–221°C, [10] m.p. 219–221°C). Anal. Calc. for $C_{23}H_{32}O_5$: C, 71.01; H, 8.30; O, 20.59. Found: C, 71.09; H, 8.24; O, 20.47. I.R. $\bar{\nu}$ max. 1730; 1660; 1630; 1240 cm^{-1}.

3β,17β-Diacetoxy-5α-androstan-7-one (*X*). Compound IX (3.37 g) was placed in an hydrogenating

flask with 50 ml of methanol, 0.2 ml of pyridine and 1.5 g of 10% palladium on charcoal. When the theoretical quantity +10% of H_2 was fixed, the solution was filtered. The solid was washed with hot acetone. The organic solutions were collected and evaporated to dryness.

Crystallization from methanol gave the analytical compound X, 2.23 g, m.p. 195–196° (lit. [9] m.p. 195–197°, [10] m.p. 192–193°).

A second crop, 330 mg, m.p. 191–193°, was obtained by addition of water to the mother-liquor. Anal. Calc. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.77; O, 20.48. Found: C, 70.60; H, 8.82; O, 20.65. I.R. $\bar{\nu}$ max. 1730; 1690; 1250 cm^{-1}. n.m.r. ($CDCl_3$) δ ppm 0.83 (C_{18}methyl, 3H, *S*) 1.13 (C_{19}methyl, 3H, *S*); 2.09 (diacetoxy, 6H, *S*) 4.70 ($H_{3\alpha}$, $H_{17\alpha}$, 2H, m).

17β-Acetoxy-3β-hydroxy-5-androsten-7-one (XI). 3β,17β-Diacetoxy-5-androsten-7-one (1 g) was dissolved in methanol (60 ml). The solution was stirred and cooled at 0°. 400 mg of KOH in 2.0 ml of water were added. After 2 h, t.l.c. showed the complete disappearance of the original product, then 1M HCl was added to neutrality.

By evaporation, 650 mg of analytical compound, m.p. 232–234° were obtained.

A second crop was a mixture of partially and totally saponified products. Anal. Calc. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.72. Found: C, 72.69; H, 8.75. I.R. $\bar{\nu}$ max. 3450; 1730; 1655; 1630; 1240 cm^{-1}. n.m.r. ($CDCl_3$) δ ppm 0.81 (C_{18}methyl, 3H, *S*) 1.20 (C_{19}methyl, 3H, *S*); 2.04 (3β-acetoxy, 3H, *S*) 4.63 ($H_{17\alpha}$, 1H, m); 5.68 (H_6, 1H, *S*).

17β-Acetoxy-3β-hydroxy-5α-androstan-7-one (XII). (a) From 17β-acetoxy-3β-hydroxy-5-androsten-7-one (XI). Compound XI (305 mg) was dissolved in a mixture of 12 ml of methanol, one drop of pyridine and 80 mg of 10% palladium on charcoal. When the theoretical +5% quantity of hydrogen was absorbed, the mixture was filtered and the solid washed with hot acetone.

After evaporation to dryness, the residue was crystallized from aqueous-methanol, giving the compound (XII), 210 mg, m.p. 184–186°. Anal. Calc. for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found: C, 72.24; H, 9.33. I.R. $\bar{\nu}$ max. 3450; 1730; 1690; 1250 cm^{-1}.

(b) From 3β,17β-diacetoxy-5α-androstan-7-one (X). The monosaponification was carried out in the same manner as for compound XI. 3β,17β-Diacetoxy-5α-androstan-7-one (1 g) gave 0.8 g of XII.

17β-Acetoxy-3β-hydroxy-5α-androstan-7-one-7-(O-carboxymethyl)oxime (XIII). 17β-Acetoxy-3β-hydroxy-5α-androstan-7-one (450 mg) (XII) was dissolved in 15 ml of anhydrous pyridine. Carboxymethoxylamine hemihydrochloride (600 mg) was added, and the mixture was stirred at room temperature for 8 days.

After evaporation to dryness, the residue was taken up with chloroform, washed with 1M HCl, water, dried and evaporated to dryness.

Crystallization from aqueous methanol gave 317.5 mg of crystals, m.p. 225° (dec.). Anal. Calc. for $C_{23}H_{35}O_6N$: C, 65.53; H, 8.37; N, 3.32. Found: C, 65.61; H, 8.26; N, 3.38. I.R. $\bar{\nu}$ max. 3390; 1720; 1640; 1220 cm^{-1}. n.m.r. ($CDCl_3$) δ ppm 0.76 (C_{18}methyl, 3H, *S*) 0.97 (C_{19}methyl, 3H, *S*); 2.03 (acetoxy, 3H, S) 4.57 (carboxymethyl, 2H, *S*); 5.95 (H acid).

17β-Acetoxy-5α-androstane-3,7-dione-7-(O-carboxymethyl)oxime (XIV). Compound XIII (637 mg) was dissolved in 225 ml of acetone and the solution was stirred and cooled to 10°. Jones reagent (1.5 ml) (26.72 g of CrO_3, 23.0 ml of Conc. H_2SO_4, water to 100 ml) was added. After 12 min, a few milliliters of ethanol was introduced. The mixture was poured into water and chloroform. After extraction, the organic layer was evaporated to dryness. The residue was crystallized from aqueous methanol and gave 630 mg of XIV, m.p. 225° (dec.). Anal. Calc. for $C_{23}H_{33}O_6N$: C, 65.85; H, 8.92; N, 3.33. Found: C, 65.61; H, 8.26; N, 3.58. I.R. $\bar{\nu}$ max. 3300; 1740; 1700; 1640 cm^{-1}. n.m.r. ($DMSOd_6$) δ ppm 0.70 (C_{18}methyl, 3H, *S*) 1.04 (C_{19}methyl, 3H, *S*); 1.91 (acetoxy, 3H, *S*) 4.37 (carboxymethyl, 2H, *S*).

17β-Hydroxy-5α-androstane-3,7-dione-7-(O-carboxymethyl)oxime (XV). Compound XIV (100 mg) was dissolved in 2.5 ml of methanol. A solution of 40 mg

Table 1.

Steroid	% cross reactivity (50%) DHT-1α-CM : BSA*	DHT-6-CMO : BSA*	DHT-7-CMO : BSA
5α-Dihydrotestosterone	100	100	100
Testosterone	23	60	23
17β-Hydroxy-5β-androstan-3-one	31.5		
5α-Androstan-3α,17β-diol		23	0.5
5α-Androstan-3β,17β-diol		7	0.4
4-Androsten-3,17-dione	0.6	1.9	3.0
4-Androsten-3β,17β-diol	1.2	3.4	
3α-Hydroxy-5α-androstan-17-one	0.17	1.4	<0.1
3α-Hydroxy-5β-androstan-17-one	0.1	0.1	
3β-Hydroxy-5α-androstan-17-one		0.3	
17α-Hydroxy-4-androsten-3-one		0.12	<0.1
3β-Hydroxy-5-androsten-17-one	<0.1	0.25	<0.1
5-Pregnene-3,20-dione		<0.03	<0.1
11β,17,21-Trihydroxy-4-pregnene-3,20-dione		<0.03	<0.1
5α-Pregnane-3β,20-diol			<0.1
17-Hydroxy-4-pregnene-3,20 dione			<0.1
Estrone		<0.03	
Estradiol			<0.1

* These antisera were obtained and calculations of cross reactions were made by B. Kouznetzova, Institut Pasteur, Paris, Laboratoire de Radioimmunologie analytique (directed by Dr F. Dray).

of KOH in 0.5 ml of water was added and the mixture was kept 12 h at room temperature. After addition of formic acid and evaporation to dryness, the residue was dissolved in ethyl acetate, washed with distilled water, and evaporated.

Crystallization from 0.3 ml of ethyl acetate gave 47.8 mg of white crystals, m.p. 200–201°. Anal. Calc. for $C_{21}H_{31}O_5N$: C, 66.83; H, 8.28; N, 3.71. Found: C, 66.85; H, 8.32; N, 3.91. I.R. $\bar{\nu}$ max. 3310; 1730; 1640 cm $^{-1}$. n.m.r. ($CDCl_3$) δ ppm 0.72 (C_{18}methyl, 3H, *S*) 1.05 (C_{19}methyl, 3H, *S*); 3.10 ($H_{17\alpha}$, 1H, m) 4.50 (CH_2oximino, 2H, *S*).

17β-Hydroxy-5α-androstane-3,7-dione-7-(O-carboxymethyl)oximino–BSA conjugate (XVI). The condensation of compound XV with BSA was carried out as for DHT-6-CMO-BSA.

Preparation of antisera. Two rabbits for DHT-7-CMO: BSA, five rabbits for DHT-6-CMO:BSA, three rabbits for DHT-1α-CM:BSA were used for immunization.

The primary injection (1mg) of the conjugate incorporated in 0.5 ml of NaCl (0.9%) and 0.5 ml of Freund's complete adjuvant was injected at multiple subcutaneous sites [11]. The same day, 0.2 ml of *Bordetella pertussis* was also injected. About six weeks after the primary injection, 3 × 0.1 mg of antigen were injected (at a single subcutaneous site (0.1 mg), intramuscularly (0.1 mg), intravenously (0.1 mg)). Plasma was collected two or three weeks after the third injection. The titres of 1:3,000 for anti-DHT-7-CMO:BSA serum, 1:20,000 for anti-DHT-6-CMO: BSA serum and 1:10,000 for anti-1α-CM:BSA serum were obtained.

RESULTS

Calculation of the degree of cross reaction was made as indicated by Abraham[12] at the 50% level and also cross reactions at 1 ng[13] were given for anti-DHT-7-CMO:BSA. All rabbits produced highly specific antisera. Among the steroids tested testosterone showed cross reactions with anti-DHT-1α-CM:BSA (23%), anti-DHT-6-CMO:BSA (60%), anti-DHT-7-CMO:BSA (23%). The anti-DHT-1α-CM:BSA cross reacted with 5β-DHT (31.5%), the anti-DHT-6-CMO:BSA with the 3α,17β-diols (23%) and the anti-DHT-7-CMO:BSA with androstenedione (3.0%). All other steroids showed minor cross reactions (Table 1). The results with anti-5α-DHT-7-CMO:BSA support the results obtained by Exley and Baker[14].

DISCUSSION

The two linkages which are used in the synthesis of the three antigens described do not have the same physical properties: the (*O*-carboxymethyl) oxime linkage induces a structural change on the ring where it is bound, this change is transmitted to ring A when this linkage is fixed to the ring B as in the two antigens described here. This is reflected in the results of cross reaction. The smallest cross reaction with testosterone was obtained with anti-DHT-1α-CM:BSA (23%) and anti-DHT-7-CMO:BSA (23%). This comparatively high cross reaction may be due to the steric hindrance of the characteristic (i.e. C-5) site by the methylene group of the carboxymethyl linkage or of the carboxymethyl oxime linkage. The difference between testosterone and 5α-DHT is thus masked. This is supported also by the Bauminger results[15] from anti-5α-DHT-1α-carboxyethylthioether:BSA. This antiserum cross reacts with testosterone (10%) and 5β-DHT (3%). Our anti-5α-DHT-1α-CM:BSA gives 31.5% cross reaction with this steroid. This difference may be due to the radius of sulphur (1.8 Å) which is smaller than that of the methylene group (2.0 Å) and which produces less steric hindrance at C-5. The binding of (*O*-carboxymethyl) oxime at C-6 or C-7 entails a change in the configuration of rings B and A. The DHT in these haptens has a configuration similar to that of testosterone. Thus the cross reactions obtained with the antisera raised from these antigens are greater or equivalent (60% and 23% respectively) to that raised by anti-5α-DHT-1α-CM:BSA (23%). The increased cross reaction of anti-5α-DHT-7-CMO:BSA compound as compared with anti 5α-DHT-6-CMO:BSA with respect to testosterone can be accounted for by the increase of steric hindrance of the A/B junction in the latter case.

Thus, the 5α-DHT-7-CMO:BSA gives a more specific antiserum for the radioimmunoassay. The cross reaction with testosterone necessitates chromatography of the sample.

Table 2.

Steroid	% of cross reactivity (1 ng) antiserum to 5α-DHT-7CMO:BSA
5α-Dihydrotestosterone	100
3β-Hydroxy-5-androsten-17-one	1
5α-Androstan-3α,17β-diol	14
5α-Androstan-3β,17β-diol	20
5-Pregnene-3,20-dione	0
4-Androsten-3,17-dione	24
17α-Hydroxy-5-pregnene-3,20-dione	2
17β-Hydroxy-4-androsten-3-one	77
3α-Hydroxy-5α-androstan-17-one	1
11β,17,21-Trihydroxy-4-pregnene-3,20-dione	0
5α-Pregnane-3β,20-diol	1
17α-Hydroxy-4-androsten-3-one	5
Estradiol	0

Acknowledgements—This investigation was supported by research grants from D.G.R.S.T. (No. 73-7-1043) and from Institut National de la Santé et de la Recherche Médicale (No. 76–1–134–4).

REFERENCES

1. Midgley A. R. and Niswender G. D.: In *Steroid Assay by Protein Binding* 2nd Karolinska Symposium, in *Research Methods in Reproductive Endocrinology*. Geneve (1970), *Acta endocr., Copenh.* **64** (1970) 320.
2. for example: Exley D., Johnson M. W. and Dean P. D. G.: *Steroids* **18** (1971) 605–620.
3. for example: Weinstein A., Lindner H. R., Friedlander A. and Bauminger S.: *Steroids* **20** (1972) 789–811.
4. a. Condom R. and Emiliozzi R.: *C.R. hebd. Séanc. Acad. Sci., Paris* ser. C **277** (1973) 983–984. b. Condom R., Duval D. and Emiliozzi R.: *C.R. hebd. Séanc. Acad. Sci., Paris* ser. C **276** (1973) 303–305. c. Condom R. and Emiliozzi R.: *Steroids* **23** (1974) 483–498. d. Condom R.: *C.R. hebd. Seánc. Acad. Sci., Paris* ser. C **281** (1975) 139–141.
5. Vaughan J. R.: *J. Am. Chem. Soc.* **73** (1951) 3347–3349.
6. Tamaoki H., Murase Y., Minato S. and Nakanishi K.: *J. biol. Chem.* **62** (1967) 7–14.
7. Rosenkranz G., Velasco M. and Sondheimer F.: *J. Am. Chem. Soc.* **76** (1954) 504.
8. Barnikol-Oetler K., Zepter R. and Heller K.: *J. Prakt. Chem.* **27** (1965) 218–223.
9. Heusler K. and Wettstein A.: *Helv. chim. Acta* **35** (1952) 284–289.
10. Ringold H. J.: *J. Am. Chem. Soc.* **82** (1960) 961–963.
11. Vaitukaitis J., Robbins J. B., Nieschlag E. and Ross G. T.: *J. clin. Endocr. Metab.* **33** (1971) 988–991.
12. Abraham G. E.: *J. clin. Endocr. Metab.* **29** (1969) 866–876.
13. de Lauzon S., Cittanova N., Desfosses B. and Jayle M. F.: *Steroids* **22** (1973) 747–761.
14. Exley D. and Baker T. S.: *J. steroid Biochem.* **7** (1976) 109–112.
15. Bauminger S., Kohen F., Lindner H. R. and Weinstein A.: *Steroids* **24** (1974) 477–488.