Studies on anabolic steroids. 10. Synthesis and identification of acidic urinary metabolites of oxymetholone in a human

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Two major unconjugated acidic metabolites of oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one, **1***), namely, 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid (***2***) and 3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid (***6a***), were detected by gas chromatography/mass spectrometry in urine samples collected after oral administration of* **1** *to a human volunteer. Reference steroid* **2** *was synthesized and identified. The identification of urinary metabolite* **6a** *was based on the synthesis of its stereoisomers and the isomerization of the methyl ester* **6b** *to its 2-epimer, 3α,17β-dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid methyl ester (***9b***). The mechanisms accounting for the formation of these acidic metabolites are discussed. (Steroids* **57:**453–459, 1992)

Keywords: oxymetholone; acidic metabolites; gas chromatography/mass spectrometry; nuclear magnetic resonance; steroids

Introduction

Oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one, **1**), was first synthesized by Ringold et al. in 1959[1] and introduced into clinical usage a few years later.[2] Oxymetholone has been widely used in the treatment of anemia[3–5] and in androgen therapy.[6,7] Liver toxicity and carcinogenicity of oxymetholone have been noticed in clinical practice.[8–13] Little information was available in the literature about the metabolism of oxymetholone in humans until Adhikary and Harkness[14] and MacDonald et al.[15] reported the isolation of two neutral urinary metabolites produced from the reduction of the 2-hydroxymethylene and 3-keto groups. Previous studies from this laboratory[16] showed that 17α-methyl-5α-androstane-3α,17β-diol (**4**) was present in a urine sample obtained following the administration of oxymetholone. This implied that the 2-hydroxymethylene group of the parent steroid was eliminated in the course of its biotransformation. It was then presumed (Scheme 1) that oxidation of the 2-hydroxymethylene group leads to the intermediate β-keto acids **5a** and/or **8a**, which are sensitive to decarboxylation to form metabolite **3** (17β-hydroxy-17α-methyl-5α-androstan-3-one). The latter keto steroid is then readily reduced by 3α-hydroxysteroid dehydrogenase to give **4**. The intermediate steroids **5a** and **8a** may also be further metabolized to form stable acidic metabolites. To further investigate the oxidative metabolic pathway of oxymetholone, and to shed some light on the relationship between its biotransformation routes and hepatic toxic side effects, series of experiments have been conducted to identify the acidic urinary metabolites of oxymetholone. This report describes the syntheses of appropriate reference steroids and assignment of their stereochemistry. The mechanism accounting for the formation of acidic metabolites **2** and **6a** will also be discussed.

Experimental

Materials and methods

Oxymetholone and its tablets (Anapolon 50) were obtained from Syntex Laboratory Ltd. (Palo Alto, CA, USA). Other steroids

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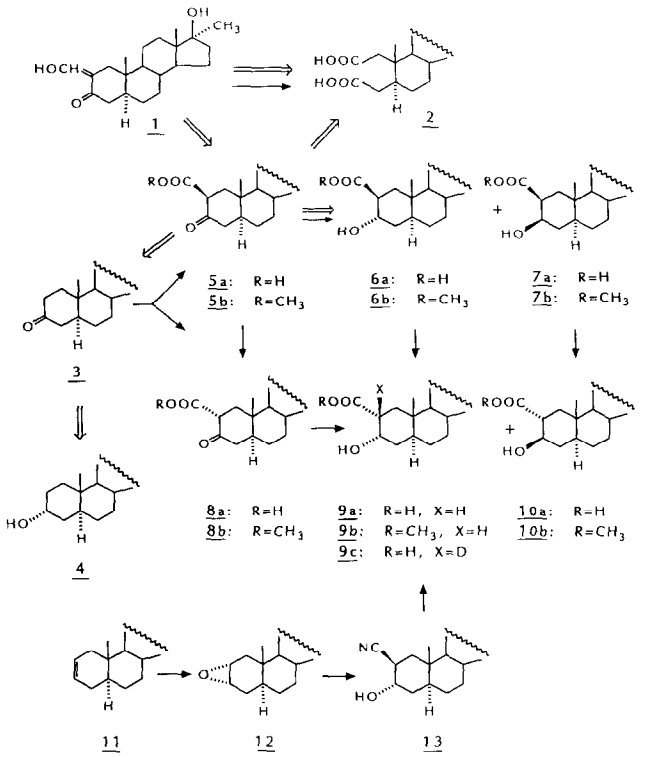

Scheme 1 Biotransformation and synthetic routes accounting for metabolites **2** and **6a**. Single arrows (→) indicate synthetic reactions; double arrows (⇒) indicate biotransformation routes.

were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Steraloids (Wilton, NH, USA). Their purities were assessed by gas chromatography/mass spectrometry (GC/MS) analysis of their trimethylsilyl (TMS) derivatives. The biochemicals and materials used in this study for extraction of urinary metabolites have been described previously.[17,18] Sodium metal in kerosine, dimethyl carbonate, sodium borohydride ($NaBH_4$), magnesium methyl carbonate (MMC) in dimethylformamide (DMF) (2.0 M), lithium diisopropylamide (LDA) in heptane/tetrahydrofuran/ethylbenzene (2.0 M), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), the MNNG-diazomethane kit (1 mmol scale), 3-chloroperbenzoic acid (mCPBA) (50–60%), and diethylaluminum cyanide in toluene (1.0 M) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Aluminum oxide (neutral) was from Camag, Switzerland (distributed by Terochem Scientific, Edmonton, Alberta, Canada). Inorganic salts (analytic grade) were from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA). Organic solvents were purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada). Deuterium oxide (D_2O, minimum isotope purity, 99.9 atom% D) and methyl alcohol-OD (CH_3OD, minimum isotope purity, 99 atom% D) were from MSD-Isotopes, Merck Frosst Canada Inc. (Montreal, Canada). All solvents were redistilled from calcium hydride. Prior to use, mCPBA was treated with phosphate buffer (pH 7.5) and then extracted with CH_2Cl_2. The organic layer was washed with water, dried with Na_2SO_4, and then evaporated in vacuo. The residue was ready for use.[19]

Melting points (MPs) were measured on an electrothermal melting point apparatus and are reported without correction. Infrared (IR) spectra were recorded in *nujols* on a Perkin Elmer 267 infrared spectrophotometer. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXA 300 or a Bruker WH 400 spectrometer. Low-resolution mass spectra (LRMS, EI) were recorded on a Hewlett Packard 5890-5970 gas chromatograph/mass selective detector (GC/MSD). High-resolution mass spectra in both positive or negative ion mode (HRMS, CI [NH_3 as reagent gas] or FAB [glycerol as matrix]) were recorded on a Kratos MS50TC mass spectrometer.

Steroid administration

After collection of blank urine samples, Anapolon 50 (50 mg, single dose) was orally administered to one healthy male volunteer (33 years of age, 60 kg). The postadministration urine samples were collected for at least 6 days and were immediately stored at −20 C until they were analyzed.

Extraction of acidic metabolites and gas chromatography/mass spectrometry analysis

A 10-ml urine sample was applied on a Sep-Pak C_{18} Cartridge (prewashed with 5 ml of methanol and 5 ml of water). The cartridge was then successively washed with 5 ml of water and 2 ml of hexane. The steroids were eluted with 5 ml of methanol and the eluate was then evaporated to dryness under a slow stream of nitrogen at 40 C. The residue was dissolved in 1 ml of water and the pH was adjusted to 8.0 with aqueous $KHCO_3$. The solution was then extracted with 2 × 5 ml of diethyl ether and the combined etheral phases were analyzed for unconjugated neutral steroid metabolites. The remaining aqueous phase was acidified with 1 M HCl to pH 1 to 2 and was extracted with ether (2 × 5 ml). The organic phases were combined, dried over Na_2SO_4, and evaporated to dryness under nitrogen. The TMS-ester/TMS-ether and methyl ester/TMS-ether derivatives of the steroids of interest were prepared prior to GC/MS analysis according to methods previously described.[18] A typical GC chromatogram of a urinary extract showing the presence of major metabolites **2** and **6a** is presented in Figure 1. The corresponding mass spectra of these two metabolites (as two different derivatives) are presented in Figures 2 and 3.

Syntheses of reference steroids

Metabolite 2 (17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid)

$NaIO_4/KMnO_4/K_2CO_3$ oxidation[20]: Oxymetholone **1** (200 mg, 0.6 mmol) was suspended in a 10-ml aqueous solution of $NaIO_4$ (770 mg), $KMnO_4$ (20 mg), and K_2CO_3 (900 mg). The mixture was stirred for 3 hours at room temperature. The resulting precipitate was filtered and the aqueous phase was extracted with diethyl ether (2 × 10 ml). The aqueous phase was then acidified to pH 1 to 2 with HCl and extracted with ether (2 × 30 ml). The combined ethereal extracts were washed with water, dried over Na_2SO_4, and evaporated in vacuo. Crystallization of the residue in acetone and ethyl acetate afforded 68 mg of **2** (yield, 32%).

Peracid (mCPBA) oxidation[21]: To a 2-ml solution of **1** (33 mg, 0.1 mmol) in $CHCl_3$ cooled with an ice bath, mCPBA was added dropwise (30 mg in 2 ml of $CHCl_3$). The mixture was then stirred for 5 hours at room temperature. The mixture was diluted with 10 ml of CH_2Cl_2, washed with water, dried over Na_2SO_4, and evaporated to dryness. The presence of both **2** and unreacted **1** in a ratio of approximately 1 : 2 was shown by GC/MS analysis of the residue.

17β - Hydroxy - 17α - methyl - 2,3 - seco - 5α - androstane - 2,3-dioic acid **2:** MP, 255 to 257 C. IR, ν_{max}, 3,300 to 2,500 (br, H bonded OH); 1,716, 1,695 (2,3-seco diacid) cm^{-1}. HRMS (FAB, negative ion), 351.2157 ($[M-1]^-$, 100%) for $C_{20}H_{31}O_5$ (theoretical value, 351.2171). LRMS (EI, as two derivatives), see Figure 2. ^1H NMR (400 MHz, CD_3OD), δ 0.83 (s, 3H, 18-H_3),

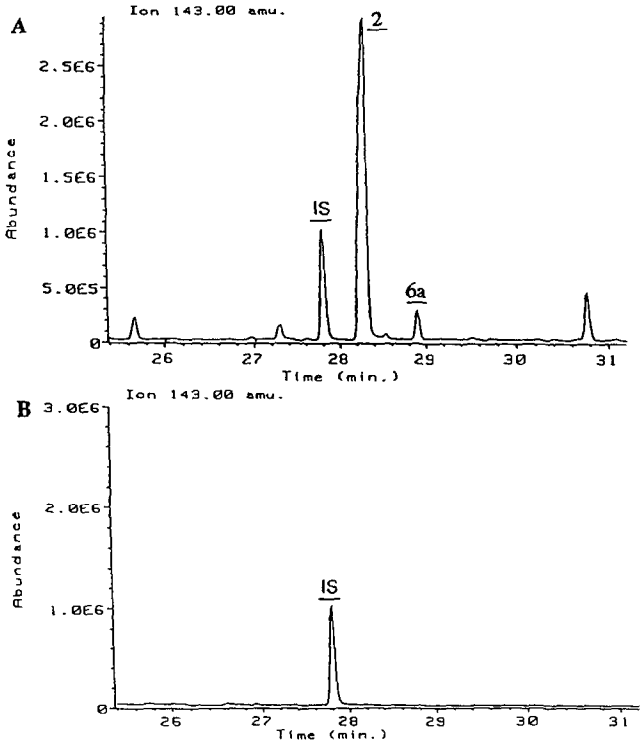

Figure 1 Reconstructed ion current chromatograms (m/z 143) from GC/MS analysis of (A) the unconjugated acidic metabolites as TMS-ester/TMS-ether derivative from a urine sample collected 2 hours after the oral administration of oxymetholone, and (B) the extract from a blank urine sample as the same derivative. IS, Internal standard (oxandrolone). The mass spectra corresponding to labeled GC peaks (**2** and **6a**) are given in Figures 2 and 3. Unlabeled metabolite peaks will be discussed in a separate paper. The GC/MS conditions are the same as those described in ref. 18.

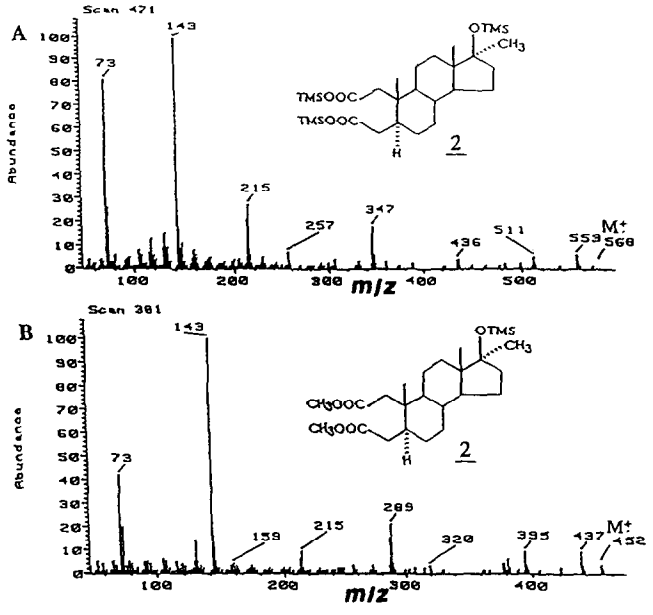

Figure 2 Electron-impact mass spectra of metabolite **2** as (A) TMS-ester/TMS-ether and (B) methyl-ester/TMS-ether derivatives.

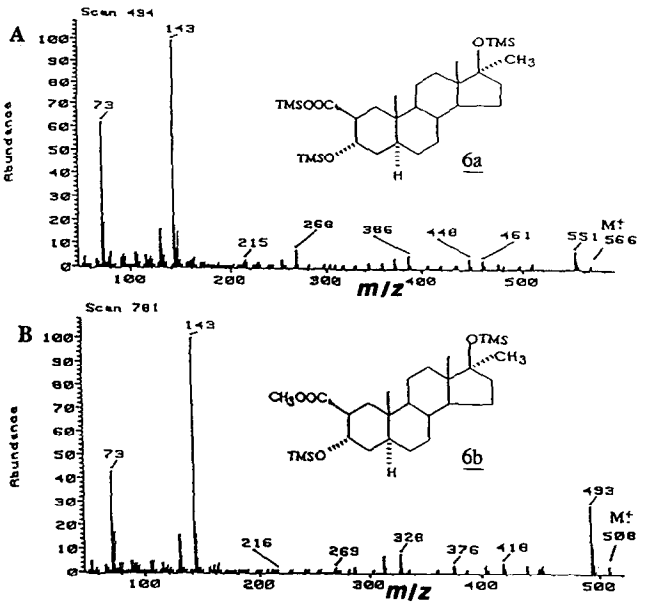

Figure 3 Electron-impact mass spectra of metabolites (A) **6a** and (B) **6b** as TMS derivatives.

0.84 (s, 3H, 19-H_3), 1.17 (s, 3H, 20-H_3), 1.92, 1.96(d-d, 1H, J_1 15.1 Hz, J_2 11.1 Hz, 4-H_a), 2.11 (t-t, 1H, 5-H), 2.30, 2.44 (AB d-d, 2H, 1-H_2, J 14.2 Hz), 2.66, 2.70 (d-d, 1H, J_1 15.1 Hz, J_2 2.4 Hz, 4-H_b). ^{13}C NMR, δ 14.6 (C-18), 16.1 (C-19), 26.1 (C-20), 36.8 (C-4), 41.5 (C-5), 42.0 (C-1), 82.2 (C-17), 175.1, 177.4 (C-2 or C-3).

Isomers of metabolite 6a (3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid)

Carboxylation of **3** *with magnesium methyl carbonate*[22,23]: A magnesium methyl carbonate solution (2.0 M, 5 ml) was added dropwise to a 20-ml solution of **3** (304 mg, 1 mmol) in DMF under argon. The mixture was then heated at 140 C overnight. Evaporation of the solvent in vacuo gave a yellowish residue, to which 50 ml of diethyl ether was added. Concentrated HCl was then added to hydrolyze the chelated magnesium salt of the β-keto acid.[22] After hydrolysis the mixture was extracted with 2 × 50 ml of ether. The combined ether phases were back-extracted with 0.5 M aqueous $KHCO_3$ (2 × 30 ml). The aqueous phase was then reacidified and extracted with ether. Evaporation of the solvent in vacuo afforded a pale yellow residue (175 mg, yield 50%). Analysis with GC/MS (TMS derivative) showed a single broad peak, indicating the presence of a mixture of the 2α- and 2β-carboxylic acids **5a** and **8a**. LRMS (tri-TMS derivative): m/z 549 ($[M-15]^+$, 86%), 459 ($[M-TMSOH]^+$, 7%), 143 (D-ring fragment,[24] 100%). The residue was submitted to the reactions described below without further purification.

Reduction of **5a** *and* **8a** *with* $NaBH_4$: $NaBH_4$ (0.1 M in isopropanol, 10 ml) was added with stirring into a 10-ml solution of the above-described mixture of **5a** and **8a** (120 mg) in isopropanol. The mixture was stirred for 3 hours at room temperature. After the reaction was terminated by adding 2 M HCl, the products were extracted with diethyl ether. The solvent was evaporated and the residue was methylated with diazomethane.[25] Analysis with GC/MS showed that the reaction afforded a mixture of the isomeric diols **6b, 9b,7b,** and **10b,** among which **9b** and **10b** were the most abundant (Figure 4A). Only **6b** had a retention time identical to that of the corresponding urinary metabolite. Attempts to isolate these four isomers by high-performance liquid

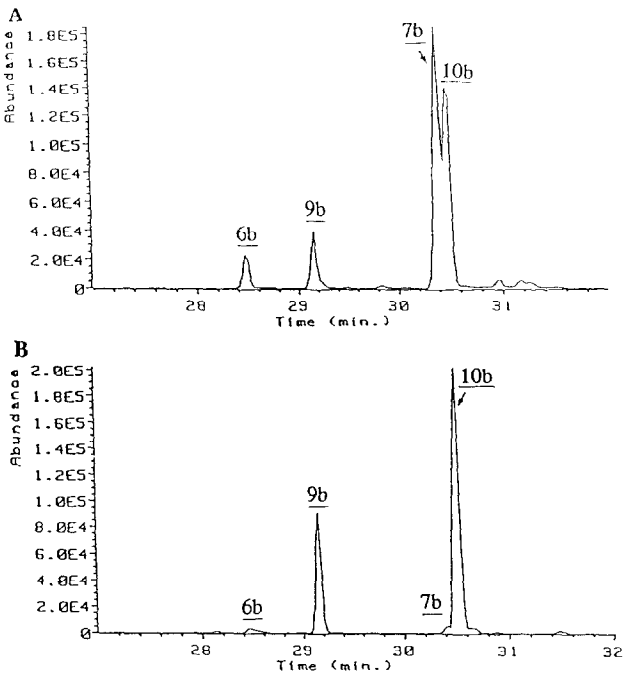

Figure 4 Reconstructed total ion current chromatograms from GC/MS analysis of (A) the four synthetic isomers (**6b, 9b, 7b,** and **10b**) obtained from MMC carboxylation of **3** and $NaBH_4$ reduction, and (B) the two isomers (**9b** and **10b**) obtained from the isomerization of **5b** and **8b** followed by $NaBH_4$ reduction, or carboxylation of **3** with methyl carbonate followed by $NaBH_4$ reduction. Trace amounts of **6b** and **7b** can be observed.

chromatography (Perkin Elmer series 3 liquid chromatograph, Whatman Partisil 10 semi-preparative silica column, hexane/ethyl acetate [10 : 4 v/v] as mobile phase) only gave **9b** and **10b** as chromatographically pure compounds. No fraction containing **6b** and **7b** as chromatographically homogeneous compounds could be obtained, presumably because **6b** and **7b** were isomerized under the chromatographic conditions. After crystallization from aqueous methanol 8 mg of **9b** and 35 mg of **10b** were obtained.

Methylation and isomerization of **5a** *and* **8a**: The methylation of a mixture of **5a** and **8a** (50 mg) with diazomethane provided **5b** and **8b,** which were then dissolved in 5 ml of tetrahydrofuran. To this solution, 100 μl of a 2.0 M LDA solution was added dropwise under argon and temperature was maintained at -5 C. The mixture was stirred at -5 C for 2 hours and then quenched with aqueous NH_4Cl. The 2α-carboxylic methyl ester **8b** obtained was reduced with $NaBH_4$ as described in method 1-A. Analysis of the reduction products with GC/MS showed that **9b** and **10b** were the major products and only traces of **6b** and **7b** were detected, probably due to incomplete isomerization at C-2 (Figure 4B).

Carboxylation of **3** *with methyl carbonate*[26]: A 10-ml dioxane solution of **3** (304 mg, 1 mmol) kept under argon was reacted with dimethyl carbonate (0.6 ml) and sodium methoxide in methanol (50 mg of sodium in 0.7 ml absolute methanol). The reaction mixture was refluxed for 6 hours, then allowed to cool to room temperature and poured into 30 ml of 10% aqueous acetic acid. The mixture was extracted with diethyl ether (2 × 50 ml) and the ether layer was dried with Na_2SO_4 and evaporated in vacuo. The keto ester **8b**,[26] which was contaminated with a large amount of **3** (70%), was then reduced with $NaBH_4$. Analysis of the reduction mixture with GC/MS gave a chromatogram similar to that shown in Figure 4B, illustrating that, besides 70% of reduced **3**, compounds **9b** and **10b** were the main reduction products.

Epoxidation of **11** *with mCPBA*[27]: A 3-ml $CHCl_3$ solution of mCPBA (54 mg) was slowly added to a 3-ml, ice-cold $CHCl_3$ solution of 17β-hydroxy-17α-methyl-5α-androst-2-ene **(11)** (58 mg, 0.2 mmol). The mixture was stirred at room temperature for 5 hours and then diluted with 10 ml of CH_2Cl_2. The resulting solution was successively washed with 10% aqueous $Na_2S_2O_3$, 0.5 M aqueous $KHCO_3$, and water. Evaporation of the organic phase in vacuo afforded crude **12** (58 mg; yield, 95%) as a white solid that was characterized by GC/MS (TMS-ether derivative). LRMS: m/z 376 ($[M]^{+\cdot}$, 8%), 361 ($[M-15]^+$, 50%), 286 ($[M\text{-}TMSOH]^{+\cdot}$, 7%), 229 (10%), 143 (D-ring fragment,[24] 100%).

Opening of epoxide **12** *with cyanide*[27,28]: To the preceding epoxide **12** (58 mg in 3 ml of toluene) was added 1 ml of diethyl aluminum cyanide (1 M in toluene). The reaction mixture was stirred for 4 hours at room temperature and then poured into a 2-M NaOH-ice mixture and extracted with diethyl ether (2 × 20 ml). The combined ether phase was then washed with water and dried over Na_2SO_4. Evaporation of ether in vacuo gave 62 mg of crude **13** (yield, 98%), which was characterized by GC/MS. LRMS (TMS ether derivative): m/z 475 ($[M]^{+\cdot}$, 3%), 460 ($[M-15]^+$, 31%), 418 (D-ring fragment,[24] 16%), 405 (D-ring fragment,[24] 35%), 385 ($[M\text{-}TMSOH]^{+\cdot}$, 10%), 370 (9%), 280 (6%), 143 (100%).

Hydrolysis of nitrile **13** *with* H_2O *or* D_2O[29]: Nitrile **13** (30 mg) was dissolved in 1 ml of acetone and 160 mg of $Ba(OH)_2$ in 5 ml of H_2O was added. Alternatively, **13** (30 mg) was dissolved in 1 ml of methyl alcohol-OD (CH_3OD) and 160 mg of $Ba(OH)_2$ in 5 ml of D_2O was added. The two mixtures were heated at 80 C for 48 hours. After work-up (acidification with HCl and extraction with ether), 4.2 mg of **9a** (yield, 13%) and 4.0 mg (yield, 10.6%) of the deuterated hydroxy acid **9c** were obtained. Analysis of **9a** and **9c** with GC/MS confirmed 100% deuterium incorporation, presumably at C-2 of **9c.** LRMS of **9a:** 566 ($[M]^{+\cdot}$, 1%), 551 ($[M-15]^+$, 8%), 386 ($[M\text{-}2TMSOH]^{+\cdot}$, 7%), 269 ($[M\text{-}2TMSOH\text{-}COOTMS]^+$, 10%), 268 ($[M\text{-}2TMSOH\text{-}HCOOTMS]^{+\cdot}$, 8%), 143 (100%). LRMS of **9c** (TMS derivative): m/z 567 ($[M]^{+\cdot}$, 1%), 552 ($[M-15]^+$, 8%), 387 (7%), 270 (10%), 269 (8%), 143 (100%).

3α,17β-Dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid methyl ester (**9b**): MP, 120 to 122 C. IR, ν_{max} 3,100 to 3,600(br, OH), 1,720 (2-COOMe) cm^{-1}. HRMS (CI), m/z 365.2678 ($[M+H]^+$, 44%) for $C_{22}H_{37}O_4$ (theoretical value, 365.2692), 346 ($[M-18]^{+\cdot}$, 83%), 331 ($[M-18-15]^{+\cdot}$, 100%), 328 ($[M-2\times18]^{+\cdot}$, 70%). ^1H NMR (400 MHz, $CDCl_3$), δ 0.82 (s, 3H, 18-H_3), 0.83 (s, 3H,19-H_3), 1.18 (s, 3H, 20-H_3), 2.58 (br d, 1H, *J* 12.4, 2β-H), 3.67 (s, 3H, 2-$COOCH_3$), 4.29 (br s, 1H, 3β-H). ^{13}C NMR, δ 12.1 (C-19), 14.7 (C-18), 26.1 (C-20), 75.1 (C-3), 82.3 (C-17), 173.2 (2α-*C*OOMe).

3β,17β-Dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid methyl ester **(10b):** MP, 142 to 144 C. IR, ν_{max} 3,100 to 3,600 (br, OH), 1,720 (2-COOMe) cm^{-1}. HRMS (CI), m/z 365.2708 ($[M+H]^{+\cdot}$, 45%), for $C_{22}H_{37}O_4$ (theoretical value, 365.2692), 346 ($[M-18]^{\times\cdot}$, 82%), 331 ($[M-18-15]^{+\cdot}$, 100%), 328 ($[M-2\times18]^{+\cdot}$, 68%). ^1H NMR (400 MHz, $CDCl_3$), δ 0.83 (s, 3H, 18-H_3),0.88 (s, 3H, 19-H_3), 1.17 (s, 3H, 20-H_3), 2.47 (m, 1H, 2β-H), 3.67 (s, 3H, 2-$COOCH_3$), 3.72 (m, 1H, 3α-H). Spin decoupling, irradiation at 3.72 ppm gave a broad doublet at 2.47 ppm with virtual coupling constant *J* 11.2 Hz, while irradiation at 2.47 ppm gave a broad doublet at 3.72 (*J* 8.9 Hz). ^{13}C NMR, δ 13.0 (C-19), 14.7 (C-18), 26.1 (C-20), 72.3 (C-3), 82.2 (C-17), 172.9 (2α-*C*OOMe).

Isomerization of urinary metabolite **6a**

Five 10-ml aliquots of postadministration urine were applied on five Sep-Pak C_{18} cartridges and the acidic urinary metabolites

were extracted as described above. The combined extracts were then methylated with diazomethane. Analysis with GC/MS showed the presence of **6b**. The ethereal solution of this methylated urinary extract was then mixed with 1.5 g of basic aluminum oxide (prepared from 50 g of neutral aluminum oxide mixed with 5 ml of 10% sodium methoxide, then dried and activated at 125 C for 4 hours). The resulting mixture was left in a sealed tube at room temperature for 24 hours and was then extracted with methanol. Analysis of the methanolic extracts with GC/MS showed that approximately 50% of **6b** had been isomerized to **9b**.

Results and discussion

Identification of urinary metabolites

A typical GC/MS profile of the major acidic urinary metabolites of oxymetholone **1** is shown in Figure 1. Comparison with blank urine samples indicates the presence of several peaks, two of which correspond to the major acidic metabolites **2** and **6a**. Their respective chemical structures have been elucidated by various synthetic approaches. Other minor acidic metabolites that are also produced from oxymetholone biotransformation in humans will be reported elsewhere.[30]

Metabolite 2. The mass spectra of methyl and TMS ester derivatives of metabolite **2** are presented in Figure 2. The characteristic ions at m/z 143, 395 and 511 ($[M-57]^{+\cdot}$), 382, and 498 ($[M-70]^{+\cdot}$) arising from the fragmentation of the D-ring[24] indicate that this moiety of oxymetholone skeleton has not been subjected to any biotransformation. A difference of 116 amu between the molecular ions of these derivatives indicates the presence of two carboxyl groups. These data suggested that **2** is a 2,3-seco-2,3-dioic steroid formed by the oxidative degradation of the oxymetholone C_2-C_3 bond. Although the occurrence of such a steroidal metabolite has never been reported in the literature, a 2,3-seco-2,3-dioic acid has been previously prepared in the cholestane series to characterize the A ring of cholestanol.[31] Two different oxidation methods[20,21] were used to prepare seco diacid **2** from oxymetholone **1**. Treatment of **1** with a mixture of $NaIO_4/KMnO_4/K_2CO_3$ was proven to be the most successful approach and afforded **2** in reasonable yield. Gas chromatography/mass spectrometry features of synthetic diacid **2** were identical to those of the urinary metabolite **2**. The HRMS and NMR data were also in agreement with the structure of 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid proposed for compound **2**. This metabolite could be detected in the postadministration urine sample until 32 hours after administration of oxymetholone. The overall excretion of **2** accounts for approximately 1.52% of the oral dose (detail quantitation data will be reported separately).

Metabolite 6a. The structural elucidation of metabolite **6a** has been more difficult to achieve. As in the case of metabolite **2**, the mass spectra of **6a** and **6b** as their TMS derivatives (Figure 3) indicated the presence of two hydroxyl groups and one carboxylic acid function and that the D ring was unchanged. It was also noticed that there was a two-mass unit difference between this metabolite and the presumed intermediate **5a** or **8a**. This indicates that **6a** was probably produced by the reduction of the 3-keto function of **5a** or **8a**. However, these data did not provide any indication about the stereochemistry at C-2 and C-3. We knew from our previous studies[32] and the literature[33] that the reduction of the 3-keto function of 5α-androstanes by 3α-hydroxy steroid dehydrogenase mainly yields 3α-hydroxy steroids in humans. Templeton and Michiel[34] reported a similar biotransformation in the rabbit whereby the 2-hydroxymethylene function of 17β-hydroxy-2-hydroxymethylene-5α-androstan-3-one, a model steroid of oxymetholone, was transformed into a 2α-carboxylic acid group. According to the above information from the literature, it seemed likely that metabolite **6a** had 2α-carboxy and 3α-hydroxy functions. To assess this proposed structural assignment, a series of syntheses was carried out.

The first strategy involved the use of readily available mestanolone **3** as starting material so that carboxylation could provide the keto acids **5a** and/or **8a**, since functionalization of 5α-androstan-3-ones A ring is usually oriented at C-2.[35] Treatment of **3** with MMC gave a mixture of the β-keto acids **5a** and **8a**, which on reduction with $NaBH_4$ afforded a mixture of the four isomers **6a**, **9a**, **7a**, and **10a** (Figure 4A) and among which only the methyl and TMS ester derivatives of **6a** exhibited retention times identical to those of the corresponding derivatives of the urinary metabolite **6a**. Isolation of the methyl ester derivatives of these individual isomers by high-performance liquid chromatography only gave the 2α-isomers **9b** and **10b**. This result may be rationalized by the fact that the axial 2β-carboxy function is sensitive to isomerization during the chromatographic separation of the original mixture of isomers, probably because of the steric strain[36] that is induced at C-2 from the angular methyl group at C-10. This hypothesis was further supported by data from the isomerization reaction of a mixture of **5b** and **8b** with LDA and subsequent reduction of the 3-keto group, which, as expected, predominantly yielded the 2α-isomers **9b** and **10b** (Figure 4B).

In a second synthetic approach to **6b**, methyl carbonate was used to stereoselectively introduce a 2α-carboxylic group[26] to give the keto ester **8b** as sole product. The reduction of **8b** with $NaBH_4$ provided **9b** and **10b** in a ratio similar to that illustrated in Figure 4B. Proton NMR data showed that the 2β-H in both **9b** and **10b** was axial (2α-carboxy) because of the strong spin-spin couplings with the neighboring C-1 axial protons ($J_{a\text{-}a}$ 11.2 to 12.4 Hz).[37] Data from these synthetic approaches indicated that both **9a** and **10a** bear a 2α-carboxy function. Therefore, one may infer that the urinary metabolite **6a** bears a 2β-carboxy group (axial) since its methyl and TMS ester derivatives possess GC retention features that are different from those of the corresponding derivatives of **9** and **10**.

Attention was then turned to a synthetic approach that provided more chiral control at C-2. Epoxidation

of the 2,3-unsaturated steroid **11** with mCPBA gave the α-epoxide **12** as sole product.[21]) Treatment of **12** with diethylaluminum cyanide gave 3α-hydroxy-2β-nitrile **13**[27,28] through trans diaxial opening of the oxirane function. Because of the isomerization of the 2β-functional groups, basic hydrolysis of the 2β-nitrile **13** solely afforded the thermodynamically stable 2α-carboxylic acid **9a.** Isomerization of the 2β-substituent during basic hydrolysis of **13** was demonstrated by deuterium incorporation at C-2 when the hydrolysis was carried out in D_2O that afforded compound **9c.** Although this approach to prepare **6a** was not successful, it demonstrated that steroid **9a** bears 2α-carboxy and 3α-hydroxy functions. Proton NMR data also indicated that **9b** has a 3β-H (equatorial) because of the weak spin-spin couplings with neighboring protons ($J_{e\text{-}a}$ or $J_{e\text{-}e}$ were less than 2 Hz),[37] whereas **10b** has a 3α-H (axial $J_{a\text{-}a}$ 8.9 Hz). At this stage, we propose that the urinary metabolite **6a** (Figure 1A) bears 2β-carboxy and 3α-hydroxy functions.

Definitive evidence for the proposed structure of **6a** was obtained by isomerization of the corresponding methyl ester of the urinary metabolite **6b** to **9b.** The isomerization of urinary **6b** was achieved under mild conditions using basic alumina as reagent. The isomer thus obtained exhibited identical chromatographic and mass spectral properties as those of compound **9b** prepared from other synthetic routes. Thus, compound **6a** was identified as 3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid.

It is of interest to note that metabolite **6a** bears 2β-carboxy and 3α-hydroxy functions, which are both axially oriented and, theoretically, thermodynamically less stable than the corresponding 2α,3α and 2α,3β isomers. The formation of **6a** can be rationalized by the fact that steroid hormones are frequently metabolized with a high degree of specificity to yield unique metabolites.[38] A possible mechanism accounting for the formation of **6a** could involve the formation of the β-keto acid **5a** from **1** in which the β-orientation of the 2-carboxylic group would be set and maintained by the specificity and stereoselectivity of the binding of oxymetholone with the oxidative enzyme. The 3-keto group then would be reduced by 3α-OHSDH to give **6a.** This proposed biosynthetic route is further supported by the occurrence of the neutral metabolite **3,** which arises from the decarboxylation of its immediate precursor **5a.**[30] This indicates that the oxidation of the hydroxymethylene group and the reduction at C-3 probably occur in a sequential manner (Scheme 1).

The 2-aldehyde functions in both formebolone[18] and oxymetholone appear to be quite labile and can easily degrade to acidic breakdown products. This lability is not surprising, particularly for oxymetholone, given the presence of a unique conjugated β-dicarbonyl moiety that is prone to decarboxylation and to biologic and chemical oxidation. The 2,3-seco-2,3-dioic acid **2** can be formed either from direct oxidation of oxymetholone or from oxidation of the intermediate β-keto acid **5a.** Data from the synthetic work described above indicate that both reactions could probably proceed via a sequence that used the enol form of oxymetholone 3-keto group[39] as substrate for epoxidation to give an oxirane that subsequently can be rearranged and/or oxidized to yield **2.** Similar oxidative reactions involving the double bond of enol functions have been reported.[40]

In conclusion, the structure of the two major acidic urinary metabolites of oxymetholone has been elucidated. The stereochemistry of the A-ring substituents of compound **6a** was determined using several synthetic approaches. The isomeric β-hydroxyacids **6a, 7a, 9a,** and **10a** synthesized in this study can be regarded as useful synthetic intermediates and model compounds for comparative spectrometric studies for structure elucidation of other metabolites of oxymetholone in humans. Finally, it is worth mentioning that the occurrence of a 2,3-seco-2,3-dioic steroid such as compound **2** as a metabolite of the metabolism of an endogenous or synthetic steroid has never been reported in the literature.

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