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PREPARATION AND ANTIGENIC PROPERTIES OF TESTOSTERONE-4-BOVINE SERUM ALBUMIN CONJUGATES*

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SUMMARY

New hapten-carrier conjugates were prepared from 4-hydroxytestosterone 4-hemisuccinate and 4-hemiglutarate by coupling with bovine serum album employing the mixed anhydride technique. The specificity of anti-testosterone antisera elicited in the rabbits by immunization with these antigens was tested by cross-reaction studies with the related steroids in the radioimmunoassay procedure and discussed from the stereochemical point of view.

INTRODUCTION

Since the first report by Nugent and his coworkers[1], a number of attempts have been made on the preparation of anti-testosterone (17β-hydroxy-4-androsten-3-one) antisera for use in radioimmunoassay by coupling the hapten to the carrier protein. The specificity of such antibodies is markedly influenced by the position on the testosterone molecule used for conjugation to the carrier. It has also been suggested that the stereochemistry of the steroid derivative in the antigen including coplanarity and rigidity is one of the important factors which determine the specificity of antisera [2, 3]. The position C-4 in the testosterone molecule appears to be an attractive site for attachment of the carrier because the bridge linked to trigonal carbon provides the characteristic feature in stereochemistry and hydroxylation at this position in the metabolism of testosterone has not yet been reported. The present paper deals with the synthesis of new type of haptens possessing a bridge at C-4 in testosterone and the specificity of antisera raised in the rabbit against these haptens in the radioimmunoassay procedure.

MATERIALS AND METHODS

Synthesis of haptens

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were determined in chloroform solution. Nuclear magnetic resonance (n.m.r.) spectra were run on a JEOL Model PS-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviation used s = singlet, m = multiplet, and t = triplet. Ultraviolet (U.V.) spectra were measured in 0.05 M phosphate buffer (pH 7.0).

4-Hydroxytestosterone bis(tert-butyldimethylsilyl) ether(II). To a solution of 4,5-epoxy-17β-hydroxyandrostan-3-one (**I**) (1.6 g) in methanol (32 ml) was added 50% H_2SO_4 (7.5 ml), and the reaction mixture was allowed to stand at room temperature for 12 h. After neutralization with 50% NaOH the methanol was evaporated under reduced pressure, and the resulting solution was extracted with ethyl acetate. The organic phase was washed with water and dried over anhydrous Na_2SO_4. After the usual preparation the residue obtained was chromatographed on silica gel (50 g). Elution with hexane–ethyl acetate (2:1, V/V) gave the crystalline product (960 mg), which in turn was recrystallised from acetone to give 4-hydroxytestosterone as colorless needles: m.p. 216–219°C (reported m.p. 222–223°C) [4]. To a solution of the product in dimethylformamide (2.6 ml)–pyridine (1.3 ml) were added imidazole (4.8 g) and *tert*-butyldimethylsilyl chloride (2.4 g), and the reaction mixture was stirred at room temperature for 12 h. The resulting solution was diluted with ether, washed with water, and dried over anhydrous Na_2SO_4. After evaporation of the solvent the residue was chromatographed on silica gel (50 g). Elution with hexane–ethyl acetate (30:1, V/V) and recrystallization of the eluate from methanol gave **II** (850 mg) as colorless needles: m.p. 113–114°C. $[\alpha]_D^{18} + 54.1°$ ($c = 0.15$). n.m.r. ($CDCl_3$) δ ppm 0 (6H, *s*, 17-OSi$(CH_3)_2$), 0.12 and 0.18 (each 3H, *s*, 4-OSi $(CH_3)_2$), 0.75 (3H, *s*, 18-CH_3), 0.89 (9H, *s*, 17-OSi-*t*-Bu), 0.95 (9H, *s*, 4-OSi-*t*-Bu), 1.20 (3H, *s*, 19-CH_3), 3.15 (1H, *m*, 6ξ-H), 3.58 (1H, *t*, $J = 8$ Hz, 17α-H). Anal. Calcd. for $C_{31}H_{56}O_3Si_2$: C, 69.87; H, 10.59. Found: C, 69.64; H, 10.85.

4-Hydroxytestosterone 17-tert-butyldimethylsilyl ether (III). To a solution of **II** (800 mg) in dioxane

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(5 ml)-methanol (5 ml) was added 5% KOH (1 ml), and the resulting solution was stirred at room temperature overnight under a N_2 gas stream. The reaction mixture was neutralized with acetic acid, diluted with ethyl acetate, washed with water, and dried over anhydrous Na_2SO_4. After evaporation of the solvent the residue was chromatographed on silica gel (40 g). Elution with hexane–ethyl acetate (5:1, V/V) and recrystallization of the eluate from methanol gave **III** (530 mg) as colorless needles: m.p. 164–165°C. $[\alpha]_D^{16}$ + 67.0° (c = 0.18). n.m.r. ($CDCl_3$)δ ppm 0.01 (6H, s, 17-OSi$(CH_3)_2$), 0.75 (3H, s, 18-CH_3), 0.88 (9H, s, 17-OSi-t-Bu), 1.18 (3H, s, 19-CH_3), 3.50 (1H, t, J = 8 Hz, 17α-H). Anal. Calcd. for $C_{25}H_{42}O_3Si$: C, 71.72; H, 10.11. Found: C, 71.64; H, 10.19.

17β-tert-Butyldimethylsilyloxy-4-hydroxy-4-androsten-3-one hemisuccinate (***IV***). To a solution of **III** (200 mg) in pyridine (2 ml) was added succinic anhydride (400 mg), and the solution was refluxed for 24 h and then concentrated. The resulting solution was diluted with ether and the insoluble material was removed by filtration. The filtrate was washed with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent the residue was chromatographed on silica gel (10 g). Elution with hexane–ethyl acetate–acetic acid (50:100:0.15) gave **IV** (220 mg) as a colorless oil. n.m.r. ($CDCl_3$) δ ppm 0 (6H, s, 17-OSi$(CH_3)_2$), 0.74 (3H, s, 18-CH_3), 0.88 (9H, s, 17-OSi-t-Bu), 1.25 (3H, s, 19-CH_3), 2.72–2.95 (4H, -$COCH_2CH_2CO$-), 3.58 (1H, t, J = 8 Hz, 17α-H).

17β-tert-Butyldimethylsilyloxy-4-hydroxy-4-androsten-3-one hemiglutarate (***V***). Treatment of **III** (200 mg) with glutaric anhydride (400 mg) followed by purification of the product in the manner as described above gave **V** (210 mg) as a colorless oil. n.m.r. ($CDCl_3$) δ ppm 0 (6H, s, 17-OSi$(CH_3)_2$), 0.76 (3H, s, 18-CH_3), 0.90 (9H, s, 17-OSi-t-Bu), 1.27 (3H, s, 19-CH_3), 2.52–2.72 (4H,-$COCH_2CH_2CH_2CO$-), 3.58 (1H, t, J = 8 Hz, 17α-H).

4-Hydroxytestosterone 4-hemisuccinate (***VI***). To a solution of **IV** (60 mg) in acetone (6 ml) was added conc. HCl (0.3 ml), and the solution was stirred at room temperature for 1 h. The resulting mixture was diluted with ethyl acetate, washed with water, and dried over anhydrous Na_2SO_4. After evaporation of the solvent the residue was chromatographed on silica gel (7 g). Elution with ethyl acetate–acetic acid (100:1) and recrystallization of the eluate from ether gave **VI** (42 mg) as colorless granules: m.p. 163.5–164.5°. $[\alpha]_D^{17}$ + 88.4° (c = 0.16). n.m.r. ($CDCl_3$) δ ppm 0.82 (3H, s, 18-CH_3), 1.29 (3H, s, 19-CH_3), 2.27–2.96 (4H,-$COCH_2CH_2CO$-), 3.69 (1H, t, J = 8 Hz, 17α-H). Anal. Calcd. for $C_{23}H_{32}O_6 \cdot 1/4\ H_2O$: C, 67.54; H, 8.01. Found: C, 67.47; H, 7.87. U.V. λ_{max} 254 nm (ε 15000).

4-Hydroxytestosterone 4-hemiglutarate (***VII***). Acid hydrolysis of **V** (200 mg) followed by purification of the product in the manner as described above gave **VII** (160 mg) as colorless semicrystals. n.m.r. ($CDCl_3$) δ ppm 0.81 (3H, s, 18-CH_3), 1.29 (3H, s, 19-CH_3), 2.50–2.72 (4H,$COCH_2CH_2CH_2CO$-), 3.70 (1H, t, J = 8 Hz, 17α-H). U.V. λ_{max} 253 nm (ε 13000).

Materials

[1,2,6,7-^{3}H]-Testosterone (86.4 Ci/mmol) was supplied from the Radiochemical Centre (Amersham). Bovine serum albumin (BSA) and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, MO) and Iatron Laboratories (Tokyo), respectively. The steroid specimens were synthesized in these laboratories and kindly donated from Teikoku Hormone Mfg. Co. (Tokyo). All solvents and chemicals used were of analytical grade.

Preparation of antigens

To a solution of the haptens (**VI, VII**) (30 mg) in dry dioxane (0.7 ml) were added tri-*n*-butylamine (0.02 ml) and isobutyl chlorocarbonate (0.01 ml) at 11°C and stirred for 30 min. To this solution was added BSA (90 mg) in water (7.2 ml)–dioxane (1.4 ml)–1N NaOH (0.08 ml) under ice-cooling and stirred for 3 h. The resulting solution was dialyzed against cold running water overnight and the turbid protein solution was brought to pH 4.5 with 1N HCl. After being allowed to stand at 4°C overnight the suspension was centrifuged at 3000 rev./min for 20 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the manner as described above. Lyophilization of the solution afforded the testosterone-4-BSA conjugates (approx 90 mg) as fluffy powder. Thirty-two mol of **VI** and 34 mol of **VII**, respectively, were incorporated into 1 mol of BSA, as determined by U.V. spectrometric analysis.

Immunization of rabbits

The antigen (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into a domestic female albino rabbit subcutaneously at multiple sites along the back. This procedure was repeated once a week for 3 weeks and then once every fortnight. Blood was collected 6 months after the initial injection from the rabbit and centrifuged at 3000 rev./min for 10 min. The antiserum thus prepared was stored at 4°C and used in the assay at the initial dilution of about 1:15000.

Measurement of radioactivity

The samples were counted on a Packard Tri-Carb Model 3380 liquid scintillation spectrometer employing modified Bray's scintillant, composed of 2,5-diphenyloxazole (4 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (900 mg), naphthalene (60 g), and sufficient dioxane to make the total volume 1 litre.

Assay procedures

All dilutions of standards, tracer, and antiserum were performed in 0.01 M phosphate buffer (pH 7.4) containing gelatin (0.1%), NaCl (0.9%), and NaN_3 (0.01%). To a series of standard solutions (0, 21, 42,

Table 1. Specificity of antisera raised in rabbits against testosterone-4-BSA conjugates

Steroid	% Cross-reactivity (50%) 4-S*	4-G*
Testosterone	100	100
5α-Dihydrotestosterone	64	62
5β-Dihydrotestosterone	9.8	8.0
5α-Androstane-3β,17β-diol	4.5	2.6
5α-Androstane-3α,17β-diol	7.8	5.1
5β-Androstane-3β,17β-diol	1.3	0.95
5β-Androstane-3α,17β-diol	1.3	1.3
5-Androstene-3β,17β-diol	1.9	0.49
4-Androstene-3β,17β-diol	13	8.1
4-Androstene-3α,17β-diol	3.8	3.2
Androstenedione	1.0	2.6
Epitestosterone	0.093	0.16
Dehydroepiandrosterone	0.038	0.062
Androsterone	1.1	0.17
3β-Hydroxy-5α-androstan-17-one	1.4	0.27
Androstanedione	5.9	1.3
19-Nortestosterone	13	6.3
Estradiol	0.017	0.003
Estrone	0.001	0.001
Estriol	<0.001	<0.001
Progesterone	0.038	0.002
5α-Pregnane-3,20-dione	0.055	0.008
Deoxycorticosterone	0.013	0.003
Cortisol	<0.001	<0.001
Cortisone	<0.001	<0.001
Cholesterol	<0.001	<0.001

* 4-S = 4-hydroxytestosterone 4-hemisuccinate-BSA, 4-G = 4-hydroxytestosterone 4-hemiglutarate-BSA.

104, 208, 624, and 832 pg of testosterone) in buffer (0.1 ml) were added [^{3}H]-testosterone (ca. 10000 d.p.m., 0.5 ml) and diluted antiserum (0.1 ml), and incubated at 4°C overnight. To the incubation mixture was added a dextran (0.06% w/v)–charcoal (1% w/v) suspension (0.5 ml), vortexed, allowed to stand at 0°C for 10 min, and then centrifuged at 4°C, 2000 rev./min for 10 min. The supernatant was transferred by decantation into a vial containing a scintillation solution (10 ml) and radioactivity was counted.

Cross-reaction study

The specificity of antisera raised against the testosterone-BSA conjugates was tested by cross-reaction studies with 25 kinds of purified steroids related to testosterone (Table 1). The relative amounts required to reduce the initial binding of [^{3}H]-testosterone by half, where the mass of unlabeled testosterone was arbitrarily chosen as 100%, were calculated by the standard curves.

RESULTS

The synthetic route leading to 4-hydroxytestosterone 4-hemisuccinate(**VI**) and 4-hydroxytestosterone 4-hemiglutarate(**VII**) as new haptens was developed and outlined in Fig. 1. The key step was the selective cleavage of the phenolic *tert*-butyldimethylsilyl ether at C-4 without disturbing the alcoholic silyl ether at C-17 under the basic conditions reported in the previous paper [5]. The haptens (**VI, VII**) were covalently linked to BSA yielding testosterone-4-BSA conjugates by the mixed anhydride method developed by Erlanger *et al.*[6]. The sera obtained from the rabbits immunized with these antigens for six months showed significantly increased activity to testosterone. The dose-response curves were constructed with approximately 1:105,000 dilution of antisera. When logit transformation was used to construct the curves, plots of logit per cent bound radioactivity *vs.* logarithm of the amount of unlabeled testosterone showed a linear relationship.

The specificity of antisera was assessed by ascertaining the ability of various related steroids to compete with [^{3}H]-testosterone for binding to antibody. The per cent cross-reaction of antisera was determined according to the method of Abraham[7]. The results on cross-reactions of anti-testosterone antisera with twenty-five kinds of related steroids are listed in Table 1. It seems likely that the cross-reactivities of these two antisera are substantially the same, but antiserum raised against the hemiglutarate-BSA conjugate is somewhat more specific than the other. The antisera proved to be sufficiently specific with an exception of 5α-dihydrotestosterone which showed 62% cross-reaction. In sharp contrast 5β-dihydrotestosterone exhibited only 8% of the activity relative to testosterone. Other related C_{19} steroids that exhibited less than 10% cross-reaction include 5α-androstane-3β,17β-diol (2.6%), 5α-androstane-3α,17β-diol (5.1%), and 4-androstene-3β,17β-diol (8.1%).

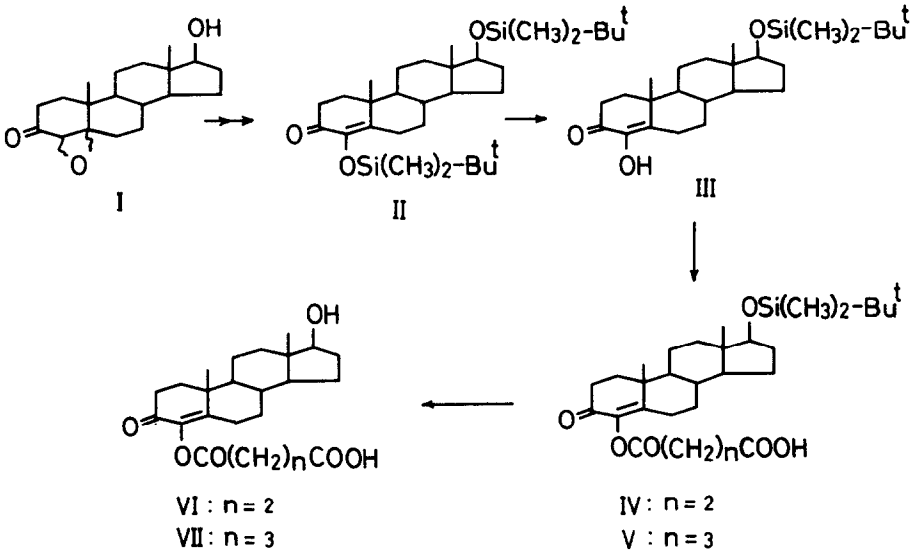

Fig. 1. Synthesis of 4-hydroxytestosterone 4-hemisuccinate and 4-hemiglutarate.

DISCUSSION

Numerous investigators have carried out the preparation of specific antisera by coupling the testosterone molecule through its various sites to a carrier protein. The cross-reactivities of anti-testosterone antisera elicited by antigens having a linkage through the positions neighboring to C-4 with the selected steroids are listed in Table 2. It is of interest that the specificity of antisera prepared by the testosterone-4-BSA conjugates is comparable to that of antisera by the C-3 [8, 9], C-6 [10], and C-7 [11] conjugates despite the use of a linkage through the site near the Δ^4-3-oxo structure for preparation of the hapten-carrier conjugate. The effects of substitution and stereochemistry on the specificity of antisera have previously been discussed by several workers. Grover and Odell[2] proposed a hypothesis that a steroid derivative which is capable of holding the coplanarity of the hapten molecule would tend to produce specific antibody. Similarly, the specificity of antisera elicited by immunization with the estradiol 3-hemisuccinate-BSA conjugate was explained in terms of the rigidity of the bridge [3]. These attractive explanations would in part rationalize the higher specificity of antisera raised against the testosterone-4-BSA conjugates. The restriction of the free rotation of the ester bond in the bridge at C-4 due to the interaction with the α,β-unsaturated function and the steric hindrance by the methylene group at C-6 as evident from the CPK model would cause the preferred conformation of the testosterone molecule to be rigid and in a plane perpendicular to the carrier protein. It is sufficiently substantiated that for the production of specific antiserum the steroid should be coupled to a carrier protein at a site remote from the inherent functional groups in such a way that the structural characteristics are left available as antigenic determinant. The present finding strongly implies that the distance between the functional groups and the surface of a carrier protein is actually important for determining the specificity of antiserum. To the best of our knowledge hydroxylation at C-4 in the biotransformation of testosterone and other Δ^4-3-oxo steroids such as progesterone and cortisol has not yet been reported. In these respects the C-4 position appears to be a suitable site for the attachment of a carrier protein in preparation of the hapten-carrier conjugate.

Table 2. Per cent cross-reaction of antisera raised against testosterone-BSA conjugates with selected steroids

	% Cross-reactivity (50%)						
	4-S	4-G	3-CMO*	3-CMO†	6α-CM	6β-CM	7α-CMTE
Testosterone	100	100	100	100	100	100	100
5α-Androstane-3α,17β-diol	7.8	5.1	26	2.8	4.9	4.3	17
5α-Androstane-3β-17β-diol	4.5	2.6	6	1.21	2.0	6.8	7
5β-Androstane-3α,17β-diol	1.3	1.3	—	—	1.3	0.4	—
5β-Androstane-3β,17β-diol	1.3	0.95	2	—	—	—	—
5-Androstene-3β,17β-diol	1.9	0.49	1.3	—	—	—	—
5α-Dihydrotestosterone	64	62	77	27	95	75	42
5β-Dihydrotestosterone	9.8	8.0	—	22	—	—	5

* Reference 7, † Reference 8. 3-CMO = Testosterone 3-(*O*-carboxymethyl)oxime-BSA, 6α-CM = 6α-Carboxymethyltestosterone-BSA, 6β-CM = 6β-Carboxymethyltestosterone-BSA, 7α-CMTE = 7α-Carboxymethylmercaptotestosterone-BSA. Other abbreviations are the same as in Table 1.

Further studies on the preparation and antigenic properties of the Δ^4-3-oxo steroid-4-BSA conjugates are being conducted in these laboratories and the details will be reported in the near future.

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