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A STUDY OF 19-*O*-CARBOXYMETHYL ETHER AND 19-HEMISUCCINATE DERIVATIVES OF TESTOSTERONE: THEIR IMMUNOGENICITY AND USE AS IODINATED RADIOLIGANDS FOR RADIOIMMUNOASSAY OF TESTOSTERONE

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Summary—Testosterone 19-*O*-carboxymethyl ether (T19C) and 19-hemisuccinate (T19H) derivatives were synthesised, and coupled to bovine serum albumin (BSA) or porcine thyroglobulin (PT) for immunogens or to iodohistamine for radioligands. The immunogenicity of these conjugates in mice was compared with those of testosterone 3-*O*-carboxymethyloxime and 15β-thioethyl conjugates. Of 10 immunogens studied, those linked to PT gave the highest antiserum titres and more sensitive standard curves. The site of conjugation (19 or 3 position) had little effect on immunogenicity. Cross-reactivity with 5α-dihydrotestosterone (DHT) was in the range 22–100%, for the 19-linked immunogens.

Antisera to T19C and T19H conjugated to PT were then raised in rabbits and characterised with 4 radioligands. Homologous assay systems in which the chemical bridge was identical in immunogen and ^{125}I-radioligand gave the highest antiserum titres but the poorest assay sensitivity while those heterologous with respect to bridge or site gave the most sensitive standard curves. Rabbit antisera to both T19C and T19H immunogens showed good specificities with respect to DHT (range of cross reactions 0.78–21.1%), androstenedione, AN (range 0.45–2.3%) and progesterone, Po (range 0.05–1.4%) with all radioligands. The best assay system employed an antiserum to the T19H-PT immunogen with heterologous radioligand [^{125}I]T19C. It had a detection limit of 15pg/tube and low cross-reactivity with DHT (0.78%), Po (0.3%) and AN (0.43%).

We conclude that 19-linked derivatives of testosterone conjugated to PT are good immunogens and the antisera when combined with [^{125}I]testosterone radioligands which are heterologous with respect to the chemical bridge, provide highly specific assays for testosterone with potential for clinical application.

INTRODUCTION

In a previous study, using a 15β-thioalkyl derivative of testosterone (T) we produced monoclonal antibodies with less than 2.8% cross-reactivity with 5α-dihydrotestosterone (DHT), although cross-reactivity with androstenedione (AN) and progesterone (Po) was unacceptably high [1]. These findings are similar to those of Rao *et al.* [2], who raised polyclonal antisera to a 15β-carboxyethylmercaptotestosterone–bovine serum albumin conjugate and employed [^{3}H]T as a radioligand. Overall cross-reactivity with AN and Po was improved using a 19-*O*-carboxymethyl ether derivative [3] although cross-reactivity with DHT ranged from 5.8–34.2%. This prompted us to study T19-linked derivatives as immunogens in mice for the production of monoclonal antibodies with improved specificity towards DHT and AN and to evaluate the use of T19-linked conjugates as iodinated radioligands.

In the present study, we describe the synthesis of 19-*O*-carboxymethyl ether and 19-hemisuccinate derivatives of T conjugated to bovine serum albumin (BSA) at different degrees of substitution and to porcine thyroglobulin (PT) for the production of antisera in mice and rabbits. We also describe the synthesis of novel iodinated radioligands synthesised from the T19-conjugates and their comparison with [^{3}H]T and a T-3-(*O*-carboxymethyl)oximino-[^{125}I]iodohistamine in the characterisation of the antisera.

EXPERIMENTAL

Equipment and reagents for chemical synthesis

Unless otherwise stated the following instruments, conditions and chemicals were used:

Melting points were determined on a Kofler hot-stage microscope. Infra-red spectra were recorded on a Pye-Unicam SP3-200 spectrophotometer, using cells of 0.1 cm path length. Ultraviolet spectra were measured on a Shimadzu 260 spectrophotometer.

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Trivial names used: androstenedione = 4-androstene-3,17-dione: 5α-dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one.

Mass spectral data were produced by a Kratos MS 25 spectrometer. Nuclear magnetic resonance spectra were obtained using a Perkin–Elmer RB 12 spectrometer. Analytical TLC were performed on Merck precoated silica gel 60F 254 plates 5 × 10 cm (5719). Merck silica gel SI 60 (40–63 nm) was used for preparative liquid chromatography. HPLC used a Water's M6000 pump, a 5 μm ODS column 250 × 4 mm and a Perkin–Elmer LC 55 detector. GLC used a Carlo-Erba HRGC on a fused silica bonded OVI phase (0.15 μ) column 25 m × 0.33 mm at 290°C with hydrogen (2.5 kg/cm^2) as carrier gas. Ultrafiltration was done using an Amicon 50 ml cell using a PM10 membrane at 15 psi Nitrogen pressure. Scintillation counting used Biofluor (Nuclear Chicago) in a Searle Mark II counter.

All solvents were obtained from May and Baker Ltd, Dagenham, U.K., and were glass redistilled. Sodium [^{3}H]borohydride was obtained from Amersham Radiochemical Centre, Amersham, U.K. 19-Hydroxyandrost-4-ene-3,17-dione(1) was provided by Syntex, Palo Alto, California and Ciba Geigy, Basle, Switzerland. BSA (crystalline) was obtained from Miles laboratories and PT from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were purchased from Aldrich Chemical Co., and used without prior purification.

Reagents for immunoassays

All reagents were of Analar grade and purchased from Sigma Chemical Co., Poole, Dorset, U.K., unless otherwise specified. Sac-cel (anti-rabbit) was from Wellcome Diagnostics, Dartford, U.K. Norit GSX charcoal was from Hopkin and Williams, Chadwell Heath, U.K., and Dextran T70 was from Pharmacia Ltd, Hounslow, U.K. Aqua-Luma was from LKB Instruments Ltd, South Croydon, Surrey, U.K. Polystyrene assay tubes 9.5 × 63.5 mm were from Luckham Ltd, Burgess Hill, West Sussex, U.K.

Synthesis of the steroid haptens

3-Oxo-17β-hydroxy-4-androsten-19-yl carboxymethyl ether **(4).** 19-Hydroxy-4-androstene-3,17 dione **(1)** has been converted into the desired hapten **(4)** previously by Rao *et al.* [3]. However their seven stage synthesis involving tetrahydropyranyl and acetyl protecting groups only gave approx 5% product. A much more direct route, with control of the reactions by chromatographic monitoring (HPLC, GLC) seemed more appropriate. Thus (Fig. 1), direct formation of the 19-carboxymethyl derivative **(2)**, selective reduction of the ring D ketone to the alcohol **(3)** and hydrolysis of the ethyl ester group affords the hapten **(4)** in only *three* steps. In practice this route proved successful and gave an overall yield of 58%.

3-Oxo-17β-hydroxy-4-androsten-19-yl hemisuccinate **(7).** The preparation of this steroid hapten **(7)** from 19-hydroxy-4-androstene-3,17-dione **(1)** proved to be more difficult than anticipated. Direct formation of the hemisuccinate **(5a)** (Fig. 1) was easily accomplished using succinic anhydride with 4-dimethylaminopyridine catalysis [4]. Unfortunately the subsequent sodium borohydride reduction could not be conveniently controlled. The methyl ester derivative **(5b)** could be more cleanly reduced but some loss of the succinate group occurred due to the alkaline nature of the commercial sodium borohydride. Attempted selective hydrolysis of the methyl ester group of the succinate **(5b)** under a variety of conditions was unsuccessful because of the surprising ease with which the complete succinate group was removed. Choice of the *t*-butyl ester **(5c)** and reduction with calcium borohydride [5] overcame these difficulties. Final selective acid catalysed hydrolysis of the *t*-butyl ester gave the desired hemisuccinate **(7)** in over 80% yield for the four stage synthesis.

T-3-O-carboxymethyl oxime **(8).** This hapten was purchased from Steraloids.

Procedure and characterisation

19-Hydroxy-4-androstene-3,17-dione **(1)** was characterised to give m.p. 165–6°C, ν_{max} ($CHCl_3$), 3400, 1730, 1650, 1210 cm^{-1}: λ_{max} (MeOH), 242 nm, ε = 17,000, δ($CDCl_3$), 0.95 (3H, s, 18 Me) 3.95 (2 H, s, 19 CH_2), 5.85 (IH, s, 4 H) ppm, MS(EI)*m/e*, 302 (25%), $C_{19}H_{26}O_3$, 272 (100%) (CI, NH_3) 320 (3%, m + 18), 303 (100% m + 1), TLC, Et_2O/CH_2Cl_2(1/1) R_f = 0.45, GLC. R_t = 2.43 min, HPLC, 80% MeOHaq R_v = 4.2 ml.

3,17-Dioxo-4-androsten-19-ethoxycarbonylmethyl ether **(2).** A solution of ethyl diazoacetate (1.3 g) in chloroform (3.5 ml) was added dropwise with stirring under an atmosphere of nitrogen at room temperature during 1.5 h to a mixture of 19-hydroxy-4-androstene-3-17-dione **(1)** (1g) and rhodium II acetate dimer (11 mg) in chloroform (3.5 ml). The reaction was stopped after a further 15-min stirring at room temperature when GLC analysis indicated approx 60% conversion to product **(2)** and less than 10% starting material **(1)** remaining. The rhodium catalyst was removed by filtration through silica gel and the solvent was evaporated. The resultant viscous oil was chromatographed on silica gel (250 g) using diethyl ether as solvent to give the 19-ethoxycarbonyl methyl ether as an oil (0.8 g, 62%): ν_{max} ($CHCl_3$), 1740, 1670, 1630, 1200, 1120 cm^{-1}: λ_{max} (MeOH), 240 nm, ε = 12,500: δ ($CDCl_3$), 0.97 (3 H, s, 18 Me), 1.40 (3 H, t, 7.5 Hz, OCH_2 *Me*), 3.87 (2 H, dd, 7.5 H_z, 19 CH_2), 4.08 (2 H, s, OCH_2CO), 4.23 (2 H, q, 7.5 Hz, OCH_2 Me), 5.90 (1 H, s, 4 H) ppm:MS(EI)*m/e*, 388 (15%), $C_{23}H_{32}O_5$, 284 (25%), 117 (100%), (CI, NH_3), 406 (20%, m + 18), 309 (100% m + 1), 225 (95%), TLC, Et_2O/CH_2Cl_2 (1:1), R_f = 0.7, GLC R_t = 5.62 min, HPLC 80% MeOH aq. R_v = 5.2 ml.

3-Oxo-17β-hydroxy-4-androsten-19-ethoxycarbonyl methyl ether **(3).** To the ketone **(2)** (0.75 g 1.93 mM) dissolved in ethanol (20 ml) was added an ethanolic solution (2 ml) of $NaBH_4$ (40 mg). HPLC

monitoring showed essentially complete reduction after 4 h. Acetone (1 ml) was added and after 5 min the mixture was acidified with dilute H_2SO_4. After addition of water the ethanol was removed by rotary evaporation and the product extracted with ethyl acetate. The organic layer was washed with water, dried ($MgSO_4$) and the solvent removed to give a viscous oil. Purification by silica gel chromatography using a dichloromethane–diethyl ether gradient gave with 40% ether the pure alcohol **(3)** as an oil (0.7 g, 94%).

M.p. 96–8°C, ν_{max} ($CHCl_3$), 3450, 1740, 1660, 1625, 1215, 1140 cm^{-1}: λ_{max} (MeoH), 241 nm, $\varepsilon = 17{,}500$: $\delta(CDCl_3)$, 0.80 (3 H, s, 18 Me), 1.28 (3H, t, 7.5 Hz, OCH_2 *Me*), 3.85 (2 H, dd, 7.5 Hz, 19 CH_2), 4.05 (2 H, s, OCH_2CO), 4.22 (2 H, q, 7.5 Hz, OCH_2 Me), 5.88 (1 H, s, 4 H) ppm, MS(EI) *m/e*, 390 (10% $C_{23}H_{34}O_5$), 286 (15%), 117 (40%), 43 (100%), (CI, NH_3) 408 (10% m + 18), 391 (100% m + 1), TLC, Et_2O/CH_2Cl_2 (1/1) $R_f = 0.5$, GLC, $R_t = 5.8$ min, HPLC, $R_v = 5.4$ ml.

3-Oxo-17β-hydroxy-4-androsten-19-yl-carboxy-methyl ether **(4)**. The ethyl ester **(3)** (250 mg) dissolved in methanol (20 ml) was treated with the reagent (KOH 200 mg, H_2O 0.6 ml, MeOH 2 ml) at room temperature with HPLC monitoring. After 4 h the reaction was complete and the alkali was neutralised by addition of dilute sulphuric acid. The methanol was evaporated, water added and the product extracted with ethyl acetate. After drying with $MgSO_4$ the solvent was removed to yield the crude product in essentially quantitative yield. Chromatography on silica gel eluting with 1% acetic acid in ethyl acetate gave the desired hapten **(4)** which readily crystallised from dry diethyl ether. M.p. 190–2°C, ν_{max} (nujol) 3380, 1735, 1645, 1130 cm^{-1}: λ_{max} (MeOH), 242 nm, $\varepsilon = 15{,}500$: $\delta(CD_3OD/CDCl_3)$, 0.8 (3 H, s, 18 Me), 3.55 (1 H, m, 17 H), 3.80, 3.95 (2 H, d, 8 Hz, 19 CH_2), 4.04 (2 H, s, OCH_2COOH), 5.85 (1 H, s, 4 H) ppm, MS(EI)*m/e*, 362 (70% $C_{21}H_{30}O_5$), 286 (20%), 274 (65%), 43 (100%), (CI, NH_3), 363 (100% m + 1), TLC, EtoAc/2% HOAc, $R_f = 0.3$, HPLC, 80% MeOHaq $R_v = 3.0$ ml.

t-*Butyl 3,17-Dioxo-4-androsten-19-yl succinate* **(5c)**. 19-hydroxy-4-androstene-3,17-dione **(1)** (909 mg, 3.0 mM), *t*-butyl hemisuccinate (740 mg, 4.75 mM), dimethylaminopyridine (300 mg, 2.46 mM) were dissolved in dichloromethane (40 ml) and treated with dicyclohexylcarbodiimide (1 ml). HPLC monitoring showed the reaction to be complete after 30 min. The dichloromethane was evaporated and the residue extracted with diethyl ether with filtration to remove the dicyclohexyl urea. Chromatography on silica (200 g) using diethyl ether as eluant gave the *t*-butyl ester **(5)** as an oil (1.35 g, 100%).

ν_{max} ($CHCl_3$), 1740, 1665, 1620, 1210, 1140 cm^{-1}: λ_{max} (MeOH), 239 nm, $\varepsilon = 17{,}500$: δ ($CDCl_3$), 0.90 (3 H, s, 18 Me), 1.55 (9 H, s, CMe_3), 2.60 (4 H, s, $(COCH_2)_2$), 4.2 (1 H, d, 11 Hz, 19 H), 4.55 (1 H, d, 11 Hz, 19 H), 5.9 (1 H, s, 4 H) ppm, MS(EI)*m/e*, 458 (0%, $C_{27}H_{38}O_6$), 402 (35%), 284 (55%), 272 (60%), 57 (100%), (CI, NH_3), 476 (3% m + 18), 459 (40% m + 1), 403 (100%), TLC Et_2O/CH_2Cl_2 (1/1), $R_f = 0.7$, HPLC, 80% MeOHaq $R_v = 5.6$ ml.

t-*Butyl 3-Oxo-17β-hydroxy-4-androsten-19-yl succinate* **(6)**. The reagent **(5)** was prepared by mixing at −20°C calcium chloride dihydrate (148 mg) dissolved in ethanol (4 ml) with sodium borohydride (76 mg) in ethanol (4 ml). The steroidal *t*-butyl succinate **(5c)** (1.35 g, 3.07 mM) was dissolved in ethanol (30 ml), toluene (2 ml) was added as an internal standard and the solution cooled to −20°C. The reagent (6 ml) was then added. HPLC, monitoring showed that after 1 h only 25% of the starting material **(5c)** remained and over 70% of the desired product **(6)** had been formed. The reduction was terminated by addition of acetone (2 ml) to destroy excess reagent. After 5 min diluted sulphuric acid was added and most of the ethanol removed by evaporation.

After extraction with ethyl acetate and drying ($MgSO_4$) the solvent was removed to give the crude product. Chromatography on silica (250 g) using a dichloromethane–diethyl ether gradient gave 15% ether, starting material **(5c)** (270 mg, 20%), with 30% ether, pure product **(6)** [950 mg, 70%], and with 35% ether, a small quantity of over reduced product.

ν_{max} ($CHCl_3$), 3450, 1725, 1660, 1620, 1160 cm^{-1}: λ_{max} (MeOH), 239 nm, $\varepsilon = 18{,}000$: $\delta(CDCl_3)$, 0.9 (3 H, s, 18 Me), 1.55 (9 H, s, CMe_3), 2.6 (4 H, s, $(COCH_2)_2$), 3.7 (1 H, m, 17 H), 4.2 (1 H, d, 11 Hz, 19 H), 4.55 (1 H, d, 11 Hz, 19 H), 5.9 (1 H, s, 4 H) ppm, MS(EI)*m/e*, 460 (0% $C_{27}H_{40}O_6$), 404 (35%), 286 (65%), 274 (50%), 57 (100%), (CI, NH_3), 478 (5% m + 18), 461 (70% m + 1), 405 (100%)::TLC, Et_2O/CH_2Cl_2 (1/1), $R_f = 0.5$, HPLC, 80% MeOHaq $R_v = 6.4$ ml.

3-Oxo-17β-hydroxy-4-androsten-19-yl hemisuccinate **(7)**. The acidic reagent was prepared by diluting 45% hydrogen bromide in acetic acid (1.5 ml) with dichloromethane (30 ml) and cooling to 0°C. The cold reagent (31.5 ml) was added to the *t*-butyl ester **(6)** [700 mg] in dichloromethane (100 ml) at 0°C during 30 min. HPLC monitoring showed complete conversion to product after a further 30 min at 0°C. Excess anhydrous sodium bicarbonate was added to neutralise the acid and the mixture filtered. Removal of the solvent by rotary evaporation gave the product as a viscous oil in essentially quantitative yield. Final purification of the succinate **(7)** was achieved by silica gel chromatography eluting with 1% acetic acid in ethyl acetate. The product failed to crystallise.

ν_{max} ($CHCl_3$), 3000, 1720, 1660, 1610 1200 cm^{-1}: λ_{max} (MeOH), 240 nm, $\varepsilon = 14{,}500$: $\delta(CDCl_3)$, 0.78 (3 H, s, 18 Me), 2.55 (4 H, s $(CH_2COO)_2$), 3.65 (1 H, m, 17 H), 4.15 (1 H, d, 12 Hz, 19 H), 4.65 (1 H, d, 12 Hz, 19 H), 5.8 (2 H, s, 0 *H*), 5.9 (1 H, s, 4 H) ppm, MS(EI)*m/e*, 404 (30%, $C_{23}H_{32}O_6$), 286 (85%), 274 (60%), 55 (100%), (CI, NH_3), 422 (2% m + 18), 405

(55%, m + 1), 305 (100%), TLC, EtOAc/2% HOAc, $R_f = 0.35$, HPLC 80% MeOHaq $R_v = 3.0$ ml.

Preparation of the steroid–protein conjugates

The standard procedure [6] developed in these laboratories, based on the mixed anhydride method, was used to conjugate 19-linked and 3-linked T derivatives to BSA and PT. The following solutions were prepared and cooled to 5°C:

(a) the steroid, ie. 3-oxo-17β-hydroxy-4-androsten-19-yl carboxymethyl ether **(4)** (100.5 mg, 278 μM, 3×10^8 dpm) dissolved in dioxan (10 ml). The tritium label was introduced using $NaBT_4$ during the reduction of the ring D ketone,

(b) tributylamine (65 mg, 350 μM) in dioxan (1 ml),

(c) isobutylchloroformate (42 mg, 308 μM) in dioxan (1 ml),

(d) histamine (113 mg, 1000 μM) in methanol (10 ml),

(e) BSA (105 mg, 90 lysine residues) in 0.2 M K_2HPO_4–dioxan 1:1 (30 ml) × 2.

(f) PT (100 mg, 25 lysine residues) in 0.2 M K_2HPO_4–dioxan 1:1 (30 ml).

The tributylamine salt of the hapten was prepared by adding the amine solution (b) (1 ml) to the steroid solution (a) (10 ml). After mixing well, the isobutylchloroformate solution (c) (1 ml) was added in one portion and after 5, 15 and 30 min a 10 μl (0.23 μM) aliquot of the reaction mixture was quenched with the histamine solution (d) (100 μl) and analysed for its mixed anhydride content by HPLC. The final assay usually indicated >90% mixed anhydride production. The conjugates were then prepared by slowly adding the mixed anhydride solution over approx 1 h at 4°C to the protein solution. Care was taken to ensure that the solutions remained homogenous throughout the coupling procedure. Conjugates T19C1, T19H1 and T3C1 were produced from mixed anhydride solution (6.6 ml, 153 μM) and BSA solution (e) (30 ml). Conjugates T19C2, T19H2 and T3C2 were prepared from mixed anhydride solution (3.3 ml) (76 μM) and BSA solution (e) (30 ml). Conjugates T19C3, T19H3 and T3C3 were obtained by coupling mixed anhydride solution (1.65 ml, 38 μM) and PT solution (f) (30 ml). Finally the remaining mixed anhydride solution (0.42 ml) was used for coupling to histamine using solution (d) (0.5 ml, 50 μM), to study the iodination reaction. HPLC analysis of the coupling reactions by monitoring the consumption of the mixed anhydride revealed that the reactions were virtually complete after 1 h.

The conjugates were recovered by ultrafiltration (Amicon 50 ml cell, PM10 membrane), over 2.5 h washing with 7 vol of distilled water. The final concentrate was diluted to 10 ml with water and divided into 10 × 1 ml aliquots for storage. Analysis of the conjugate solution using ultraviolet spectroscopy and radioactivity measurements allowed determination of the yield and estimation of the hapten number for each conjugate as summarised in Table 1. Analysis of the ultrafiltrates showed only the presence of the original steroid hapten which was recovered after acidification by ether extraction and yields were 42–55% for each conjugate.

T15β-thioethyl-BSA. The synthesis of this conjugate was described previously [1].

Immunisation

Groups of Balb/c mice received subcutaneous (s.c.) injections of immunogen(1 mg) emulsified in Freund's complete adjuvant followed by intraperitoneal (i.p.) injections of conjugate (10 μg) in Freund's incomplete adjuvant at monthly intervals. Mice were bled 2 weeks after each i.p. injection and antiserum titre determined. The characterisation of the antisera was carried out after the third i.p. injection.

The multi-site immunisation technique was used to raise antisera in New Zealand white rabbits. Five s.c. injections of conjugate (100 μg in total) emulsified in Freund's complete adjuvant were given to each animal, followed by 5 s.c. and 5 intramuscular (i.m.) injections of conjugate (100 μg in total) in Freund's incomplete adjuvant at monthly intervals. The rabbits were bled 2 weeks after the second boost, for characterisation of antisera.

Immunoassays

Diluent. For mouse antisera, assay diluent was 0.05 M sodium phosphate buffer pH 7.4 containing 0.9% NaCl, 0.1% gelatin and 0.1% NaN_3. For rabbit antisera, assay diluent was 0.05 M sodium phosphate buffer pH 7.4 containing 0.1% BSA and 0.1% NaN_3.

Radioligands. [1,2,6,7^{3}H]T) ([^{3}H]T) (sp. act. 80–105 Ci/mmol) and T-3-(*O*-carboxymethyl)-oximino-(2-[^{125}I]iodohistamine ([^{125}I]T3CMO) (sp. act. approx 2000 Ci/mmol) were purchased from Amersham International, Amersham, U.K. T19-hemisuccinyl-[^{125}I]iodohistamine ([^{125}I]T19H) and T19-carboxymethyl ether-[^{125}I]iodohistamine ([^{125}I]T19C) were prepared as described by Hunter *et al.* [7] and purified by TLC on silica gel (Merck 5583) in chloroform–methanol–acetic acid (90:10:1, by vol). The 19-hemisuccinate extract gave 2 discrete peaks of radioactivity (R_f 0.31 and 0.59) and immunoreactivity was shown to be associated only with component of R_f 0.31. The 19-carboxymethyl extract gave a single peak (R_f 0.44) of radioactivity and immunoreactivity on TLC. The radioligands were eluted in ethanol, and approx 5 fmol radioligand (10–15,000 cpm) were added per assay tube. Approximately 8,000 cpm or 150 fmol of [^{3}H]T was added per assay tube.

Standards. T and steroids for cross-reactivity studies including DHT, AN and Po were dissolved in absolute ethanol and stored at a concentration of 1 mg/ml at −20°C. Working standards of 40 ng/ml (T), 320 ng/ml (DHT) and 800 ng/ml (AN and Po)

were prepared in diluent, stored at −20°C, and diluted serially in assay diluent for each assay.

Assay procedure. Antiserum (100 μl) was incubated with radioligand (100 μl) and 100 μl standard or buffer for 2–16 h at 4°C. For mouse antisera, separation of bound and free [^{3}H]T was achieved using 0.5 ml charcoal reagent (Norit GSX 0.25%, Dextran T70 0.025%) incubated for 15 min at 4°C followed by centrifugation (2,000 *g*) for 15 min at 4°C. The supernatant was decanted into 2.5 ml Aqua Luma and counted in a LKB Rackbeta scintillation counter. Assays employing rabbit antisera were separated using a suspension of anti-rabbit IgG coupled to cellulose (Sac-cel), with incubation for 30 min at 4°C followed by the addition of 1.0 ml assay diluent and centrifugation (2,000 g) for 15 min at 4°C. For ^{125}I-radioligands the precipitate was counted in a LKB Multigamma counter, and for [^{3}H]T the supernatant was counted as described previously.

Assessment of antisera. Antisera were stored at −20°C and diluted for use in the appropriate diluent buffer. The antibody titre was expressed as the final dilution of antibody required to bind 50% of the radioligand. This antibody concentration was used in the subsequent characterisation of sensitivity and specificity. Assay sensitivity was defined as the mass of testosterone in pg/tube producing a 10% decrease in specific binding. Relative assay sensitivities were compared from the mass of testosterone displacing 50% of bound radioligand. The specificity of each antiserum was studied with respect to the following potentially major cross-reacting steroids, DHT, AN and Po. Cross-reactivity was calculated according to Abraham[8] from the mass of each steroid required to produce 50% displacement of the radioligand. The affinity constant was calculated by the method of Muller[9] for a competitive radioimmunoassay under equilibrium conditions.

RESULTS

Comparison of mouse antisera raised to different immunogens

19-Carboxymethyl ether and 19-hemisuccinate derivatives of T were synthesised as shown in Fig. 1 and covalently coupled to BSA or PT to give the steroid–protein molar ratios shown in Table 1. T3-*O*-carboxymethyloxime was similarly coupled to BSA or PT (Table 1). The immunogenicity of each conjugate was assessed by immunising mice with each conjugate following a hyperimmune protocol with collection of serum after 3 or 4 i.p. immunisations. A similar regime has previously shown satisfactory responses [1]. The titres of the antisera shown in Table 2 although low, are typical of the responses found in mice. Three of the T19-linked immunogens gave higher titres (1:490, 1:640 and 1:3500) than a T15β-BSA immunogen which was used successfully for the production of monoclonal antibodies [1]. There was a very striking improvement in immunogenicity when thyroglobulin was used as the carrier protein as shown by groups immunised with T19C3 or T3C3. Poorest responses (titres < 1:10) were obtained with the BSA immunogens T19C1, T3C1, and T3C2 but all the mice immunised with the other conjugates responded.

For each group of antisera the mass of T which produced a 50% displacement of the [^{3}H]T radioligand is shown in Table 2. T3C3, T19C3 and T19H3 immunogens, yielded the most sensitive displacement curves (mean values of 107, 330 and 640 pg/tube respectively), with values of 60, 70 and 140 pg/tube respectively for the best individual antisera. Specificity studies (Table 3) revealed significant cross-reaction with DHT for all immunogens (mean 58–83%) while most individual antisera gave acceptable cross-reactivity with AN and Po.

Comparison of rabbit antisera with different radioligands

Three rabbits were immunised with PT conjugates T19H3 or T19C3 which had previously given antisera of acceptable titre and avidity in mice. The antisera obtained were characterised with respect to titre and displacement using the radioligands, [^{3}H]T, [^{125}I]T19H, [^{125}I]T19C and [^{125}I]T3CMO (Table 4). Titre was increased using ^{125}I-radioligands compared with ^{3}H-radioligands which may partly reflect the higher specific activity of the iodinated tracer and hence the lower mass per assay tube. Antisera raised to both T19H3 and T19C3 immunogens yielded highest titres with the corresponding homologous radioligands (1:230,000 with [^{125}I]T19H and

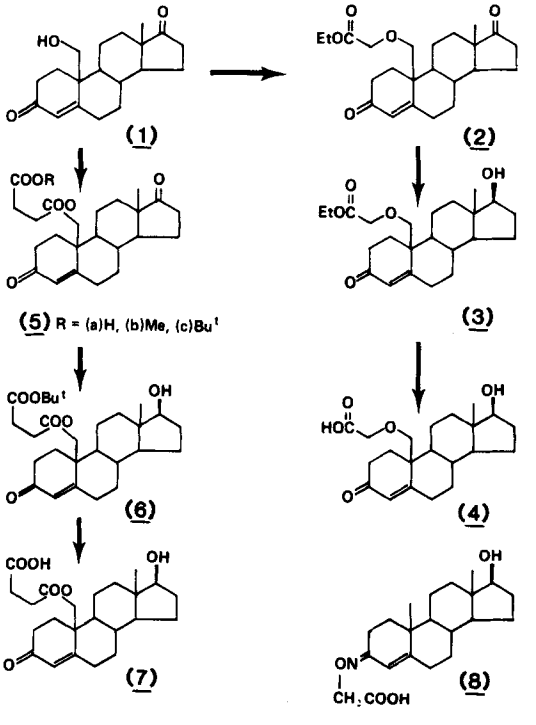

Fig. 1. Synthesis of the steroid haptens.

Table 1. Classification of the immunogens

Immunogen	Abbreviation	Steroid: protein u.v.	Steroid: protein ^{3}H	Yield (mg)
19-Linkage				
Testosterone 19-hemisuccinate-BSA	T19H1	41	40	129
Testosterone 19-hemisuccinate-BSA	T19H2	25	26	120
Testosterone 19-hemisuccinate-PT	T19H3	106	107	106
Testosterone 19-carboxymethyl ether-BSA	T19C1	29	30	122
Testosterone 19-carboxymethyl ether-BSA	T19C2	21	22	117
Testosterone 19-carboxymethyl ether-PT	T19C3	78	80	104
3-Linkage				
Testosterone 3-carboxymethyloxime-BSA	T3C1	32	—	123
Testosterone 3-carboxymethyloxime-BSA	T3C2	22	—	117
Testosterone 3-carboxymethyloxime-PT	T3C3	77	—	104
15-Linkage				
Testosterone 15β-thioethyl-BSA	T15T	45	—	—

Synthesis of 19- and 3-linked immunogens are described in the text, and preparation of the 15-linked immunogen was described previously [1]. PT is porcine thyroglobulin. *Steroid: protein is expressed as a molar ratio, analysed by u.v. or ^{3}H.

Table 2. Assessment of mouse antisera

Immunogen	Number of mice	Titre Range	Titre Mean	50% Displacement (pg/tube) Range	50% Displacement (pg/tube) Mean
T19H1	4	1:60–1:210	1:140	1500–6900	2600
T19H2	4	1:420–1:600	1:490	460–2900	2700
T19H3	5	1:30–1:1200	1:640	140–2300	640
T19C1	5	<1:10	—	ND	—
T19C2	3	1:30–1:210	1:120	460–2200	1000
T19C3	5	1:900–1:9000	1:3500	70–690	330
T3C1	4	<1:10	—	ND	—
T3C2	5	<1:10–1:100	—	ND	—
T3C3	5	1:2700–1:12000	1:6500	60–170	107
T15T	5	1:150–1:450	1:270	1300–24,500	12,000

All mouse antisera were assessed using [^{3}H]testosterone as the radioligand with separation by charcoal. ND: not determined.

Table 3. Specificity of mouse antisera raised to 19-carboxymethyl ether and 19-hemisuccinate immunogens

	Cross-reactivity (%)					
	5α-Dihydrotestosterone		Androstenedione		Progesterone	
Immunogen	Range	Mean	Range	Mean	Range	Mean
T19H1	35–100	83	<1.0–23	—	<1.0–10	—
T19H2	22–100	62	<1.0–6.5	—	<0.1–6.0	—
T19H3	40–100	76	0.2–6.0	3.1	<0.1–10	—
T19C2	37–100	58	4.0–10.0	6.0	<0.1–1.8	—
T19C3	34–100	76	<1.0–6.0	—	<0.1–6.0	—

The immunogens are defined in Table 1.

Table 4. Characterisation of rabbit antisera to T-19 hemisuccinate and carboxymethyl ether immunogens with four radioligands

		[^{3}H]T		[^{125}I]T19H		[^{125}I]T19C		[^{125}I]T3CMO	
Immunogen	Rabbits	Titre	50% Displacement (pg/tube)	Titre	50% Displacement (pg/tube)	Titre	50% Displacement (pg/tube)	Titre	50% Displacement (pg/tube)
T19H3	R1	3000	650	300,000	900	40,000	150	30,000	360
	R2	2400	470	200,000	700	45,000	500	25,000	310
	R3	3000	530	200,000	500	40,000	120	10,000	400
	Mean	2800	550	230,000	700	42,000	260	22,000	360
T19C3	R1	9000	420	100,000	270	250,000	700	12,000	220
	R2	9000	480	220,000	170	270,000	400	30,000	170
	R3	3000	400	50,000	170	60,000	550	5.000	190
	Mean	7000	430	120,000	200	193,000	550	15,700	200

The radioligands were [^{3}H]T, [^{3}H]testosterone: [^{125}I]T19H, testosterone 19-hemissuccinyl-[^{125}I]histamine: [^{125}I]T19C, testosterone 19-carboxymethyl ether-[^{125}I]histamine: [^{125}I]T3CMO, testosterone 3-carboxymethyloxime-[^{125}I]histamine. For abbreviations of immunogens see Table 1. R1–R3 refers to individual rabbits.

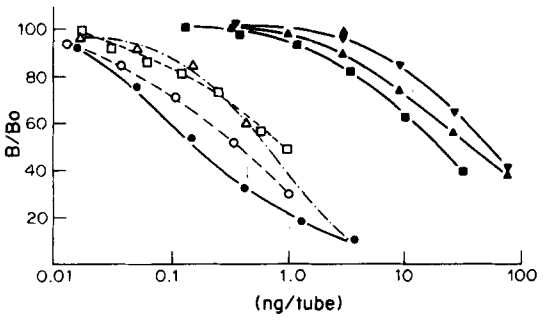

Fig. 2. A comparison of testosterone standard curves with 4 different radioligands and the specificity of the rabbit antiserum T19H-R1. The antiserum was raised to the 19-hemisuccinate immunogen, T19H3 (see Table 1) in rabbit 1, with a 19-carboxymethyl ether derivative, [^{125}I]T19C, as radioligand. Cross-reactivities are shown with testosterone (●---●), 5α-dihydrotestosterone (■---■), progesterone (▲---▲), and androstenedione (▼---▼).

Testosterone standard curves are compared using the following radioligands, [^{125}I]T19C (●—● [^{125}I]T19H (□---□) [^{125}I]T3CMO (○---○), [^{3}H]testosterone (△---△).

1:193,000 with [^{125}I]T19C respectively). For each antiserum group there was on average a 2- to 3-fold difference in the mass of testosterone giving 50% displacement with the 4 radioligands studied. For both immunogens, the most sensitive standard curves were obtained with the heterologous radioligands: for example with antisera to T19H3 in combination with [^{125}I]T19C and [^{125}I]T3CMO, and with antisera to T19C3 in combination with [^{125}I]T19H and [^{125}I]T3CMO. Assay sensitivity was poorest for each antiserum group using the homologous radioligands. For an individual antiserum (T19H3 R1), a comparison of standard curves with the different radioligands is shown in Fig. 2.

Table 5 compares the effect of different radioligands on the specificity of the rabbit antisera. Overall cross-reactivity was low averaging 5.8–14.9% for DHT, 0.64–1.1% for AN and 0.12–0.70% for Po. There was no clear difference in the specificity of groups of antisera with different radioligands but for individual antiserum such as T19H3 R1 or T19C3 R1, marked specificity differences were apparent.

The heterologous assay system, using the antiserum from rabbit 1 immunised with T19H3 and the [^{125}I]T19C radioligand gave the best combination of sensitivity and specificity achieved in the present study (Fig. 2). The affinity constant was $1.5 \times 10^9\ M^{-1}$, with a sensitivity of 15 pg/tube and a working range of 25–1500 pg/tube. The cross-reactivity with DHT, AN and Po was 0.78, 0.3 and 0.43% respectively.

DISCUSSION

We have previously described the production of T antisera using a 15β-thioalkyl conjugate and the selection of monoclonal antibodies of widely differing specificities [1]. Using this approach antiserum specifity was inadequate for the accurate measurement of testosterone in females without prior chromatographic purification of the samples. We are aware of only one report of an antibody achieving this specificity and that antibody was raised to a C_{19}-linked immunogen [3]. In this paper, we describe the synthesis of T19-hemisuccinate (T19H) and T19-carboxymethyl ether (T19C) derivatives and their conjugation to BSA or PT in parallel with a T3-carboxymethyloxime (T3C) derivative. The antisera raised with these immunogens were then characterised with radioligands prepared by linking the derivatives to iodohistamine.

The two derivatives (T19H and T19C) were synthesised by a novel, more direct route and monitored by chromatographic analysis which provided a much improved yield over the previous study [3]. Parallel synthesis of the two different derivatives from the same starting material and parallel conjugation to BSA or PT or linkage to iodohistamine gave a controlled comparison of immunogens and radioligands. The immunogenicity of T19H, T19C and T3C conjugates coupled to BSA or PT were compared in mice by characterising the antisera produced using [^{3}H]T as the radioligand. For each hapten studied, immunogens with thyroglobulin as the carrier protein produced antisera of highest titre and avidity which were also superior to antisera raised to T15-thioethyl-BSA in a previous study [1]. Similarly,

Table 5. Effect of different radioligands on the specificity of rabbit antisera

		[^{3}H]T			[^{125}I]T19H			[^{25}I]T19C		
Immunogen	Rabbits	DHT	AN	Po	DHT	AN	Po	DHT	AN	Po
T19H3	R1	8.9	1.5	0.48	6.4	1.75	1.4	0.78	0.3	0.43
	R2	11.8	0.85	0.29	8.8	0.85	0.55	10.6	2.3	1.0
	R3	24.1	0.88	0.17	9.2	0.67	0.14	21.4	0.55	0.15
Mean		14.9	1.07	0.31	8.1	1.07	0.70	10.9	1.05	0.53
T19C3	R1	1.6	1.4	0.35	1.0	1.0	0.19	8.5	1.8	0.8
	R2	8.0	0.71	0.24	8.9	0.47	0.11	8.5	0.61	0.18
	R3	10.0	1.3	0.07	7.6	0.45	0.05	6.4	0.8	0.06
Mean		6.5	1.1	0.22	5.8	0.64	0.12	7.8	1.07	0.35

DHT, 5α-dihydrotestosterone: AN, androstenedione: Po, progesterone, R1–R3 refers to individual rabbits. The immunogens are defined in Table 1 and the radioligands in the text.

for each derivative, the BSA conjugates with a lower incorporation rate were more immunogenic. Antisera raised to T19C and T19H immunogens showed high cross-reactivity with DHT (in the range of 58–83%), but acceptable cross-reactivity for AN and Po in certain mice. Nevertheless the lack of specificity with respect to DHT obtained in the mice should not limit these as a source of monoclonal antibodies, since selected monoclonal antibodies may show striking improvements in specificity [1].

The T19-linked immunogens giving the highest titre and avidity in mice (T19-H3 and T19C3) were chosen to raise antisera in rabbits. In steroid assays employing iodinated radioligands, loss of assay sensitivity may arise due to bridge recognition resulting from the presence of the same chemical bridge and site of attachment to the steroid in both the immunogen and radioligand [10, 11]. All combinations of antisera and radioligand were therefore examined in the present study to provide information on possible bridge effects and to identify the combination of assay reagents producing optimal assay sensitivity. Titres of all antisera were improved using ^{125}I—rather than ^{3}H-radioligands which may partly reflect the lower mass of the iodinated tracers. For each immunogen highest titres, but poorest assay sensitivity was obtained with homologous iodinated tracers. The most sensitive assays were obtained using ^{125}I-radioligands with a heterologous bridge, or, both heterologous bridge and site of attachment ([^{125}I]T3CMO). Titres and avidity were intermediate with the ^{3}H-radioligand. These findings are indicative of a degree of bridge recognition by the antisera and have been described previously for Po [10, 12] and for AN [11] and stress the need for selection of a radioligand which will yield optimal assay sensitivity.

The major problem with T antisera is the cross-reactivity with the reduced metabolite, DHT. We found that this cross-reactivity was much lower with rabbit antisera (0.78–24.1%) than with mouse antisera (22–100%) and in general better than that found by Rao *et al.*[3] using a T19-*O*-carboxymethylether derivative conjugated to BSA (5.8–32% in 4 rabbits). Two out of six of the rabbit antisera gave DHT cross-reactivity of 1.0% or less with selected radioligands which is similar to that obtained with polyclonal antisera raised to a 15-linked immunogen [2, 13] and with monoclonal antibodies derived from a 15-linked immunogen [1] and a 17-linked immunogen [14]. This specificity is also superior to that obtained with antisera raised to 3-linked immunogens [14, 15]. In contrast, antisera raised to the 15- and 17-linked immunogens have shown major cross-reactivity with Po and AN [1, 3, 14]. In the present study with 19-linked immunogens we have achieved low cross-reactivity with AN (0.3%) and Po (0.05%) confirming the findings of Rao *et al.* [3]. Thus our studies suggest that the 19-linked immunogens can overcome the problems of specificity encountered with 3-, 15- and 17-linked immunogens, especially if the antisera are combined with selected radioligands.

With T19-hemisuccinate and T19-carboxymethyl ether conjugates linked to PT no major differences in antiserum specificity were encountered when comparing [^{3}H]T, [^{125}I]T19H or [^{125}I]T19C as radioligands, although striking improvements in individual antisera (T19C3-rabbit 1 and T19H3-rabbit 1) were obtained with respect to their DHT specificity. Since we achieved good specificity with antisera derived from both T19H3 and T19C3, our findings suggest that the nature of the bridge may be less important than the site of linkage in determining specificity.

Of all the rabbit antisera, T19H3-rabbit 1, gave the best combination of high sensitivity and specificitiy with the corresponding heterologous bridge radioligand [^{125}I]T19C, providing a working range of 25–1000 pg/tube. This has the potential for the development of a sensitive and specific radiommunoassay of testosterone for use in clinical studies.

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