THE PREPARATION OF THREE FLUORESCENCE-LABELLED DERIVATIVES OF TESTOSTERONE

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ABSTRACT

Three fluorescence-labelled derivatives of testosterone were prepared consisting of the steroid separated from the fluorochrome by a hydrocarbon "bridge". "Bridges" of different lengths (C2 to C7) were used as the length required to avoid steric hindrance effects by the fluorochrome in studies on steroid-protein binding was unknown. The three derivatives prepared were: 17β-hydroxy-4-androsten-3-one 3-(O-(N-(2'-mercapto)ethyl)carbamoylmethyl)oxime, 17β-hydroxy-4-androsten-3-one 3-(O-(N-(3'-amino)propyl)carbamoylmethyl)oxime and 17β-hydroxy-4-androsten-3-one 3-(O-(N-(7'-amino)heptyl)carbamoylmethyl)oxime. These were then coupled with either a dansyl or a fluorescein molecule. Overall yields were sufficient and the products immunoreactive with anti-testosterone antiserum.

A number of immunoassays not requiring the use of radioactive tracers have been developed in recent years. Among these are fluorescence and fluorescence polarisation immunoassays. The latter have the further advantage that no separation of free and bound ligand is required and so such assays would be rapid and simple to automate. So far for steroids the fluorescence polarisation immunoassay of cortisol has been reported (1).

Development of an enzyme-linked immunoassay for testosterone in this laboratory (2) has shown that coupling of testosterone to another molecule via the C-3 position hardly affects the immunoreactivity of the product with an antiserum raised against testosterone-3-(O-carboxymethyl)oxime-bovine serum albumin. Therefore, with a view to developing immunoassays involving fluorescence-labelled tracers and fluorescence polarisation detection, the synthesis of fluorescent derivatives of testosterone via the C-3 position has been undertaken.

In order to avoid steric hindrance effects the attempt has been made to synthesise derivatives with the fluorochrome separated from

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the steroid by a hydrocarbon "bridge" - of length 2C-7C.

The two fluorochromes used were fluorescein isothiocyanate (FITC) and 5-(iodoacetylaminoethyl)amino-naphthalene-1-sulphonic acid(IAEDANS). The former was chosen because of the high quantum yield of fluorescein and the apparently simple method for attaching the isothiocyanate to amino groups. For the preparation of such derivatives a 1,ω-diamino-hydrocarbon could be coupled, at one end, to testosterone-3-(O-carboxy-methyl)oxime and, at the other end,to FITC. The fluorescent reagent IAEDANS was used so that syntheses could be simplified by the use of a heterogeneous bifunctional "bridge" (cysteamine). IAEDANS, intro-duced by Hudson and Weber (4), reacts with sulphydryl groups to form photostable products.

MATERIALS

Testosterone was obtained from Roussel UCLAF and 1,2,6,7-^{3}H testo-sterone (80 Ci/mmol) came from the Radiochemical Centre, Amersham). 1,7-diamino-n-heptane and 1,3-diamino-n-propane were supplied by Aldrich and cysteamine hydrochloride, tri-n-butylamine and isobutyl chloro-carbonate by Merck. 4-Phenylspiro(furan-2(3),1'-phthalan)-3,3-dione ("Fluram"/"Fluorescamine") came from Hoffman-La Roche and 5,5'dithiobis (2-nitrobenzoic acid) from Aldrich.

The fluorochrome 5-(iodoacetylaminoethyl)amino-naphthalene-sul-phonic acid (IAEDANS) and fluorescein isothiocyanate (FITC) were obtained from Molecular Probes Inc.(Texas) and Sigma respectively. The FITC, when tested chromatographically for purity on thin-layers of silica gel with chloroform:ethanol (6:4) as developing solvent, in some cases was found to be highly impure, with four major impurities (over 50% of the total colour intensity). Such samples were purified before use on a silica gel 60 (Merck) column with the same solvent mixture.

Pre-coated silica gel 60 sheets (Merck) were used for analytical T.L.C. and Whatman PLK-5 silica gel pre-coated plates were used for preparative T.L.C.

Fluorescence measurements and spectra were obtained with a Fica Model 55Mk.2 spectrofluorometer, U.V. and I.R. spectra were obtained with Perkin Elmer Model 402 and 257 spectrophotometers respectively. High pressure liquid chromatography was carried out with a Varian Model 5000 chromatograph with an MCH-reversed phase (30x0.4 cm)column. Scintillation counting was carried out with an Intertechnique Model SL 4000 counter and melting points were measured with a Reicher electric melting point apparatus.

Radioimmunoassays were performed using an anti-testosterone-3-(O-carboxymethyl)oxime-bovine serum albumin rabbit antiserum in a liquid phase assay,with dextran-coated charcoal to separate free from bound ligand (5).

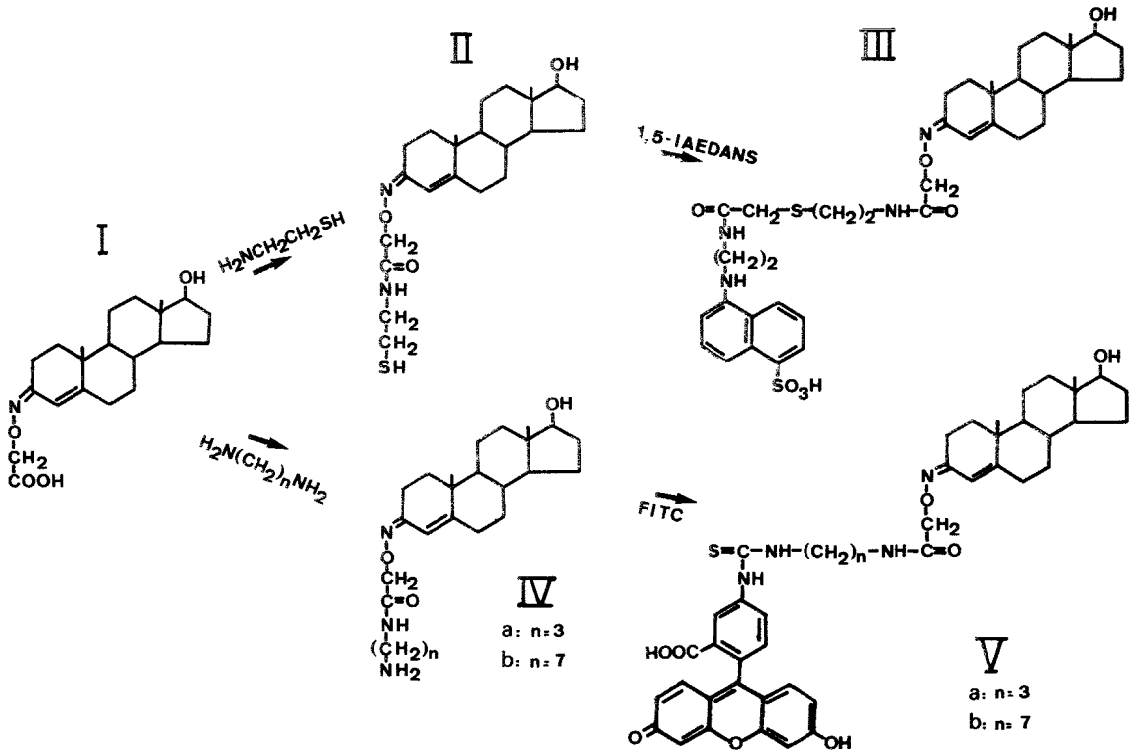
I
II
III
IV
V
$H_2NCH_2CH_2SH$
$H_2N(CH_2)_nNH_2$
1,5-IAEDANS
FITC
a: n=3
b: n=7
a: n=3
b: n=7

FIGURE 1

EXPERIMENTAL

1. 17β-hydroxy-4-androsten-3-(O-carboxymethyl)oxime and tritiated 17β-hydroxy-4-androsten-3-(O-carboxymethyl)oxime - (I).

These compounds were prepared according to the method of Erlanger et al.(5), the tritiated derivative being formed from a mixture of testosterone (1 g) and ^{3}H testosterone (0.36 µg, 100 nCi).

2. 17β-hydroxy-4-androsten-3-one 3-(O-(N-(2'-mercapto)ethyl)carbamoylmethyl)oxime - (II) - (testosterone-cysteamine).

This compound was prepared by the Erlanger method (5) from I (testosterone-3-CMO, 95 mg; ^{3}H testosterone-3-CMO, 5 mg) (total 0.26 mmol) and cysteamine hydrochloride (22 mg, 0.19 mmol). After reaction 5 ml 0.05N sodium hydroxide was added and the mixture extracted with 4x15 ml diethyl ether.

The extract was washed with 3x10 ml 0.05N hydrochloric acid, followed by 3x10 ml 0.05N NaOH. 86% of the original radioactivity was found in the organic phase. The extract was analysed by T.L.C. using benzene: ethyl acetate:acetone (1:8:1) as developing solvent. Steroids were revealed with 8% sulphuric acid in 50% ethanol and heating, free amines were revealed with 4-phenylspiro(furan-2(3),1'-phthalan)-3,3-dione (0.2 mg/ml in acetone) and free thiols were revealed with 5,5'-dithiobis-(2-nitrobenzoic acid) (0.3 mg/ml in acetone). The R_Fs of identified spots are shown in Table 1. A number of steroid spots were found but only one was associated with free thiols. Thus, despite the presence of steroidal impurities, as only one steroid in the extract had free thiols, this crude extract was used directly for coupling with the fluorochrome.

3. 17β-hydroxy-4-androsten-3-one 3-(O-(N-(3'-amino)propyl)carbamoylmethyl)oxime - (IVa) - (testosterone-3-DAP) and 17β-hydroxy-4-androsten-3-one 3-(O-(N-(7'-amino)heptyl)carbamoylmethyl)oxime - (IVb) - (testosterone-3-DAH).

Again using the Erlanger method these two compounds were formed from I (testosterone-3-CMO, 175 mg; ^{3}H testosterone-3-CMO, 5 mg) (total 0.51 mmol) with a 29-fold molar excess of either 1,3-diamino-n-propane or 1,7-diamino-n-heptane (15.3 mmol). The reactions were carried out in a nitrogen atmosphere.

After reaction the reaction mixtures were treated similarly. The dioxane was evaporated under a stream of nitrogen and the mixture was taken to pH 1 with 1N HCl, the unreacted I then being extracted with 4x10 ml ethyl acetate, which was back-washed with 5 ml 1N HCl. The combined aqueous phase was then taken to pH 11 with concentrated NaOH and extracted with 4x25 ml ethyl acetate:acetone (4:1). The organic phase was washed with 5 ml 1N NaOH, dried over anhydrous sodium sulphate and flash evaporated . Only 5% of the original radioactivity was found in this extract.

The extracts were taken up with methanol and submitted to preparative T.L.C. with chloroform:methanol:water (60:39:1) as developing solvent. Reference spots were revealed for steroids and free amines as before and the R_Fs are shown in Table 1. For both reactions, apart from the major aminated steroid spot there was a minor spot which migrated near the solvent front (R_Fs 1.00 and 0.97 respectively for the diaminopropane and diaminoheptane reactions). The major aminated steroid

was eluted from the gel with 4x20 ml developing solvent mixture and the solvent flash evaporated. For both products a total recovery of 2.7% of the original radioactivity, corresponding to 6 mg IVa and 7 mg IVb, was obtained.

4. 17β-hydroxy-4-androsten-3-one 3-(O-(1'-sulfo-5'-naphthylaminoethyl-carbamoylmethylthioethylcarbamoylmethyl)oxime - (III) -(testosterone -cysteamine-DANS).

The crude preparation of II (<0.23 mmol, by radioactivity) in 7 ml methanol was reacted with 50 mg IAEDANS (0.115 mmol) in 3 ml 0.02M phosphate buffer pH 7.0; at this pH solubilisation was complete. The reaction was left 6 hours at room temperature in the dark (IAEDANS is photosensitive), the pH being maintained at 7.0.

The solvent was evaporated and the products taken up with methanol and submitted to preparative T.L.C. with chloroform:ethanol (1:1) as developing solvent. Reference spots were revealed for steroids after viewing under UV light - the R_Fs are shown in Table 1. The spot corresponding to III was eluted from the gel with 4x10 ml methanol and the chromatographic step repeated. The overall recovery of radioactivity was 9.6%, corresponding to 19 mg III. The product was crystallised from toluene:methanol.

The UV spectrum (ethanol) is shown in Figure 2. IR spectrum (KBr): 3400 cm^{-1} (steroidal 17β-OH), 2940 cm^{-1} (steroidal CH stretch), 1665 cm^{-1} (amide), 1190 cm^{-1} (SO_3H), 785 cm^{-1} (w)(aromatic -$(CH)_3$-). Fluorescence spectrum (methanol): excitation, λ_{max} 343 nm; emission, λ_{max} 475 nm. Melting point 183-186°C.

5a. 17β-hydroxy-4-androsten-3-one 3-(O-(3',6'-dihydroxyfluoran-5-ylthio-ureidopropylcarbamoylmethyl)oxime - (Va) - (testosterone-DAP-fluorescein).

The crude preparation of IVa was taken up with 2ml methanol and 0.5 ml 1M sodium bicarbonate and mixed with 1.5 molar equivalents of FITC. The mixture was held for 18 hours at 4°C, 24 hours at 20°C and 2 hours at 30°C to ensure reaction. Analytical T.L.C. with chloroform: ethanol (7:3) showed that some IVa remained unreacted. Thus two further molar equivalents of FITC were added and the incubation procedure repeated. T.L.C. then showed the reaction to be complete.

After neutralisation with IN HCl the solvent was evaporated and the residue taken up with ethanol and methanol and submitted to preparative T.L.C. first with chloroform:ethanol (7:3), to remove traces of IVa and FITC breakdown products, and then with benzene:ethyl acetate: acetone (1:8:1) to remove unreacted FITC. Each time the product was eluted from the gel with 4x20 ml ethanol and 2x20 ml methanol and the solvent flash evaporated. The overall recovery of radioactivity was 0.84%, corresponding to 3.5 mg Va. The product was crystallised from water.

The UV spectrum (ethanol) is shown in Figure 2. The IR spectrum (KBr): 3400 cm^{-1} (steroidal 17β-OH), 2935 cm^{-1} (steroidal CH stretch), 1665 amide, 1215 cm^{-1} (phenol), 1115 cm^{-1} (ether), 860 cm^{-1} (w) (assymetric trisubstituted aromatic). Fluorescence spectrum (methanol): excitation, λ_{max} 498 nm; emission, λ_{max} 522 nm. Melting point >340°C.

5b. 17β-hydroxy-4androsten-3-one 3-(O-(3',6'-dihydroxyfluoran-5-yl-thioureidoheptylcarbamoyl methyl)oxime - (Vb) - (testosterone-DAH-fluorescein).

The crude product of IVb was treated in exactly the same way as the product IVa except that a second incubation with FITC was not necessary as all the steroid reacted with FITC in the first incubation. After the two preparative T.L.C. steps - the R_Fs are shown in Table 1 - only 0.35% of the original radioactivity was recovered, corresponding to 1.6 mg Vb. The product was crystallised from acetone:water.

The UV spectrum (ethanol) is shown in Figure 2. IR spectrum (KBr): 3400 cm^{-1} (steroidal 17β-OH), 2935 cm^{-1} (steroidal CH stretch), 1665 cm^{-1} (amide), 1215 cm^{-1} (phenol), 1115 cm^{-1} (ether), 860 cm^{-1} (w)(assymetric trisubstituted aromatic). Fluorescence spectrum (methanol): excitation, λ_{max} 498nm; emission, λ_{max} 522nm. Melting point: > 340°C.

TABLE 1

	Developing Solvent System			
	I	II	III	IV
Testosterone-3-CMO	0.00	0.62	-	0.54
Cysteamine	0.00	-	-	-
1,3-diaminopropane	-	0.00	-	0.00
1,7-diaminoheptane	-	0.00	-	0.00
Testosterone-3-cysteamine	0.60	-	1.00	-
Testosterone-3-DAP	0.00	0.65	-	0.05
Testosterone-3-DAH	0.00	0.65	-	0.10
IAEDANS	-	-	0.25	-
FITC	0.74	-	-	0.91
Testosterone-cysteamine-DANS	-	-	0.45	-
Testosterone-DAP-fluorescein	0.37	-	-	0.68
Testosterone-DAH-fluorescein	0.32	-	-	0.60

The R_Fs of the reagents and products on silica gel 60 (analytical) thin-layers with the following developing solvent systems:

I: Benzene:ethyl acetate:acetone (1:8:1)
II: Chloroform:methanol:water (60:39:1)
III: Chloroform:ethanol (1:1)
IV: Chloroform:ethanol (7:3)

6. Stability and immunoreactivity of the products.

Thin-layer chromatography using chloroform:ethanol (1:1) and high pressure liquid chromatography using methanol:water (45:55) (1.5 ml/min) as developing solvents showed that the testosterone-cysteamine-DANS was stable for at least 8 months in methanol at 4°C. Radioimmunoassay of the product showed that it was recognised by the anti-testosterone-3-CMO-bovine serum albumin antiserum with 70% of the displacement of radioactive testosterone by an equivalent amount of testosterone (Table 2).

Thin-layer chromatography of testosterone-DAP-fluorescein and testosterone-DAH-fluorescein with benzene:ethyl acetate:acetone (1:8:1) as developing solvent showed that after storage at 4°C as

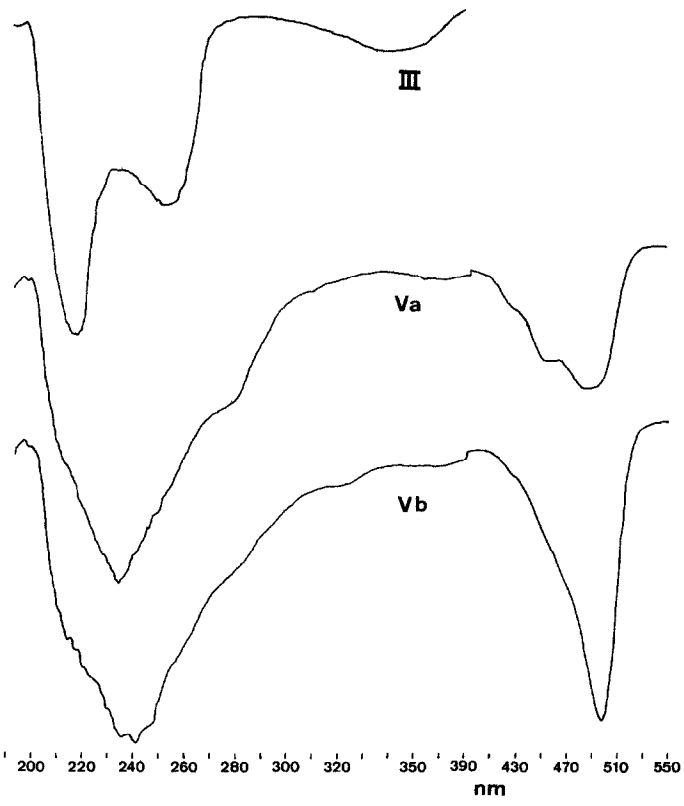

Figure 2: The UV spectra (in ethanol) of testosterone-cysteamine-DANS (III), testosterone-DAP-fluorescein (Va) and testosterone-DAH-fluorescein (Vb). The pH-dependent absorption at 498 nm by the fluorescein moiety diminished with shortening bridge length.

crystals or dissolved in methanol more polar products were formed. However, these products all contained the steroid and the fluorochrome, indicating the formation of different states or forms of the molecules. Both of the parent compounds gave rise to at least three of these different forms. The fluorescence spectrum of each of these forms remained the same as that of the parent compound.

Isolation of the different forms, storage at -15°C in methanol for 3 months and chromatographic analysis showed that, under these conditions, each form gave rise to all the others though there was a tendency for each to yield forms more polar than itself, thus it was not an equilibrium distribution.

A fresh isolation of each of the forms was carried out, the products were taken up with 0.05M phosphate buffer pH 7.0 containing 1 g/l gelatin and fluorescence intensities measured. The concentration of each form was calculated from its fluorescence relative to FITC

standards in the same buffer. Testosterone radioimmunoassays were carried out with appropriate dilutions of each form. The results are shown in Table 2. It can be seen that while both parent compounds had 10 % of the immunoreactivity of testosterone (assuming fluorescence intensity measurements gave absolute concentrations) the more polar forms were all more, and equally, immunoreactive (40-50 %) with the antiserum used here.

TABLE 2

R_F	Immunoreactivity		
	III	Va	Vb
0.45	0.7	-	-
0.32 - 0.37	-	0.1	0.1
0.14 - 0.16	-	0.4	0.5
0.10 - 0.11	-	0.4	0.5
0.00 - 0.01	-	0.5	0.5

The immunoreactivity of fluorescence-labelled testosterone derivatives after T.L.C. separation of the various forms. Developing solvents were: ethanol:chloroform (1:1) for III (testosterone-cysteamine-DANS) and benzene:ethyl acetate: acetone (1:8:1) for Va (testosterone-DAP-fluorescein) and Vb (testosterone-DAH-fluorescein). Immunoreactivities are given as a fraction of that of the same concentration of testosterone.

CONCLUSIONS

With the formation of fluorescein-labelled testosterone derivatives the major product(s) of the reaction between testosterone-3-CMO and the diaminohydrocarbon was/were (a) relatively hydrophobic steroid(s) with no free amino groups. Losses at this stage were increased by the process of removing the large excess of unreacted diaminohydrocarbon used to prevent coupling of the steroid to both ends of the "bridge" (6). Losses after coupling with FITC resulted from inefficient elution of the products after T.L.C. Use of methanol, which may react with FITC (7), in the reaction could not be avoided but did not affect yields as no unreacted aminated steroid was found after the reaction. Nevertheless, despite the low percentage yields the total quantities of the products obtained (1.6 - 3.5 mg) were amply sufficient, only small amounts (e.g. 100 pg) being required for fluorescence polarisation studies.

The coupling of IAEDANS to testosterone with cysteamine as the "bridge" gave a comparatively high yield of product (9.6%). This

product was very stable, no breakdown being detectable after 8 months in methanol at 4°C.

Fluorescence polarisation immunoassays require the use of small fluorescence-labelled molecules and these must, obviously, be recognised by the antiserum used. On the one hand analogous derivatives to those reported by Ekeke and Exley for steroids (8) were not prepared as they are of relatively high molecular weight and thus of diminished utility for polarisation studies. On the other hand preliminary experiments have shown that direct coupling of dansyl hydrazine with testosterone, as reported by Chayen et al.(9) for 17-ketosteroids, gave a product which was poorly recognised by the anti-testosterone-3-CMO-bovine serum albumin antiserum used in this laboratory. Thus the attempts were made to synthesise derivatives in which the fluorochrome is spaced from the steroid by a hydrocarbon "bridge".

It has been found that the testosterone-cysteamine-DANS derivative had 70 % of the immunoreactivity of testosterone with our antiserum and could thus be used as the labelled antigen in immunoassays. Similarly the testosterone-DAP-fluorescein and testosterone-DAH-fluorescein were both immunoreactive and their more polar forms had 40-50 % of the immunoreactivity of testosterone. Thus, as all the forms were immunoreactive, they too could be used for immunoassays. The higher quantum yield of fluorescein compared with DANS means that immunoassays using derivatives containing the former compound would be more sensitive.

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