I - PREPARATION OF STEROID-ANTIGENS THROUGH POSITIONS OF THE STEROID NOT BEARING FUNCTIONAL GROUPS.

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ABSTRACT

Dehydroepiandrosterone (DHA) and Dihydrotestosterone (DHT) derivatives were prepared in order to obtain antibodies to these steroids. DHA and DHT were coupled to bovine serum albumin through positions which left the functional groups of the steroid free.

An intercalated bond with a carboxylic function was linked to C-7 or C-15 of DHA and C-1 of DHT.

In 1957, Erlanger et al (1) first achieved a successful preparation of steroid antigens. Until 1970, methods to obtain haptens were virtually unchanged : namely, a functional group, hydroxyl or ketone, of the steroid hormone was converted to the hemisuccinate or carboxymethyloxime. In a second step, the hapten was coupled to an immunogenic carrier, generally bovine serum albumin.

Howewer, the antibodies to these antigens are specific to the haptenic part in the particular steroid ; the resulting antibodies lose specificity at the functional group and its immediate vicinity.

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In 1970, Midgley and Niswender (2) reported that the antibodies prepared from antigens in which progesterone was linked to the antigenic carrier through C-11 had a greater specificity than those obtained from antigens in which progesterone was linked through C-3 or C-20.

These authors attributed the increased specificity of this conjugation of the steroid-antigen to the fact that both ends of the steroid retained their pre-existing functions.

By extrapolation, they suggest "that conjugates should be prepared through a position distal to structurally unique region", ideally positions 6 or 7 of steroids.

At the same time, Linder, Perel and Friedlander (3) obtained an estradiol-antigen linked at C-6.

Numerous studies have since then confirmed Midgley and Niswender's hypothesis with steroids linked at C-6 and C-7 (4-8). The resulting antibodies had great specificity, with minimal cross-reactions, and the relative activity of compounds with kindred structure was in a ratio of about 1 to 100. It is likely that functional groups of the steroid which remain free also act as antigenic determinants.

The aim of this study was to discover new sites on steroids for side chain linking to a carrier. The method consisted in temporarily protecting the functional groups and to add a side chain beyond the position of those groups.

Of course, this side chain should possess a reactive group such as a carboxylic function to be coupled onto a carrier.

We were interested in varying the linkage positions and we have reported (9) preliminary results obtained with two models of androgenic steroids: dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one = DHA) and dihydrotestosterone (17β-hydroxy-5α-androstan-3-one = DHT).

A - Preparation of 3β-hydroxy-17-oxo-5-androsten-7-one 7-(O-carboxymethyl)oxime:B.S.A. (figure 1)

3β-acetoxy-17-ethylenedioxy-5-androstene (II) was prepared by the usual method, i.e. from the 3β-hydroxy-5-androsten-17-one (DHA) (I) by formation of the ketal in benzene with ethylene glycol and p-toluenesulfonic acid followed by an acetylation.

Then (II) was oxidized with sodium chromate in a mixture of acetic anhydride-acetic acid, according to the method described by Marshall et al. (10). The resultant compound (III) was precipitated in an aqueous solution of sodium bicarbonate to avoid deketalisation, and recrystallised.

Condensation of (III) with carboxymethoxylamine hemi hydrochloride followed by a deketalisation and a saponification gave the hapten (V).

The condensation of (V) to bovine serum albumin (B.S.A.) according to the method of Vaughan (11) gave the antigen (VI).

The number of steroids incorporated per mole of B.S.A. was measured according to H. Tamaoki et al. (12) utilising nitrotropone, and indicated 24 moles of steroid per mole of B.S.A..

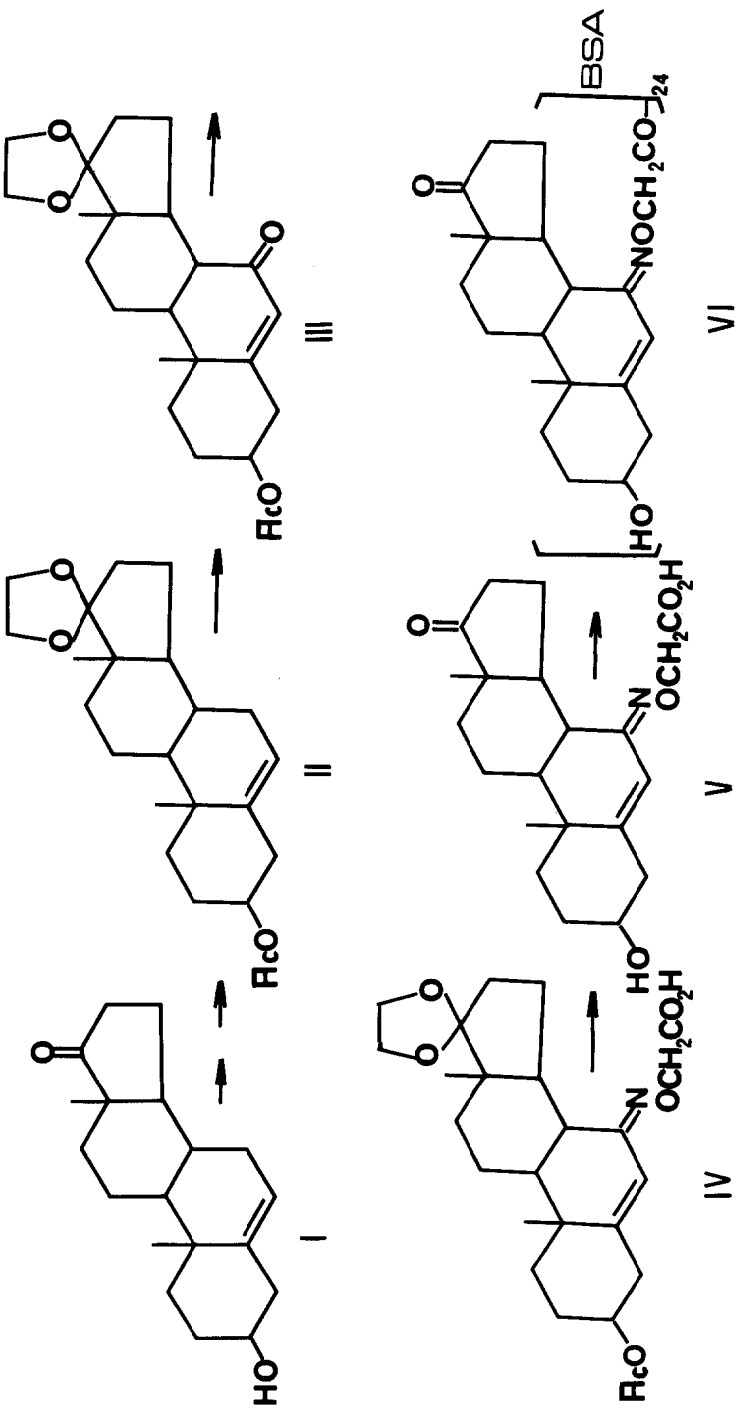

FIGURE 1

Optical density was measured taking as reference the molar extinction coefficient of nitrotropone-B.S.A. as 5.10^3 M^{-1} cm^{-1} at 420 nm.

B - Preparation of 3β-hydroxy-15α-carboxymethyl-5-androsten-17-one:B.S.A. (figure 2)

The method to obtain 3β-hydroxy-5,15-androstadien-17-one (X) was the same as that utilised by us (12) and by Sykes and Kelly (13).

After the formation of ethylene ketal from DHA (I), bromine introduced to position 16, using phenyl trimethylammonium bromide perbromide (P.T.T.) in anhydrous tetrahydrofuran, according to the method described by Marquet et al. (14).

Bromoketal (VIII) was dehydrobrominated by potassium tert-butylate in dimethylsulfoxide, giving the diene (IX). Ethylenic ketone (X) was obtained by hydrolysis of (IX) with p-toluenesulfonic acid in aqueous acetone.

Addition of diethyl sodiomalonate to (X) was followed by saponification; decarboxylation in refluxing dioxane in presence of strong acid led to compound (XI).

To elucidate the stereochemistry of the 15-carboxymethyl group, the 17-ketone was reduced with potassium borohydride to the diol (XII)++. A single isomeric compound was formed. The reduction gave rise to probably the 17β-alcohol.

Not any lactone could be obtained from the compound (XII).

++ This compound can be used as a hapten.

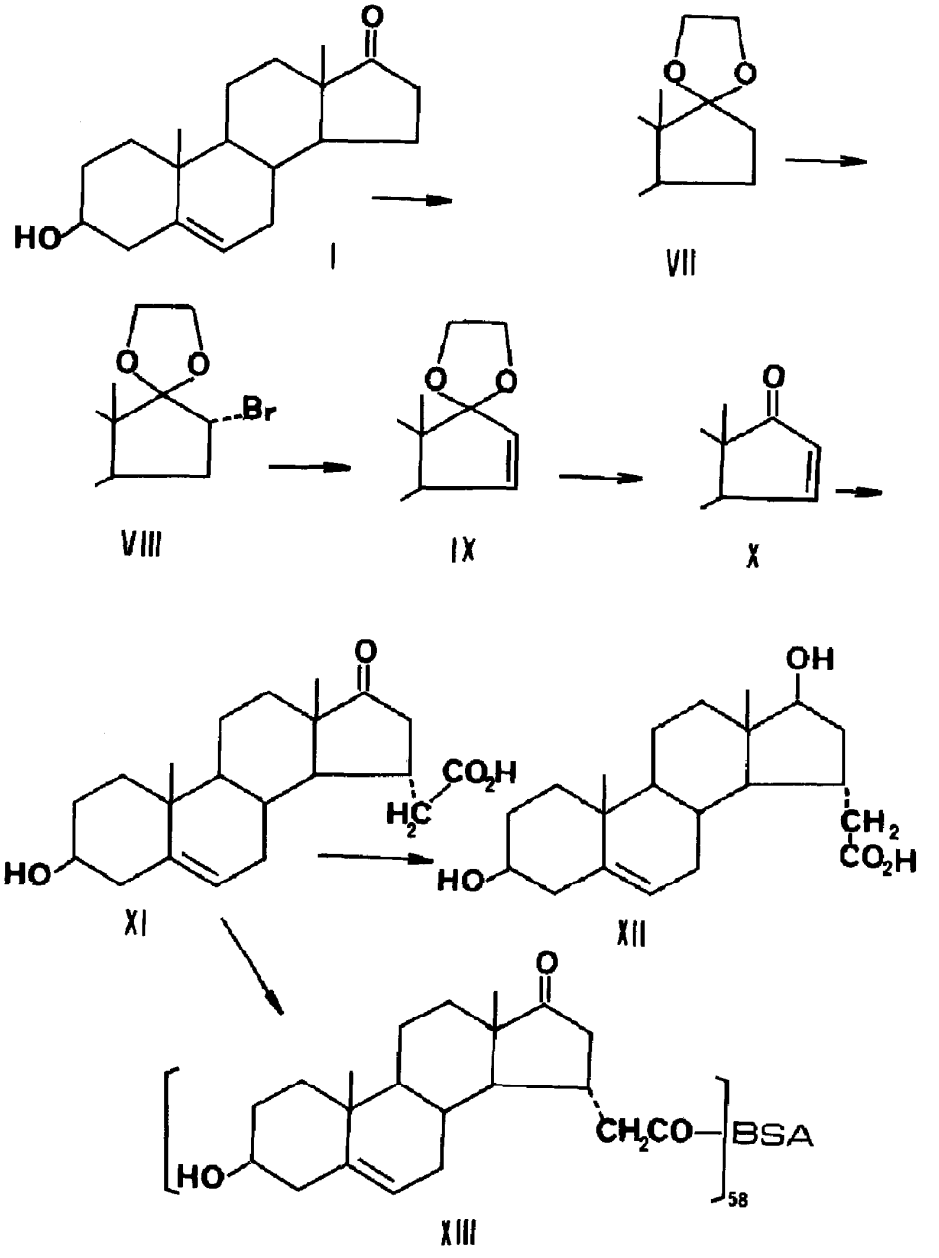
HO
O
I
VII
Br
VIII
IX
X
XI
XII
XIII
CO2H
H2C
OH
CH2
CO2H
CH2CO
BSA
58

FIGURE 2

Because a lactone can only result from C-15 and C-17 cis functional groups, we conclude that the 15-carboxymethyl group is probably 15α (trans to 17β).

Condensation of the hapten (X) with B.S.A. was carried out in the same manner as for compound (V). Incorporation was measured, as previously described, and indicated 58 moles of steroid per mole of B.S.A..

C - Preparation of 17β-hydroxy-1α-carboxymethyl-5α-androstan-3-one:B.S.A. (figure 3)

1α-carboxymethyl-17β-hydroxy-5α-androstan-3-one (XV) was prepared by a Michaël reaction utilising diethylmalonate and sodium methylate in methanol.

Addition was followed by saponification and decarboxylation as previously described (for compound XI). The addition gave only one of both possible isomers.

Determination of the stereochemistry of this product (XV) was carried out with the mixture of isomers obtained by reduction of the 3-ketone with potassium borohydride. It is known that this reduction results in the formation of an intermediate compound on the less hindered side (15).

Otherwise it has been reported (16) that, in the case of 3-oxo steroids, the reduction by potassium borohydride gave 3β-hydroxy compounds in 90% yields.

In our case, the 3α-hydroxy isomer was predominant (about 80%). It was less polar, in thin layer chromatography, than the 3β-hydroxy isomer.

With a carboxymethyl link situated in 1β position lactonisation can not occur either with 3α or 3β hydroxy

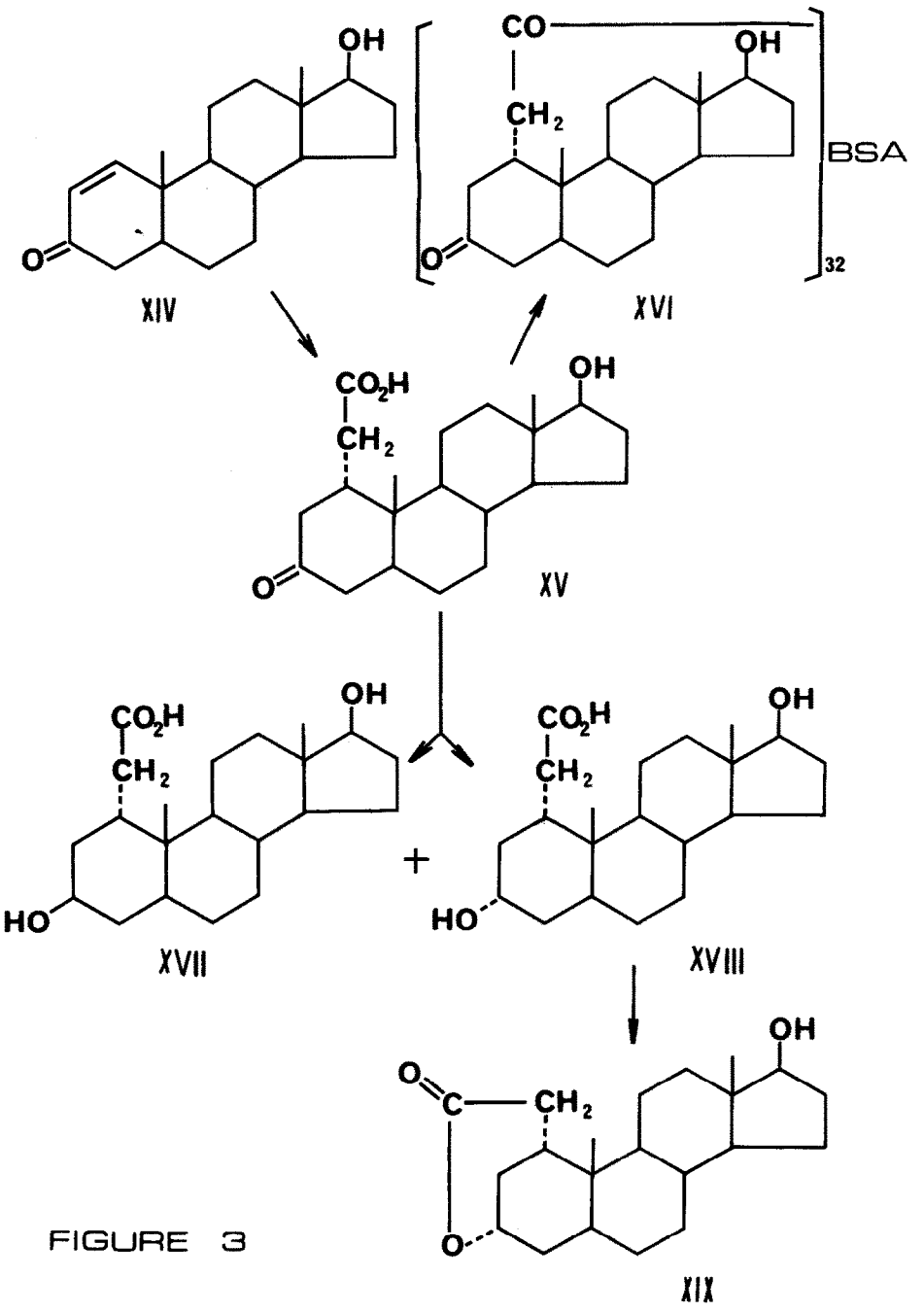
OH
O
XIV
CO
CH2
OH
O
BSA
32
XVI
CO2H
CH2
OH
O
XV
CO2H
CH2
OH
HO
XVII
+
CO2H
CH2
OH
HO
XVIII
OH
O
C
CH2
O
XIX

FIGURE 3

compounds; if the 1α-carboxymethyl product has been formed, lactonisation will only result from the 3α-hydroxy isomer.

When the mixture of both isomers was subjected to conditions of lactonisation, thin layer chromatography showed that the spot corresponding to compound (XVII) disappeared whereas a new spot appeared. This product was isolated and identified as the lactone (XIX).

Thus the carboxymethyl group is 1α. It is not surprising because the approach at the C-1 position of (XIV) by the malonyl anion is easier from the α side than from the β side.

These results are in agreement with those of Kočor et al. (17) in which Michaël addition with steroidal 3-keto 1,4,6-trienes gave 1α-substitued steroids.

Condensation of (XV) with B.S.A. was achieved in the same manner as for the others antigens. The incorporation was 32 moles of steroid per mole of B.S.A..

EXPERIMENTAL

All melting points were determined on a Leitz microscope with heating stage and were uncorrected.

Infrared spectra were obtained on a Perkin Elmer 254 (KBr pellets).

Nuclear Magnetic Resonance spectra were obtained from a Varian A 60 with tetramethylsilane as the internal standard.

Optical rotations were obtained from a Jouan-Roussel automatic polarimeter with dioxane as the solvent.

Microanalysis were done by Service de Microanalyse du C.N.R.S. 94-THIAIS- France.

3β-acetoxy-17-ethylenedioxy-5-androsten-7-one (III)

To 3β-acetoxy-17-ethylenedioxy-5-androstene (II) (10g) in acetic anhydride (20 ml) and acetic acid (40 ml) was added, with stirring, at room temperature, 13g of anhydrous sodium chromate. After dissolution, the mixture was kept at 40°C during 18 hours. Then, the solution was slowly poured in water saturated with sodium bicarbonate.

The product was filtred off, washed with water, dried and crystallised from methanol, giving 2.6g (III), m.p. 168-170°C.

Recrystallisation from the same solvent gave 1.75g of analytical product, m.p. 175-177°C.

Anal. Calcd for $C_{23}H_{32}O_5$ C, 71.10 ; H, 8.30 ; O, 20.59
Found C, 70.85 ; H, 8.16 ; O, 20.65

$[\alpha]_D$ - 574 (c 0.6)

I.R. $\bar{\nu}_{max}$ 2950; 2880; 1810; 1740; 1670; 1640 cm^{-1}.

N.M.R. ($CDCl_3$) δ_{ppm} 0.83 (C_{18}methyl, 3H, s); 1.25 (C_{19}methyl, 3H, s); 2.03 (acetoxy, 3H, s); 3.95 (ketal, 4H, s); 5.7 (H olefinic, 1H, m).

3β-hydroxy-7-(O-carboxymethyl) oximino-5-androsten-17-one (V)

The ketone (III) (810 mg) was dissolved in pyridine (20 ml) with carboxymethoxylamine hemi hydrochloride (850 mg).

The mixture was stirred at 40°C for 24 hours. Pyridine was distilled off under reduced pressure. The residue was dissolved in ether and washed with 1N HCl, then with water to neutrality.

The solution was dried over Na_2SO_4 and evaporated to dryness. The residue (945 mg) was dissolved in acetone (50 ml) and p-toluenesulfonic acid (25 mg) in 5 ml of water was added. The solution was kept for 24 hours at room temperature. Acetone was distilled off and the residue was dissolved in a mixture of tert-butanol (50 ml) and 10 ml of 8N sodium hydroxyde. This mixture was stirred at 25°C for 24 hours.

Tert-butanol was distilled off under reduced pressure,

the product was dissolved in water (50 ml) and extracted with ether (3x50 ml). The aqueous solution was acidified with formic acid and extracted with ether (2x60 ml).

The organic solutions were combined, dried over Na_2SO_4 and evaporated to dryness.

The crystalline material (1.64 g) was crystallised from aqueous methanol giving (V) 517 mg, m.p. 215°C.

An analytical sample was recrystallised from the same solvent, m.p. 220°C.

Anal. Calcd. for $C_{21}H_{29}O_5N$ C, 67.18; H, 7.79; O, 21.31; N, 3.73
Found C, 67.13; H, 7.51; O, 21.60; N, 3.98

$[\alpha]_D$ -286 (c 0.7)

I.R. $\bar{\nu}_{max}$ 3400; 2940; 1740; 1640; 1090 cm^{-1}.

N.M.R. (DMSO) δ_{ppm} 0.83 (C_{18}methyl, 3H, s); 1.1 (C_{19}methyl, 3H, s); 4.5 (CH_2 oximino, 2H, s); 6.4 (H_6, 1H, m).

17-ethylenedioxy-5-androsten-3β-ol (VII)

p-Toluenesulfonic acid (0.5 g), 3β-hydroxy-5-androsten-17-one (I) (44 g), benzene (550 ml) and ethylene glycol (45 ml) were refluxed in an apparatus fitted with a reflux condenser and a provision for the exclusion of moisture for 24 hours. Then, an aqueous saturated solution of sodium bicarbonate (200 ml) was added and the reaction mixture was allowed to cool slowly to room temperature giving a first crop (31.05 g) of (VII), m.p. 167-168°C.

Concentration of the mother-liquors gave a second crop (16.9 g) m.p. 162-164°C.

$[\alpha]_D$ -177 (c 1.3)

N.M.R. (CCl_4) δ_{ppm} 0.8 (C_{18}methyl, 3H, s); 1.06 (C_{19}methyl, 3H, s); 3.81 (ethylenedioxy, 4H, s); 5.36 (H_6, 1H, m).

16α-bromo-17-ethylenedioxy-5-androsten-3β-ol (VIII)

The ketal (VII) (10.2 g) was dissolved in anhydrous tetrahydrofuran (200 ml). P.T.T. (13.4 g) was added. The resulting mixture was stirred for 2 hours after which time the solution was colourless. Pyridinium hydrobromide was filtered off and the solvent evaporated to dryness.

The residue was dissolved in a mixture of chloroform-ether (100 ml; 1:4, v/v) and washed with water to neutrality. The organic solution was dried over Na_2SO_4 and evaporated to dryness. The residue was crystallised from aqueous methanol to give the bromo compound (VIII) (6.5 g), m.p. 145-150°C.

An analytical sample was recrystallised three times from methanol, m.p. 169-171°C.

Anal. Calcd. for $C_{21}H_{31}BrO_3$ C, 61.31; H, 7.59; O, 11.67
Found C, 61.65; H, 7.56; O, 11.68

$[\alpha]_D$ -186 (C 1.0)

N.M.R. ($CDCl_3$) δ_{ppm} 0.88 (C_{18}methyl, 3H, s); 1.00 (C_{19}methyl, 3H, s); 3.45 (ketal, 4H, s); 5.30 (H_6, 1H, m).

17-ethylenedioxy-3β-hydroxy-5,15-androstadiene (IX)

The bromo ketal (VIII) (29.7 g) was dissolved in dimethylsulfoxide (400 ml). Freshly resublimed potassium tert-butoxide (16.75 g) was added and the reaction flask was stoppered and left to stand at 40°C for 15 hours.

The solution was poured into dry ether (1 l) and washed with water to neutrality, dried, and evaporated to dryness. The residue was crystallised from methanol to give the diene (IX) (20.62 g), m.p. 162-164°C.

Anal. Calcd for $C_{21}H_{30}O_3$ C, 76.32; H, 9.15; O, 14.53
Found C, 76.16; H, 9.15; O, 14.83

$[\alpha]_D$ - 202 (c 1.3)

N.M.R. (CCl_4) δ_{ppm} 0.83 (C_{18}methyl, 3H, s); 1.0 (C_{19}methyl, 3H, s); 3.83 (ethylene ketal, 4H, caracteristical multiplet of ethylene ketal with a vicinal double bond); 5.3 (H_6, 1H, m); 5.57 (H_{15}, 1H, quartet); 6.0 (H_{16}, 1H, doublet J_{15-16}= 6 Hz).

3β-hydroxy-5,15-androstadien-17-one (X)

The ketal (IX) (500 mg) was dissolved in acetone (25 ml). A solution of p-toluenesulfonic acid (25 mg) in water (2.5 ml) was added and the solution was kept at 15°C for 24 hours. Then, water (7.5 ml) was added and the solution was evaporated. Material which crystallised during evaporation was filtered off, washed and dried to give (X), m.p. 187-195°C. An analytical sample was recrystallised from ethyl acetate, m.p. 196-200°C.

Anal. Calcd for $C_{19}H_{26}O_2$ C, 79.68; H, 9.15; O, 11.17
Found C, 79.16; H, 9.13; O, 11.35

$[\alpha]_D$ -278 (c 0.85)

N.M.R. (DMSO) δ_{ppm} 0.95 (C_{18}methyl, 3H, s); 1.0 (C_{19}methyl, 3H, s); 4.53 (H_3, 1H, m); 5.33 (H_6, 1H, m); 6.0 (H_{15}, 1H, quartet, J_{15-16}= 6 Hz, J_{14-15}= 3 Hz); 7.67 (H_{16}, 1H, quartet, J_{15-16}= 6 Hz, J_{14-16}= 1.5 Hz)

15α-carboxymethyl-3β-hydroxy-5-androsten-17-one (XI)

Ketone (X) (1.72 g) was dissolved in methanol (10 ml). Diethylmalonate (1.3 g) and a solution (5 ml) of sodium methylate in methanol (185 mg of Na) was added.

The solution was kept at room temperature for 29 hours. Acetic acid was added and evaporation gave a product which was redissolved in chloroform, washed with water to neutrality, dried and evaporated to dryness. The product (2.62 g) was dissolved in tert-butanol (40 ml) and stirred with a solution (20 ml) of sodium hydroxide (8N) for 16 hours. Tert-butanol was evaporated and the product extracted with chloroform. The aqueous solution was acidified with formic acid and washed with a mixture of chloroform-ether (4:1; v/v). The organic solution was evaporated giving a semi-crystalline product (1.3 g). This product was dissolved in a mixture of dioxane (30 ml), water (8 ml) and formic acid (3 ml), and refluxed for ten hours.

Evaporation gave a product which was recrystallised from ethyl acetate to give 660 mg (XI), m.p. 230-232°C.

An analytical sample was recrystallised from methanol, m.p. 238-240°C.

Anal. Calcd for $C_{21}H_{30}O_4$ C, 72.80; H, 8.73; O, 18.47
Found C, 72.52; H, 8.63; O, 18.59

$[\alpha]_D$ + 70 (c 0.6)

I.R. $\bar{\nu}_{max}$ 3300; 2940; 1880; 1780; 1270; 1140 cm^{-1}.

N.M.R. (DMSO) δ_{ppm} 0.95 (C_{18}methyl, 3H, s); 1.06 (C_{19}methyl, 3H, s); 5.37 (H_6, 1H, m).

15α-carboxymethyl-5-androsten-3β,17β-diol (XII)

The acidic compound (XI) (250 mg) was dissolved in methanol (40 ml) and potassium borohydride (180 mg) was added

with stirring. After 5 min, few drops of acetic acid was added.

The solution was evaporated, the residue was dissolved in ether and washed with water. The organic solution was evaporated to dryness, giving a product which was crystallised from methanol-ethyl acetate giving (XII), m.p. 253-255°C.

Anal. Calcd for $C_{21}H_{32}O_4$ C, 72.38; H, 9.26
Found C, 72.55; H, 9.13

$[\alpha]_D$ + 36 (c 0.7)

I.R. $\bar{\nu}_{max}$ 3500-3200; 2900; 1740; 1680; 1440; 1270; 1040 cm^{-1}.

1α-carboxymethyl-17β-hydroxy-5α-androsta-3-one (XV)

17β-hydroxy-5α-androst-1-en-3-one (XIV) (1.9 g) was dissolved in methanol (15 ml). Condensation was carried out with the same method as for the compound (X).

The residue obtained after condensation was dissolved in ethanol (50 ml) and a solution (15 ml) of sodium hydroxide (8N) was added. The mixture was heated under reflux for 15 min. After decarboxylation, the product (1.6 g) (XV) was crystallised from ether (976 mg), m.p. 118-120°C. An analytical sample was recrystallised from benzene, m.p. 208-211°C.

Anal. Calcd for $C_{21}H_{32}O_4$ C, 72.38; H, 9.26
Found C, 72.26; H, 9.29

$[\alpha]_D$ + 57 (c 0.7)

I.R. $\bar{\nu}_{max}$ 3400; 2920; 1700; 1360; 1100 cm^{-1}.

N.M.R. (DMSO) δ_{ppm} 0.66 (C_{18}methyl, 3H, s); 1.12 (C_{19}methyl, 3H, s); 3.15 (H_{17}, 1H, m); 6.65 (CH_2link., 2H, m).

Preparation of lactone XIX

By reducing (XV) with potassium borohydride, a mixture of two compounds was obtained. This mixture (84 mg) was dissolved in dioxane (30 ml) with HCl (0.5 ml) and refluxed for 2.5 hours. The solution was evaporated to dryness and subjected to preparative thin layer chromatography.

The less polar product (32 mg) was crystallised from ether to give the lactone (XIX) (20 mg), m.p. 227-229°C.

Anal. Calcd for $C_{21}H_{33}O_3$ C, 75.86; H, 9.70; O, 14.44
Found C, 75.62; H, 9.71; O, 14.66

I.R. $\bar{\nu}_{max}$ 3440; 2920; 1680; 1380; 1230; 1040 cm^{-1}.

PRELIMINARY RESULTS

Antibodies of high affinity were obtained injecting separately derivatives VI, XIII and XVI to rabbits.

Antibodies obtained from VI and XIII showed a particular great specificity.

These results will be soon published by Dr Dray and coll.

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The following trivial names have been used :
Dehydroepiandrosterone (DHA) :3β-hydroxy 17-oxo androst-5-ene.
Dihydrotestosterone (DHT) :3-oxo 17β-hydroxy (5α) androstane.