PREPARATION AND ANTIGENIC PROPERTIES OF 5α-DIHYDROTESTOSTERONE-11-(O-CARBOXYMETHYL) OXIME-BSA CONJUGATE

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ABSTRACT

The C-11 (O-carboxymethyl) oxime derivative of 5αdihydrotestosterone (5αDHT) has been prepared. Due to steric hindrance at C-11, a novel two step procedure was used to introduce the (O-carboxymethyl) oxime at this position. Condensation of this oxime to bovine serum albumin afforded a conjugate which produced anti-5αDHT sera inoculated rabbits. Apart from a 30% cross reaction with testosterone, the antisera was reasonably specific for 5αDHT.

INTRODUCTION

Production of steroid antisera for radioimmunoassay has placed emphasis on the design of the steroid antigens. The site of conjugation of steroid to the protein carrier has shown to be a crucial factor in determining the specificity of resulting antisera. Steroids coupled at positions which leave their functional groups free, notably C-1, C-6, C-7 and C-11 have generally favoured the production of more specific antisera. (1 to 3).

Reasonably specific antisera exist for oestrogens, but unfortunately due to the close structural similarity of 5α-dihydrotestosterone (5α DHT) and testosterone (T), antisera raised against T antigens cross react significantly (> 20%) with 5α DHT (4 to 10). The converse is also true for 5αDHT antisera; including 5αDHT-1carboxymethyl-thioether-BSA (11) and 5αDHT-19-hemisuccinate-BSA (10).

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Attempts in this laboratory to couple 5αDHT at C-3 and C-7 have shown that such coupling elicited antisera with a 50% cross reaction with testosterone (12,13). Later consideration, that these sites of attachment were probably too close to the crucial C-5 configuration, have led us to choose C-11 to link 5αDHT to protein. However, the C-3 and C-7 carboxymethyl oxime conjugates produced antisera with favourable cross reactions towards other androgens.We have used the carboxymethyl oxime bridge in the present work, hoping to maintain this advantage.

This communication describes the synthesis of 5αDHT-11-(O-carboxymethyl) oxime-bovine serum albumin conjugate and is the first report of a steroid antigen with this type of linkage at C-11. The steric hindrance of the C-11 position was such that we could not directly introduce the (O-carboxymethyl) oxime bridge. A novel approach had to be used, of first forming the ordinary oxime and then reacting with sodium chloroacetate. The specificity of the antisera elicited by this antigen in rabbits is given.

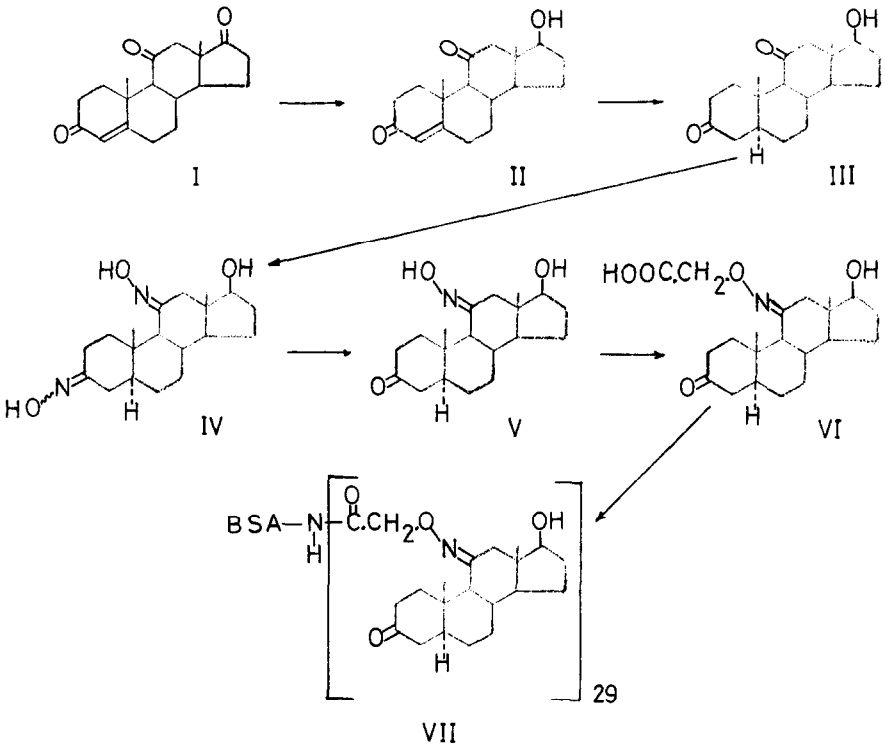
SYNTHESIS

The preparation of 5αDHT conjugate is shown in Fig.I. Androst-4-ene-3, 11, 17-trione (I) was chosen as a convenient, relatively cheap starting material. This was converted in high yield to 17β-hydroxy-androst-4-ene-3, 11-dione (II) by a microbiological reduction using Saccharomyces cerevisiae. The method was a modified version of Herzog et al (14). Catalytic hydrogenation was used to saturate the Δ-4 bond and the resulting mixture of 5α and 5β isomers were separated by preparative layer chromatography.

Many attempts to react carboxymethoxylamine hemihydrochloride with the 11-keto group failed.

However it was found that hydroxylamine hemi-

FIGURE 1. SYNTHESIS STEPS I to VII.



hydrochloride would react with 17β-hydroxy-5α-androstane-3,11-dione (III) to give the corresponding dioxime material. The 3-oxime group was selectively removed by treatment with pyruvic acid (15) and the resulting 17β-hydroxy-5α-androstane-3,11-dione-11-oxime reacted with sodium chloroacetate in ethanolic sodium hydroxide to give 17β-hydroxy-5α androstane-3,11-dione-11-(O-carboxymethyl) oxime (VI). A modified version of Erlanger et al (16) was used to conjugate this material to bovine serum albumin to form VII.

EXPERIMENTAL

17 β-hydroxy-androst-4-ene-3,11-dione (II)

Androst-4-ene-3,11,17-trione (I) (0.45g) and 10g of yeast extract (Oxoid) were added to a solution of sucrose (200g in 500ml) contained in a 2L conical flask. This suspension was autoclaved at 15psi for 45 minutes. To the cooled solution were added 100ml ethanol and 100g of baker's yeast (Saccharomyces cerevisiae). The pH of this mixture was adjusted to 4.7 by the addition of dilute sulphuric acid.

The flask was plugged with cotton wool and incubated in a flat-bed shaker at 27°C, thus allowing agitation of contents but maintaining anaerobic conditions. Hourly adjustments of the pH to 4.5-5.0 were made for the first 10 hours, by the addition of dilute ammonium hydroxide, then at 8 hour intervals for a total incubation time of 48 hours.

The incubation mixture was centrifuged and the supernatant reduced in vacuo to 200ml. The cell mass was extracted by refluxing for two 30 minute periods with 1 litre portions of methanol. Methanolic extracts were then taken to dryness and the residues combined with the supernatant before making up to 600ml with distilled water.

The aqueous solution was extracted three times with ethylene dichloride. The organic phase was washed to neutrality with water then dried over anhydrous sodium sulphate followed by rotary evaporation. Recrystallization from acetone-hexane yielded 435mg crystals, mp 183-185°C. $(\alpha)_D^{23°C}$ =+167°. Thin layer chromatography (TLC) on silica gel (Merck GF_{254} precoated plastic plates, 0.25mm) showed one spot. ν_{max} 1710cm^{-1} (11 ketone), 1672cm^{-1} and 1619cm^{-1} (4-3-one).

17β-hydroxy-5α-androstane-3,11-dione (III)

500mg of 10% palladium on charcoal and 1g of 17β-hydroxy-androst-4-ene-3,11-dione (II) were added to 200ml of ethanol contained in a 500ml reaction flask of a Parr hydrogenator. Hydrogenation was carried out for 3 hours at 5psi. After filtering off the catalyst the crude product was applied as a band to 20 preparative silica gel plates (Merck PF_{254}, 20x20x0.075cm). Four developments were made in benzene:hexane: ethyl acetate:acetic acid (100:100:200:8). Bands were observed as opaque regions after spraying the plates with distilled water. A main band containing the required product and running at 9-11cm from the origin was scraped off the plates and shaken well with 200ml tetrahydrofuran, 100ml hexane and 50ml water. The organic phase was filtered and the aqueous slurry further extracted with ethyl acetate. The total organic extracts were taken to dryness and recrystallized from ethyl acetate-hexane.

Yield = 49%. mp = 193-196°C. $(\alpha)_D^{20°C}$ = +71.5°. ν_{max} 1708-1712cm^{-1} (diketone). λ_{max}(EtOH) = 285nm, E_{max} = 83M^{-1}cm^{-1}.
Anal Calculated for $C_{19}H_{28}O_3$. C,75.0; H,9.2. Found C,74.3; H,8.4.

17β-hydroxy-5α-androstane-3, 11-dioxime (IV)

Compound III (109mg) was dissolved in 4.5ml of dry pyridine, 480 mg of powdered hydroxylamine hydrochloride was added and the mixture refluxed under an atmosphere of nitrogen for 6 hours. After leaving in a vacuum desicator overnight the reaction volume was further reduced by rotary evaporation. 20ml of water was added to the resulting oil and vigorously shaken to give a white precipitate, which was then harvested on a sinter and washed well with water. The dry weight was 113mg.

Examination of this material by TLC (System 1: - toluene: dioxane: acetic acid, 100:50:2 and System 2:- benzene: ethyl acetate, 3:1) showed two, almost resolved spots, both different from the starting material. The same TLC systems were able to distinguish between the syn and anti isomers of 17β-hydroxy-5α-androstan-3-oxime.

A portion of the precipitate was recrystallised from dichloromethane-hexane. Two types of crystals were observed; one mp = 269-275°C, the other mp = 279-280°C, presumably corresponding to the syn and anti oxime isomers at the C-3 position. (A sample of 17β-hydroxy-11-oxo-5α-androstane-3-oxime also showed two types of crystal mp = 211-213°C and 230-233°C respectively and gave two spots on TLC with Systems 1 and 2).

$(\alpha)_D^{25°C} = +93.6°$. $\nu_{max} = 1678\text{-}1690 cm^{-1}$ (C=N).

17β-hydroxy-5α-androstane-3, 11-dione-11-oxime (V)

Compound 1V (110mg) was dissolved in 2ml of acetic acid and 1ml of water. Pyruvic acid (200µl), previously redistilled under vacuum, was added and the mixture heated on a steam bath, under an atmosphere of nitrogen, for 20 minutes. 1.5ml of water was added and heating continued for 3 hours. A further 2ml of water was added to the stirred mixture which was then cooled on ice for 30 minutes.

The white precipitate was filtered off giving a dry weight of 82mg. Recrystallisation from ethyl acetate - pet. ether gave 52mg. TLC showed only one spot, which could indicate that only one isomer of the C-11 oxime had been formed. mp = 201-204°C. $(\alpha)_D^{25°C} = +131°$.

$\nu_{max} = 1690\text{-}1705 cm^{-1}$. $\lambda_{max} = 285nm(EtOH)$, $E_{max} = 43 M^{-1}cm^{-1}$.

Anal Calculated for $C_{19}H_{29}N_1O_3$. C,71.4; H,9.1; N,4.4. Found C,70.1; H,9.1; N,3.9.

The Zimmerman reaction (17), which gives a characteristic absorption for 5α-3-one keto steroids at 540nm, was used to confirm that the C-3 oxime had been selectively removed. Compound 1V gave a negative result for this test.

17β-hydroxy-5α-androstane-3, 11-dione-11-(O-carboxymethyl) oxime (V1)

Prior to the synthesis of (V1), tests were made using various derivatives of 5αDHT in order to verify that O-carboxymethylation would take place at the C-11 oxime, and not at the C-17β hydroxy position.

2mg quantities of these derivatives were subjected to the reaction conditions of O-carboxymethylation using 25mg sodium chloroacetate and refluxing for 2hr (for actual conditions see below). Reactions were monitored by TLC. The results of these tests are shown in Table I.

TABLE I

Effect of the chloroacetic acid reaction on various derivatives of 5αDHT

Test	Derivative of 5αDHT	Na $OOCCH_2C\ell$ Present	Products if different from starting material
1	Underivatised (i.e. 5αDHT)	+	-
2	C-11-keto	+	-
3	C-3-oxime	+	3-(O-carboxy-methyl oxime)
4	C-3-oxime	-	Partially 5αDHT
5	C-3-(O-carboxymethyl) oxime	+	Partially 5αDHT
6	C-11-oxime	-	-
7	C-11-oxime	+	11-(O-carboxy-methyl) oxime

Tests 1 and 2 indicate that C-3-keto, C-11-keto and C-17β are not reactive under these conditions. Comparison of test 4 with test 6 demonstrates the stability of the C-11 oxime group under the basic conditions used in contrast to the C-3 oxime which is partially cleaved to yield 5αDHT.

Compound V (31mg) was dissolved in 3ml ethanol, 0.1 ml 1N sodium hydroxide was added and the solution refluxed under nitrogen for 10 minutes. 25mg of sodium chloroacetate were dissolved in 12ml ethanol before addition to the hot solution. Refluxing was continued under a gentle stream of nitrogen, until after 2 hours most of the solvent had been removed. TLC showed about 60% yield of desired product at this stage.

20ml of water was added to the residue and the pH brought to 2.0 with 1N HCl, followed by extraction with 4 x 25ml ethyl acetate. The pooled organic phase was then extracted with 4 x 50 ml sodium hydroxide solution, pH.9. The pooled aqueous layers were chilled on ice and brought to pH 2 with 1N HCl before extracting with 4 x 50ml ethyl acetate. The organic phase was washed with water then taken to dryness. The residue was dissolved in a few drops of ethanol and recrystallised from ethyl acetate-hexane, giving 9mg. mp = 180-183°C.

ν_{max} = 1725cm^{-1} (carboxyl), 1710cm^{-1} (3-keto), 1690 cm^{-1} (C=N).

Anal Calculated for $C_{21}H_{31}N_1O_5$. C,66.8; H,8.2; N,3.7. Found C, 66.9; H,7.8; N,3.1.

17β-hydroxy-5α-androstane-3, 11-dione-11-(O-carboxymethyl) oxime-bovine serum albumin (VII)

Compound VI (7.5mg) was dissolved in a mixture of 0.8ml dry dioxane, 0.4ml dry dimethyl formamide, 6µl tri-N-butylamine and 20µl of N-methyl morpholine, slight warming was necessary. Isobutylchloroformate (2.7µl) in 0.14ml dry dioxane was added and the solution stirred at 1-2°C for 30 minutes.

This mixed anhydride preparation was slowly added to a stirred, previously prepared solution of bovine serum albumin (34.6mg) in 0.692ml of water and 0.208ml dioxane at 0°C. The apparent pH was maintained at 8.5 by the addition of a few drops of 0.1N sodium hydroxide from time to time. After one hour the mixture was kept at -15°C for 24 hours. The conjugate was then purified by the acetone precipitation procedure (16). After lyophilisation 29.1mg of conjugate was recovered.

Estimation of number of haptens per molecule of BSA

The Zimmerman reaction (17) was modified as follows to estimate hapten number. Weighed portions of conjugate (approx. 2mg) were dissolved in 0.2ml 2.5N potassium hydroxide, and 0.2ml ethanol by gentle warming. As a control

the conjugate was replaced by 2mg BSA. 0.2ml of an ethanolic solution of 1,3-dinitro benzene (2% w/v) were added to tests and controls. After 5 minutes 2ml ethanol were added and absorbances measured at 540nm.

Standard curves were constructed over the range 0-2mg/ml (ethanol) using 5α-3-one steroids viz. 17β-hydroxy-5α-androstan-3-one, 17β-hydroxy-5α-androstane-3, 11-dione and 17β-hydroxy-5α-androstane-3, 11-dione-11-oxime. To 2mg of BSA was added 0.2ml 2.5N KOH followed by 0.2ml steroid standard and 0.2ml 1,3-dinitro benzene solution. After 5 minutes 2ml ethanol added and absorbances measured at 540nm. (E_{540nm}=3 x 10^3 $cm^{-1}M^{-1}$).

Ratio of moles hapten to moles conjugate were calculated using difference in absorbance of tests over control at 540nm. The hapten ratio was found to be 29.

Preparation of antisera. Two male rabbits weighing 2-3 kg were immunised 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 1:3000 was obtained after about 6 months.

Characterisation 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 characterisation of one antiserum only is presented. The results of the cross reactivity of various related steroids tested for their ability to compete with 10pg radioactively labelled 5αDHT for binding sites of the antiserum are shown in Table II. 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) (4.2%) or with the 3α or 3β, 17β diols (all less than 3%). 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 2.4%. The only major cross

reactant was testosterone (30%) which has a similar overall skeletal shape to 5αDHT.

TABLE II

Percentage cross reactions of the 5αDHT antiserum

Steroid	Percentage cross reaction
5α-Dihydrotestosterone	100
Testosterone	30.4
17α-hydroxy-5α-androstan-3-one	4.2
17α-hydroxy-4-androsten-3-one	3.4
5α-androstane-3β, 17β-Diol	2.7
17β-hydroxy-5β-androstan-3-one	2.4
17β-hydroxy-17α methyl-5α-androstan-3-one	0.4
5α-androstan-11-one	< 0.3
5α-androstane -3α, 17β-Diol	0.23
5α-androstan-3, 17-Dione	0.15
4 - androstene-3, 17-Dione	0.14
5α-pregane-3, 20-Dione	0.12
5α-androstane-3, 11, 17-Dione	0.08
4 - pregnene -3, 20-Dione	0.05
17α-hydroxy-4-pregnene-3, 20 Dione	0.02
5β-pregnane-3, 20-Dione	0.012
3β-hydroxy-5-androsten-17-one	0.0075
11β, 17α 21 trihydroxy-4-pregnene-3, 20-Dione	0.0025

DISCUSSION

This report describes the first chemical synthesis of a steroid-(O-carboxymethyl)-oxime-BSA conjugate linked at the C-11 position. Conjugates using C-11α and C-11β hemisuccinates of steroids have previously been prepared (5, 6 & 18), but the stereochemical hindrance and concomitant lack of reactivity of C-11 oxo compounds may have deterred previous workers from using the carboxymethyl oxime conjugation. The described two stage synthesis of the carboxymethyl oxime bridge may be applicable to other sterically hindered oxime formations. The selective de-oximation of the C-3 oxime by treatment of the dioxime with pyruvic acid is a useful technique and should enable simpler syntheses of certain other steroidal carboxymethyl oxime antigens.

Unfortunately the antisera raised by the C-11 conjugate is not highly specific, it possesses a 30% cross reaction with testosterone (T). The 5αDHT-1α-carboxymethyl-thioether-BSA conjugate used by Bauminger et al. (21) raised antisera claimed to have as little as 10% cross reaction with testosterone. However, antisera raised by similar C-1 conjugation for 4-androstene-3, 17-dione (22) showed 50% cross reaction with 5α-androstane-3, 17-dione (the equivalent epimer as between 5αDHT and T). This suggests that Bauminger et al. antisera was probably a unique collection and the cross reaction is probably in general higher than 10%. This antisera also cross reacts with 5α-androstane-3α, 17β diol at

the 16% level. Thus it appears that all antisera raised against 5αDHT to date cross reacts seriously with testosterone whether conjugation is made at C-1, C-3, C-7, C-11, C-17, or C-19. The antibody site appears to have difficulty in distinguishing between compounds which differ merely by the C_4-C_5 bond. This is reflected in the fact that all androgens and progesterones and other Δ^4-3-one compounds after conjugation to protein, do not raise really specific antisera of the calibre of that raised for the oestrogens (23).

Apart from the major cross reaction with testosterone, the C-11 oxime raised antisera is reasonably specific to all other androgens tested. Unlike antisera raised by C-1, C-3, and C-19 · 5αDHT conjugates which possess substantial cross reactivity with androstandiols in particular 5α androstane-3α, 17β diol, the present antisera like that of the C-7 antigen has very low cross reactivity (0.23% only for the C-11) for the 5α-diol, which is known to exist in plasma in significant amounts (24). The C-11-(O-carboxymethyl) oxime-BSA conjugate raised antisera which is slightly more specific than that obtained with our previous C-7 conjugate (13), and this makes it one of the most specific antisera yet devised for 5αDHT.

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