METABOLISM OF 17β-HYDROXY-2α-METHYL-5α-ANDROSTAN-3-ONE IN THE RABBIT

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Abstract - The neutral urinary excretion products of 17β-hydroxy-2α-methyl-5α-androstan-3-one from the rabbit dosed orally were investigated. Together with oxidation-reduction of the oxygen functions at C-3 and C-17 hydroxylation occurred at C-15, C-16, and at the 2α-methyl positions of the steroid nucleus.

INTRODUCTION

17β-Hydroxy-2α-methyl-5α-androstan-3-one (drostanolone) (I) is a synthetic steroid which has been used clinically (together with its 17α-methyl derivative) for its anabolic properties (1) and as its propionate ester in the treatment of androgen responsive breast carcinomas (2). We have studied the metabolism of this substance in the rabbit to identify its major metabolic products and to determine the possible in vivo formation of this substance or a common metabolite from 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (3).

EXPERIMENTAL

Proton magnetic resonance spectra were recorded on a Varian HR 220 instrument by the Canadian 220 MHz NMR Centre, Ontario Research Foundation, Sheridan Park, Ontario. GLC was carried out on a Varian Aerograph Ser. 1800 chromatograph equipped with a hydrogen flame ionization detector and a pyrex glass column packed with 2% OV-17 on Chromosorb GHP

80/100 mesh, internal diameter 2 mm, length 180 cm. The detector temperature was 275°, column temperature 240° and the carrier gas (N_2) flow rate was 30 ml/min. Hydroxylated steroids were treated with trimethylsilyl chloride in pyridine before injection so that 1 µl contained 25 µg of steroid. Retention times are relative to 5α-cholestane. Mass spectra of components of mixtures were recorded on samples collected in capillary tubes from a GLC stream splitter attachment. Elemental analyses were performed by Mr. G. Crouch, School of Pharmacy, University of London, England. Solvent systems for TLC: I_a, 25% ethyl acetate-hexane; I_b, 50% ethyl acetate-hexane. Acetylations were carried out in acetic anhydride-pyridine solution at room temperature, overnight. Other apparatus and chromatographic procedures are given in ref. 4.

Administration of 17β-hydroxy-2α-methyl-5α-androstan-3-one: Three mature male albino rabbits, maintained on a Purina rabbit chow diet and water ad. libitum, were housed singly in cages designed for efficient separation of urine and faeces. A controlled illumination environment of 12 hrs light and 12 hrs darkness was maintained. The animals were each dosed three times at two-day intervals with a finely divided slurry of I (0.7 g) in propylene glycol (10 ml) by oral administration. A total of 3.85 l of urine was collected over 7 days under a layer of toluene and stored daily at -5°. Urine collected for a further 48 hours (1.2 l) gave a crude residue (53 mg) which showed no metabolite peaks on GLC. Blank urine (3.1 l) from rabbits dosed with propylene glycol (10 ml) as above was collected similarly prior to dosing. No gross alteration in the appearance, food intake or behaviour of the dosed animals was observed.

Isolation of neutral steroids (enzymatic hydrolysis): The pooled urine (3.85 l) was adjusted to pH 5.0 with glacial acetic acid and incubated with bovine liver β-glucuronidase (800 FU/ml) at 37° for 72 hrs followed by ether extraction (4x). The combined ether layers were washed successively with 0.5N-HCl, IN-NaOH, water, and dried over sodium sulfate. Evaporation of the solvent at reduced pressure yielded a crude residue (1.84g). A control experiment yielded urine (3.1 l) giving a crude residue (183 mg).

Oxidation of crude neutral steroids: A portion (100 mg) of the total crude urinary extract on oxidation with Jones reagent (5) gave a crystalline substance (77 mg). TLC in System I_a and I_b showed one major spot with identical R_f (0.69 System I_a) with 2α-methyl-5α-androstane-3,17-dione and a minor spot (R_f, 0.22, System I_a). GLC showed one major peak (RRT, 1.05) identical to 2α-methyl-5α-androstane-3,17-dione (87%) and minor peaks (RRT, 1.22, 1.33, 1.58). Recrystallization of the total oxidized product from methanol gave 2α-methyl-5α-androstane-3,17-dione (II), m.p. 150-151° [lit.(6) m.p. 152-154°], i.r. νmax 1743 (5-membered ring C=O), 1706(6-membered ring C=O) cm^{-1}; m.s., m/e: 302(M^+). Mixed melting point with an authentic

sample was not depressed. Comparison of i.r. and m.s. showed them to be identical. GLC/m.s. of the major peak gave a m.s. identical with the 3,17-dione.

Separation of ketonic and non-ketonic materials: The remaining crude material (1.74 g) was treated with Girard T-reagent (4) to give a crude non-ketonic fraction (1.05 g) and a ketonic fraction (661 mg).

Ketonic Fraction:

3α-Hydroxy-2α-methyl-5α-androstan-17-one (III): The ketonic fraction contained mainly one substance (TLC, GLC). A portion (443 mg) of the crude ketonic material was chromatographed on ethyl acetate washed alumina (8 g). From the fractions eluted with benzene (253 mg) a crystalline substance was obtained, which on recrystallization from methanol gave 3α-hydroxy-2α-methyl-5α-androstan-17-one (III), (32 mg), m.p. 195-196°; R_f, 0.56 (System Ia); RRT, 0.52; i.r.νmax 3500 (OH), 1730 (5-membered ring C=O, H-bonded); p.m.r.δ: 0.80(C-10 methyl), 0.85 (C-13 methyl), 0.90, d, J=7Hz, (2α-CH_3), 3.77, m, $W_{\frac{1}{2}}$=7Hz, (3βH) ppm; m.s., m/e: 304(M^+).

Anal. Found: C, 78.94, H, 10.52; $C_{20}H_{32}O_2$ requires C, 78.90; H, 10.59.

The mass spectra of more polar fraction eluted with ether and methanol indicated the presence of diolone (m/e, 320) and dionol (m/e, 318) substances. Rechromatography of these fractions on ethyl acetate treated alumina did not yield pure substances.

2α-Methyl-5α-androstane-3,17-dione (II): Oxidation of 3α-hydroxy-2α-methyl-5α-androstan-17-one (III) with Jones reagent gave 2α-methyl-5α-androstane-3,17-dione (II), m.p. 152.5-153.5°, (identical i.r. and m.s. spectra with an authentic sample). Mixed melting point was not depressed.

Non-ketonic fraction: The crude non-ketonic material (982 mg) was dissolved in 20% petroleum ether/benzene and chromatographed on alumina. A total of 170 fractions of 50 ml each were collected with solvents of increasing polarity. Fractions of similar components (by GLC) were combined and from the fractions containing mainly one substance, the following metabolites were isolated by repeated recrystallization.

2α-Methyl-5α-androstane-3α,17α-diol (IV): Fraction eluted with ether and 2.5% MeOH/ether which contained mainly one substance (TLC, GLC) on recrystallization from MeOH yielded IV (34 mg); m.p. 151°; R_f 0.44 (System Ia), RRT 0.29; i.r.νmax 3430 (OH) cm^{-1}; p.m.r.δ: 0.64 (C-13 methyl), 0.80 (C-10 methyl), 0.93, d, J=7Hz, (2α-CH_3), 3.69, d, J=6Hz, (17βH), 3.75, m, $W_{\frac{1}{2}}$=7Hz, (3βH), ppm; m.s., m/e: 306(M^+).

Anal. Found: C, 78.50; H, 11.21. $C_{20}H_{34}O_2$ requires C, 78.38; H, 11.18.

2α-Methyl-5α-androstane-3β,17α-diol (V): Fractions eluted with 10-25% ether/benzene (43 mg) were recrystallized from MeOH to yield V (18 mg), m.p. 215-216°; R_f 0.458 (System Ia); RRT, 0.149; i.r.νmax. 3230-3400 (OH) cm^{-1}; p.m.r.δ: 0.64 (C-13 methyl), 0.82(C-10 methyl), 0.97, d, J=7Hz, (2α-CH_3), 3.11,

sextet, $W_{\frac{1}{2}}$=27Hz, (3αH), 3.71, d, J=6Hz, (17βH) ppm; m.s., m/e: 306(M^+).
Anal. Found: C, 78.09; H, 11.12. $C_{20}H_{34}O_2$ requires C, 78.38; H, 11.18.
2α-Methyl-5α-androstane-3β,17β-diol (VI): Fractions eluted with 5% ether/benzene contained mainly one substance (TLC and GLC). This substance was separated on a thick-layer silica plate developed 3x in System Ib) and further purified by filtering through ethyl acetate washed alumina in benzene to yield VI. R_f, 0.47 (System Ia); RRT, 0.50; i.r.νmax 3450 (OH) cm^{-1}; m.s., m/e: 306(M^+). Comparison with an authentic sample showed them to be identical (R_f, RRT, i.r. m.s.).
2α-Methyl-5α-androstane-3α,15α,17α-triol (VIIa): Fractions eluted with 5% MeOH/ether (63 mg) showed the presence of mainly one peak (RRT 0.36). Recrystallization from methanol yielded VIIa (8.2 mg), m.p. 215-216°; R_f, 0.05 (System Ia); i.r.νmax. 3520 and 3450 (OH) cm^{-1}; p.m.r.δ: 0.69 (C-13 methyl), 0.81 (C-10 methyl), 0.93, d, J=7Hz, (2α-CH_3), 3.65, d, J=6Hz, (17βH), 3.76, m, $W_{\frac{1}{2}}$=7Hz, (3βH) 4.01, sextet, (15βH) ppm; m.s., m/e: 304(M^+-H_2O).
Anal. Found: C, 72.97; H, 10.48. $C_{20}H_{34}O_3 \cdot \frac{1}{2}H_2O$ requires C, 72.47; H, 10.63.
2α-Methyl-5α-androstane-3α,15α,17α-triol triacetate (VIIb): Acetylation of VIIa gave a non-crystalline product VIIb, R_f, 0.38 (System Ib); p.m.r.δ: 0.80 (C-13 methyl), 0.81 (C-10 methyl), 0.83, d, J=7Hz, (2α-CH_3), 2.007, 2.045, 2.068 (OAc), 4.75, d, J=6Hz, (17βH), 4.92, m, 2H, (3βH overlapping with the 15βH) ppm; m.s., m/e: 388(M^+-HOAc). Jones oxidation of VIIa yielded non-crystalline substance, m.s., m/e: 316(M^+).
2α-Methyl-5α-androstane-3α,16α,17α-triol (VIIIa): The mother liquor from IXa was applied to a thick-layer preparatory plate, developed three times (System Ia) and separated into three bands. From the least polar band (R_f, 0.475, System Ia, developed 3x) a crystalline substance was obtained which on recrystallization from methanol gave VIIIa (2 mg), m.p. 240°; RRT, 0.55; p.m.r.δ: 0.71 (C-13 methyl), 0.81 (C-10 methyl, 0.96, d, J=7Hz, (2α-CH_3), 3.64, d, J=6Hz, (17βH), 3.78, m, $W_{\frac{1}{2}}$=7Hz, (3βH), 4.44, m, (16βH), ppm; m.s., m/e: 322(M^+).
Anal. Found: C, 72.95; H, 10.36. $C_{20}H_{34}O_3 \cdot \frac{1}{2}H_2O$ requires C, 72.47; H, 10.63.
2α-Methyl-5α-androstane-3α,16α,17α-triol triacetate (VIIIb): The combined residues from the isolation of VIIIa were acetylated and upon further separation on thick-layer silica plates gave a non-crystalline product (VIIIb) from the least polar band corresponding to the acetate of VIIIa; R_f, 0.48 (System Ib, developed 2x); p.m.r.δ: 0.80 (C-13 methyl, 0.82 (C-10 methyl), 0.84, d, J=7Hz, (2α-CH_3), 2.000, 2.057, 2.089 (OAc), 4.93, m, $W_{\frac{1}{2}}$=7Hz, (3βH), 5.00, d, J=6Hz, (17βH), 5.36, m, (16βH) ppm; m.s., m/e: 388(M^+-HOAc).
2α-Hydroxymethyl-5α-androstane-3α,16α,17α-triol (IXa): Fractions eluted with 25-100% methanol/ether and HOAc:MeOH:Et_2O (5:10:85) were combined (172 mg). These fractions showed

three fractions on GLC (RRT: 0.55, 0.66 and 0.84) and on recrystallization from methanol yielded a substance (RRT, 0.84), IXa (18 mg), m.p. 258-260°; R_f. 0.025 (System Ia, developed 3x), i.r.νmax. 3100-3500 (OH); m.s., m/e: 388(M^+).

2α-Acetoxymethyl-5α-androstane-3α,16α,17α-triol triacetate (IXb): The second band from the thick layer separation of the acetylated material (see VIIIb) yielded a non-crystalline product (IXb) R_f, 0.275 (System Ib, developed 2x); p.m.r.δ: 0.82 (C-10 and C-13 methyl) 2.002, 2.030, 2.045, 2.086 (OAc), 3.83, 3.98, J_{AB}=11Hz, $J_{A2\beta}$=9Hz, $J_{B2\beta}$=6Hz, 2H, (-CH_2OAc at C-2), 5.00, d, J=6Hz, (17βH), 5.07, m, $W_{\frac{1}{2}}$=7Hz, (3βH), 5.36, m, $W_{\frac{1}{2}}$=17Hz, (16βH)ppm; m.s., m/e: 446(M^+-HOAc).

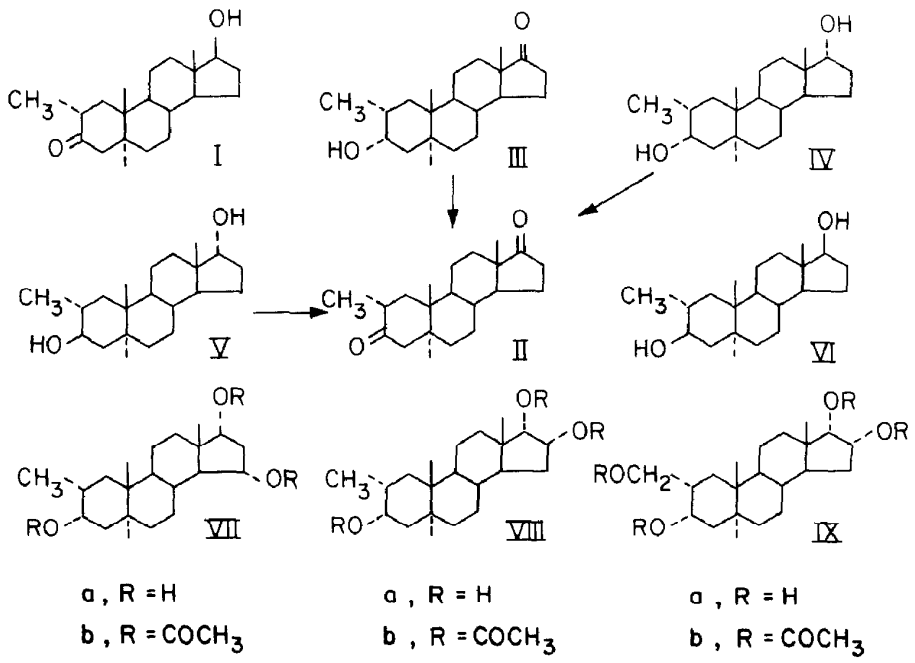
2α-Methyl-5α-androstane-3,17-dione (II): (I) (55 mg), was oxidized with Jones reagent to give a crude product (46 mg). Recrystallization from methanol yielded (II), m.p. 152-153°, [lit. (6) m.p. 152-154°]. R_f, 0.69 (System Ia); RRT, 1.05; i.r.νmax (CCl_4) 1706 (6-membered ring C=O), 1744 (5-membered ring (C=O); m.s., m/e: 302(M^+).

2α-Methyl-5α-androstane-3β,17β-diol (VI): (I) (775 mg) was reduced by sodium borohydride in ethanol to give a product (611 mg), m.p. 165-170°. Recrystallization from methanol yielded VI, m.p. 174-175° [lit. (7) m.p. 175-177°], R_f, 0.47 (System Ia), RRT, 0.50, i.r.νmax 3450 (OH) cm^{-1}; m.s., m/e: 306(M^+).

5α-Androstane-3β,16α,17α-triol 3-acetate and triacetate: The triol monoacetate was prepared from 3β-acetoxy-5α-androst-16-ene (8) by the method of Brutcher and Bauer (9) using the work-up procedure of Baran (10), m.p. 147-149° [lit. (8) m.p. 153.5-154°]; p.m.r.δ: 0.69(C-13 methyl), 0.83(C-10 methyl), 2.03 (3βOAc), 3.59, d, J=5Hz(17βH), 4.41, m, $W_{\frac{1}{2}}$=15Hz(16βH), 4.67 m, $W_{\frac{1}{2}}$=25Hz (3αH)ppm: Triacetate, m.p. 160-3° [lit. (11) m.p. 168-9°]; p.m.r.δ: 0.84(C-10 and C-13 methyl) 2.00, 2.03, 2.09 (3β-,16α-,17α-OAc) 4.67, $W_{\frac{1}{2}}$=25Hz, (3αH), 4.98, d, J=5.5Hz (17βH), 5.34, m, $W_{\frac{1}{2}}$=16Hz, (16βH) ppm.

RESULTS AND DISCUSSION

The crude neutral steroid extract from glucuronidase hydrolysis of urine from rabbits dosed orally with 17β-hydroxy-2α-methyl-5α-androstan-3-one (I) was divided into a ketonic (40%) and a non-ketonic fraction (60%) with Girard T-reagent. This extract showed a weight increase over the control equivalent to 26% of the dosed weight. The GLC trace of these two fractions indicated no alteration in the steroid material with respect to the untreated product.



Jones oxidation of the crude extract yielded a single peak on GLC accounting for 87% (relative percentage of the peak areas) of the material present compared with a control experiment. The mass spectrum of this peak was identical with authentic 2α-methyl-5α-androstane-3,17-dione (II). Recrystallization of the oxidation product yielded the 3,17-dione (II) which was identified by comparison with an authentic sample prepared from oxidation of (I).

The ketonic fraction consisted mainly of one substance by TLC and GLC (70%). On column chromatography over alumina a crystalline product was obtained. The infrared spectrum of this substance showed the presence of hydroxyl absorption and a five-membered ring carbonyl group. The mass spectrum

indicated a molecular ion isomeric with I. The p.m.r. spectrum showed the presence of a C-13 methyl signal indicative of a 17-carbonyl function and a C-10 methyl signal together with a downfield proton (3.77 ppm, $W_{\frac{1}{2}}$=7Hz) in agreement with a 3α-hydroxyl group (12). A doublet (0.90 ppm, J=7Hz) showed the presence of the 2α-methyl function. Jones oxidation yielded 2α-methyl-5α-androstane-3,17-dione (II). These results establish this substance as 3α-hydroxy-2α-methyl-5α-androstan-17-one (III).

The non-ketonic portion on column chromatography over alumina yielded fractions from which the following substances have been obtained. Two diol compounds were identified as 2α-methyl-5α-androstane-3α,17α-diol (IV) and 2α-methyl-5α-androstane-3β,17α-diol (V) on the following evidence. Both substances on Jones oxidation yielded 2α-methyl-5α-androstane-3,17-dione (II) thereby establishing the position of the two hydroxyl groups. Both substances showed the presence of a downfield proton (3.69-3.71 ppm, d, J=6Hz) corresponding to a 17α-hydroxyl group. Each substance showed a second downfield proton having the characteristic chemical shift and coupling pattern of a 3α-hydroxyl (IV, 3.75 ppm, m, $W_{\frac{1}{2}}$=7Hz) and 3β-hydroxyl (V, 3.11 ppm, m, $W_{\frac{1}{2}}$=27Hz) group (13). Comparison of the TLC, GLC and m.s. of a further fraction with an authentic sample of 2α-methyl-5α-androstane-3β,17β-diol (VI), prepared from sodium borohydride reduction of I identified VI as a minor metabolite.

From the more polar fractions 2α-methyl-5α-androstane-

3α,15α,17α-triol (VIIa) has been identified on the following evidence. Carbon-hydrogen analysis and the mass spectrum (m/e: 304, M^+-H_2O) indicated that this substance was a triol. The p.m.r. spectrum showed the presence of the 2α-methyl group and three downfield protons. The chemical shifts and coupling patterns of two of these protons were in agreement with the presence of the 3α- and 17α-hydroxyl functions. The remaining downfield proton was assigned the 15β-configuration on the basis of the coupling pattern and chemical shift (sextet, 4.01 ppm) (12). The p.m.r. spectrum of the derived triacetate (VIIb) showed three acetoxy-methyl signals and three downfield protons two of which are in agreement with the 3α- and 17α-acetoxyl groups. The third signal, which was shifted downfield by 0.91 ppm relative to the hydroxyl group, is in agreement with a hydroxyl function in the 5-membered D-ring since shifts on acetylation of <1 ppm have been associated with D-ring hydroxyl functions (14). The chemical shifts of the C-10 and C-13 methyl groups, although not unique, are also consistent with the 15α-hydroxyl group.

Evidence for the presence of a second crystalline triol identified as 2α-methyl-5α-androstane-3α,16α,17α-triol (VIIIa) is based on the following information. Chromatographic (TLC) and mass spectral data (m/e: 322, M^+) indicate that the substance is a triol. The p.m.r. spectrum of the alcohol and its derived triacetate (VIIIb) show the presence of the 2α-methyl group together with the 3α- and 17α-hydroxyl functions and one newly introduced hydroxyl function. This hydroxyl group is

assigned the 16α-configuration on the basis of the similarity of the band width at half-height and the chemical shift of the 16β-proton (12). Comparison of the p.m.r. spectra of authentic samples of 5α-androstane-3β,16α,17α-triol 3-acetate and 5α-androstane-3β,16α,17α-triol triacetate shows agreement with this assignment.

TABLE The relative percentages of the GLC peak areas of the urinary metabolites of 17β-hydroxy-2α-methyl-5α-androstan-3-one in the rabbit.

Metabolite	%
3α-hydroxy-2α-methyl-5α-androstan-17-one (III)	27
2α-methyl-5α-androstane-3α,17α-diol (IV)	20
2α-methyl-5α-androstane-3β,17α-diol (V)	6
2α-methyl-5α-androstane-3β,17β-diol (VI)	6
2α-methyl-5α-androstane-3α,15α,17α-triol (VIIa)	11
2α-methyl-5α-androstane-3α,16α,17α-triol (VIIIa)	3
2α-hydroxymethyl-5α-androstane-3α,16α,17α-triol (IXa)	11
unidentified material	16
total crude urinary extract	100

A substance eluted from the most polar fractions and isolated as an impure crystalline solid showed a mass spectrum (m/e: 388, M^+) indicative of a tetrol (IXa). Acetylation yielded a non-crystalline product (IXb) whose increased solubility allowed a p.m.r. spectrum in $CDCl_3$ to be recorded. The p.m.r. spectrum clearly showed the presence of four acetoxyl functions and the absence of a doublet associated with the 2α-methyl group. Three downfield protons were in agreement with the presence of the 3α-, 16α-, and 17α-acetoxyl functions as assigned to the triacetate (VIIIb). A signal showing an ABX coupling at 3.83-3.98 ppm and integrating for two hydrogen

atoms was assigned to the acetoxymethyl group accounting for the loss of the 2α-methyl doublet. This tetrol can be assigned the structure 2α-hydroxymethyl-5α-androstane-3α, 16α,17α-triol (IXa).

The relative percentages of each metabolite was estimated from the GLC peak areas of the total urinary extract, ketonic (39% of the total weight) and non-ketonic fractions (61% of the total weight) as shown in the Table. None of the substances identified were observed in the neutral urinary excretion products of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (3).

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