BUTTERWORTH HEINEMANN

Metabolism of anabolic steroids in humans: Synthesis of 6β-hydroxy metabolites of 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, and metandienone

Willi Schänzer, Steve Horning, and Manfred Donike

Institut für Biochemie, der Deutschen Sporthochschule Köln, Köln, Germany

*Hydroxylation at position 6β of testosterone **I** (17β-hydroxyandrost-4-en-3-one) and the anabolic steroids 17α-methyltestosterone **II** (17β-hydroxy-17α-methylandrost-4-en-3-one), metandienone **III** (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one), 4-chloro-1,2-dehydro-17α-methyltestosterone **IV** (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-one), and fluoxymesterone **V** (9-fluoro-11β, 17β-dihydroxy-17α-methylandrost-4-en-3-one) was achieved via light-induced autooxidation of the corresponding trimethylsilyl 3,5-dienol ethers dissolved in isopropanol or ethanol. The reaction further yielded the 6α-hydroxy isomer in low amounts. The 6β-hydroxy isomers of **I–V** and the 6α-hydroxy isomers of **I, III,** and **IV** were isolated and characterized by ^{1}H and ^{13}C NMR, high-performance liquid chromatography, gas chromatography, and mass spectrometry. Human excretion studies with single administered doses of boldenone (17β-hydroxyandrosta-1,4-dien-3-one), 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, metandienone, 17α-methyltestosterone, and [16,16,17-$^{2}H_3$]testosterone showed that 6β-hydroxylation is the major metabolic pathway in the metabolism of 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, and metandienone, whereas for boldenone, 17α-methyltestosterone, and testosterone, 6β-hydroxylation is negligable.* (*Steroids* **60**:353–366, 1995)

Keywords: fluoxymesterone; metandienone; 4-chloro-1,2-dehydro-17α-methyltestosterone; 6β-hydroxylation; metabolism; ^{1}H NMR; ^{13}C NMR; GC; HPLC; MS

Introduction

The misuse of anabolic steroids in sports is controlled by laboratories accredited by the Medical Commission of the International Olympic Committee. The confirmation of positive cases is based on a gas chromatographic (GC)/mass spectrometric (MS) identification of urinary excreted anabolic steroids and their metabolites.[1] For this purpose, the synthesis of 6β-hydroxy metabolites, which are used as reference compounds, is necessary.

6β-Hydroxylation of anabolic steroids in humans was published for metandienone in 1963 by Rongone and Segaloff[2] and for boldenone in 1971 by Galletti and Gardi.[3] The confirmation of the metabolites was based on comparison with synthesized reference substances. In the metabolism of 4-chloro-1,2-dehydro-17α-methyltestosterone, Schubert and colleagues[4,5] isolated a 6β-hydroxy metabolite which was also prepared by microbiological oxidation with *Absidia glauca*.[6]

6β-Hydroxylation of steroids was first described for cortisol in 1954 by Burstein et al.,[7] who isolated 6β-hydroxycortisol from urine after intravenous administration of 270 mg of cortisol to a human subject with Cushing's syndrome and compared it after acetylation with authentic 6β-hydroxycortisol diacetate monohydrate. In 1956, Nadel et al.[8] published an improved method for isolation of 6β-hydroxycortisol from urine and also identified 6β-hydroxycortisol in urine of normal human subjects who were not treated with cortisol.

The first synthesis of a 6β-hydroxy steroid with a 3-keto-4-ene structure was described in 1941 by Ehrenstein,[9] who synthesized the acetate of 6β-hydroxyandrost-4-en-3,17-

Address reprint requests to Dr. W. Schänzer, Institut für Biochemie, der Deutschen Sporthochschule Köln, Carl-Diem-Weg 6, D-50933 Köln, Germany.
Received April 25, 1994; accepted December 12, 1994.

655 Avenue of the Americas, New York, NY 10010

0039-128X/95/$10.00
SSDI 0039-128X(95)00008-E

dione via epoxidation of dehydroepiandrosterone. The 6-hydroxy configuration was first postulated as 6α, but in 1948 the structure was revised to 6β-hydroxy.[10] This exact configuration was established based on the work of Plattner and Lang in 1944[11] who determined the configuration of the 5α,6α- and 5β,6β-epoxides of cholesterol. To obtain the 6β-hydroxy structure Ehrenstein and colleagues hydrolyzed the corresponding 6β-acetate by treatment with diluted potassium hydroxide.[12,13]

In 1954 Sondheimer et al.[14] used the reaction described by Ehrenstein for the synthesis of 6β-hydroxy-3-keto-4-ene steroids but starting directly with the 3-keto-4-ene steroid. In the same year Romo et al.[15] published a two-step preparative method for the synthesis of 6β-hydroxytestosterone 17-acetate. The 3,5-dienol acetate of testosterone was prepared and then oxidized with monoperphthalic acid, yielding the 6β-hydroxytestosterone directly. A similar two-step preparative method was published in 1962.[16]

In 1967 Gardi and Lusignani utilized an autooxidation method for the synthesis of 6β-hydroxy steroids in high yield.[17] They converted steroids with androst-4-en-3-one structures to their corresponding *n*-alkyl-3,5-dienol ethers (mainly ethyl dienol ethers) and after dissolving in ethanol exposed them to sunlight.

We tried to use the method of Gardi and Lusignani to obtain 6β-hydroxy structures of the anabolic steroids 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, and metandienone (Figure 1); however, formation of *n*-alkyl-3,5-dienol ethers of androsta-1,4-dienes and 17α-methyl-17β-hydroxy steroids using acidic conditions was unsuccessful and several decomposition products were formed. Hence, we proceeded to synthesize the 6β-hydroxy structures by forming trimethylsilyl (TMS) 3,5-dienol ethers, which are easily and quantitatively formed with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)/ trimethyliodosilane (TMIS), and exposing them to light.

6β-Hydroxytestosterone **(Iβ)** was also prepared from testosterone via photolysis of the TMS 3,5-dienol ether and shown to be identical with purchased **Iβ**.

Experimental

Steroids and chemicals

Testosterone, 17α-methyltestosterone, and metandienone were purchased from Sigma (Deisenhofen, Germany). 4-Chloro-1,2-dehydro-17α-methyltestosterone (used for the excretion study) was a gift from Jenapharm (Jena, Germany, and fluoxymesterone was a gift from Ciba-Geigy (Basel, Switzerland). 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was purchased from Aldrich (Steinheim, Germany). [1α,2α-$^2H_2$]testosterone was synthesized by G. Opfermann (Institut für Biochemie, Deutsche Sporthochschule Köln, Germany) as described by Dehennion et al.[18] 4-Chloro-17α-methyltestosterone was synthesized as described by Ringold et al.[19] [16,16,17-$^2H_3$]testosterone[20] and MSTFA were synthesized in our laboratory. All other reagents and solvents were of analytical grade.

Metabolism studies

All anabolic steroids were administered orally as follows: [16,16,17-$^2H_3$]testosterone (subject A, male, 39 years, 75 kg: 20 mg), 17α-methyltestosterone **II** (subject A: 10 and 100 mg),

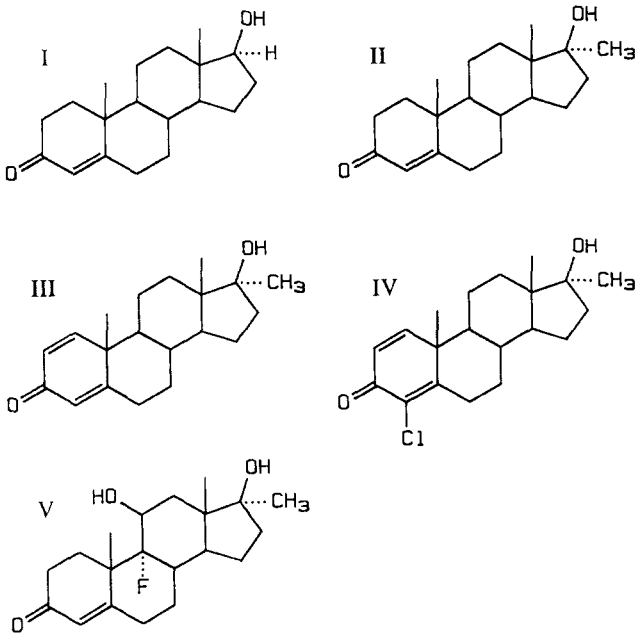

Figure 1 Structure formula of **I**, testosterone; **II**, 17α-methyltestosterone; **III**, metandienone; **IV**, 4-chloro-1,2-dehydro-17α-methyltestosterone; and **V**, fluoxymesterone.

4-chloro-1,2-dehydro-17α-methyltestosterone **IV** (subject B: male, 32 years, 62 kg: 20 mg), fluoxymesterone **V** (subject A: 22 mg and 40 mg), metandienone **(III)**[21] (subject A: 22 and 40 mg; subject B: 10 mg), and boldenone[22] (subject A: 20 and 80 mg; subject B: 40 mg).

Isolation of metabolites of anabolic steroids from urine

The anabolic steroids and their metabolites were isolated from urine as described by Donike et al.[23] with some modifications.

Unconjugated excreted steroids. To 5 mL of urine was added 0.5 g of a solid buffer sodium bicarbonate/potassium carbonate (2:1, w/w), and the unconjugated steroids were extracted with 5 mL of diethyl ether (distilled over calcium hydride). The ether layer was transferred after centrifugation and evaporated to dryness under vacuum.

Conjugated excreted steroids and metabolites. Urine (2 ml) was adsorbed on Amberlite XAD-2 polystyrene resin. The XAD-2 column (pasteur pipette, closed with a glass pearl, bed height: 2 cm) was washed with 2 mL of twice distilled water, and conjugated and unconjugated steroids were eluted with 2 mL methanol. The methanolic eluate was evaporated to dryness, and the residue was enzymatically hydrolyzed with 50 μL β-glucuronidase from Escherichia coli K12 (Boehringer, Mannheim, Germany) in 1 mL 0.2 M phosphate buffer, pH 7.0 for 1 h at 50°C. After hydrolysis, the buffer solution was alkalized with 250 μL 5% potassium carbonate solution, and the steroids were extracted with 5 mL diethyl ether. After centrifugation, the ether layer was transferred and evaporated to dryness under vacuum.

Derivatization for GC/MS

Trimethylsilylation. To obtain TMS ethers, the dry residue was derivatized with 100 μL of MSTFA/Imidazole (Imi) (100:2, v/w) and heated for 15 min at 60°C. TMS 3,5-dienol ethers were formed when the samples were derivatized with 100 μL of

MSTFA/NH_4I/dithioerythreitol (1000:2:4, v/w/w) which is equivalent to an MSTFA/trimethyliodosilane solution (1000:2, v/v),[24] and heated for 15 min at 60°C.

GC/MS

The electron impact (EI) mass spectra presented have been registered with a double focusing mass spectrometer (Finnigan MAT 95) coupled with a Hewlett Packard 5890 gas chromatograph (Hewlett Packard Ultra 1 capillary column, I.D. 0.2 mm, film thickness 0.11 μm, helium carrier gas 1 mL/min, split 1:20). The electron energy was 70 eV and the ion source temperature was 200°C.

Partial EI mass spectra, listed in Table 1, and quantification of excreted steroids were performed with a GC/MS system (GC/MSD Hewlett-Packard [GC 5890/MS 5970]), with the electron energy set at 70 eV, column: Hewlett-Packard, HP1, fused silica capillary column cross-linked methyl silicone (OV 1), 17 m, I.D. 0.2 mm, film thickness 0.11 μm. The carrier gas was helium (1 mL/min, split 1:10), and the temperature program was as follows: initial temperature 200°C, program rate 5 C/min, final temperature 300°C.

High resolution mass spectrometry (HRMS)

HRMS (Finnigan MAT 95) at a resolution of 10,000 was used to determine the exact molecular mass of the underivatized steroids.

High performance liquid chromatography/ultraviolet (HPLC/UV) detection

HPLC was performed with the HPLC 1090 Hewlett-Packard under the following conditions: photodiode detector 190–600 nm; column Merck Lichrosorb (R) RP 18, 5 μm, 10 × 0.4 cm + precolumn 2.4 × 0.4 cm; solvent (A): water, (B): acetonitrile. The flow was set at 1 mL/min, and there was a linear gradient which increased from 10% to 70% in concentration of B within 20 min.

Nuclear magnetic resonance (NMR) spectroscopy

^1H NMR and ^{13}C NMR with distortionless enhancement by polarization transfer (DEPT) 135° experiments were prepared by O. Aulenbacher, Grünenthal GmbH (Aachen, Germany). Spectra (300 MHz) were run using an AC 300 Bruker. About 20 mg of reference or synthesized substance was dissolved in deuterochloroform. Fluoxymesterone and 6β-hydroxyfluoxymesterone were less soluble in deuterochloroform and therefore were dissolved in dimethylsulfoxide-d_6.

Synthesis of reference steroids

6β-Hydroxytestosterone (Iβ)

Testosterone bis-TMS ether. Testosterone **I** (5 g, 17.4 mmol) was dissolved in 30 mL ethyl acetate. MSTFA (8 ml; 43.2 mmol) and 100 mg ammonium iodide were added, and the solution was refluxed for 30 min. The reaction mixture was cooled, diluted with 500 mL *n*-pentane, washed with 50 mL 2% aqueous potassium carbonate, dried over sodium sulfate, and evaporated to dryness. The reaction was analyzed by GC/MS and yielded 98% of the bis-TMS 3,5-dienol ether (preparation of dienol ether see Experimental). The partial EI mass spectrum of **I** bis-TMS 3,5-dienol ether is given in Table 1.

6β-Hydroxytestosterone (*I*β) and 6α-hydroxytestosterone (*I*α). The residue containing the testosterone bis-TMS ether was suspended with 200 mL isopropanol in a 500 mL beaker, and while stirring it was exposed to light (60 W spotline lamp, Phillips), at a distance of 30 cm from the solution for 10 h. To hydrolyze the 17β-O-TMS group, 130 μL of 10 N hydrochloric acid was added to the autooxidized mixture, and after 20 min it was diluted with 500 mL diethyl ether and washed with 50 mL 2% aqueous potassium carbonate. The organic phase was evaporated to dryness and separated on silica gel 60 (bed 3 × 40 cm) using ethyl acetate/*n*-pentane (60:40, v/v). Fractions containing the **Iβ** were combined and crystallized from ethyl acetate. This yielded 400 mg of pure **Iβ** with a percent yield of 7.6% and a m.p. of 214–215°C (lit.[16] 213–216), HRMS 304.203 amu (calculated 304.204 amu). For the EI mass spectrum of **Iβ** bis-TMS ether, see Figure 4A, and for NMR data, see Table 2 and Table 3.

Part of the fractions containing the 6α-hydroxytestosterone were further purified by preparative HPLC (column: Nucleosil (R) RP-18, 7 μm, 25 × 1 cm, Macherey-Nagel, Düren, Germany) using water/acetonitrile as the solvent. This yielded 32 mg of pure compound, which had a m.p. of 198–199°C, HRMS 304.204 amu (calculated 304.204 amu). The EI mass spectrum of **Iα** bis-TMS

Table 1 Partial mass spectra of TMS enol isomers of testosterone **I**, [1α,2α-$^2H_2$]testosterone, 17α-methyltestosterone **II**, and fluoxymesterone **V**

Steroid	M^+	Characteristic ions m/z								
		Further intense ions		A/B-ring fragment ions				D-ring ion		
I bis-TMS (2,4-diene)[a]	432 (28)	431 (0)	417 (22)		207 (43)	194 (62)	193 (35)	179 (36)		73 (100)
I bis-TMS (3,5-diene)	432 (86)	431 (0)	417 (12)		209 (17)	208 (14)	196 (11)	193 (11)		73 (100)
[1α,2α-$^2H_2$]**I** bis-TMS (2,4-diene)[a]	434 (26)	433 (8)[b]	419 (22)		209 (57)	196 (64)	195 (51)	181 (35)		73 (100)
[1α,2α-$^2H_2$]**I** bis-TMS (3,5-diene)	434 (86)	433 (0)	419 (12)		211 (23)	210 (23)	198 (15)	195 (13)		73 (100)
II bis-TMS (2,4-diene)[a]	446 (19)		431 (10)	341 (7)	207 (27)	194 (46)	193 (32)	179 (27)	143 (67)	73 (100)
II bis-TMS (3,5-diene)	446 (32)		431 (3)	301 (80)	209 (5)	208 (10)	196 (3)	193 (9)	143 (14)	73 (100)
V bis-TMS (2,4-diene)[a]	480 (20)		390 (2)		207 (23)	194 (23)	193 (27)	179 (14)	143 (100)	73 (70)
V bis-TMS (3,5-diene)	480 (31)		390 (27)	335 (41)	209 (4)	208 (8)	196 (2)	193 (8)	143 (13)	73 (100)
V tris-TMS (2,4-diene)[a]	552 (27)		537 (5)	219 (15)	207 (16)	194 (43)	193 (53)	179 (24)	143 (100)	73 (83)
V tris-TMS (3,5-diene)	552 (49)		462 (38)	407 (21)	209 (5)	208 (8)	196 (5)	193 (10)	143 (54)	73 (100)

[a]2,4-Dien isomer was obtained by derivatization with KAc/MSTFA and 3,5-dien isomer with MSTFA/TMIS (see Experimental).
[b]The ion 433 is also a M^+ ion caused by enolization to C-2 with elimination of deuterium at C-2α.
V bis-TMS = **V** 17β-O-TMS ether 3,5-dienol (2,4-dienol) ether.

Table 2 ^{1}H NMR (300 MHz) spectra of steroids **I–V** and their 6-hydroxylated structures

	C-4	C-6	C-18		C-19		C-20
Steroid	H	H	H_3	Shift[a]	H_3	Shift[a]	H_3
Testosterone (I)	5.73		0.80		1.20		
6β-Hydroxy-I (Iβ)	5.82	4.35 t (J = 2.9 Hz)	0.82	+0.03	1.39	+0.19	
6α-Hydroxy-I (Iα)	6.18	4.32 br m ($w_{1/2} \cong$ 20 Hz)	0.80	0	1.20	0	
17α-Methyltestosterone (II)	5.73		0.91		1.21		1.22
6β-Hydroxy-II (IIβ)	5.82	4.36 t (J = 2,8 Hz)	0.94	+0.03	1.40	+0.20	1.22
Metandienone (III)	6.08 d (J = 1.4 Hz)		0.94		1.25		1.20
6β-Hydroxy-III (IIIβ)	6.15 d (J = 1.8 Hz)	4.54 br s ($w_{1/2} \cong$ 6.7 Hz)	0.97	+0.03	1.46	+0.20	1.20
6α-Hydroxy-III (IIIα)	6.49 t (J = 1.8 Hz)	4.49 dd (J = 5.4 and 11.3 Hz)	0.93	−0.01	1.23	−0.02	1.20
4-Chloro-1,2-dehydro-17α-methyltestosterone (IV)	—		0.94		1.32		1.19
6β-Hydroxy-IV (IVβ)	—	5.50 t (J = 2.9 Hz)	0.97	+0.03	1.52	+0.20	1.21
6α-Hydroxy-IV (IVα)	—	4.76 br m ($w_{1/2} \cong$ 23 Hz)	0.93	−0.01	1.36	+0.04	1.20
6β-Hydroxy-IV[b] (IVβ)	—	5.45 d (J = 3.1 Hz)	0.84		1.44		1.06
Fluoxymesterone (V)[b]	5.65		1.01		1.50		1.05
6β-Hydroxy-V[b] (Vβ)	5.71	4.81 d (J = 2.9 Hz)	1.03	+0.02	1.65	+0.15	1.07

[a]Chemical shift difference between 6-hydroxy isomer and parent steroid.
[b]Substance dissolved in dimethylsulfoxide-d_6.
Chemical shift values are given in ppm downfield from tetramethylsilane.
Abbreviations used: br m = broad multiplet; br s = broad singlet; d = doublet; dd = double doublet; t = triplet; $w_{1/2}$ = half-height width. Values noted without an abbreviation refer to singlet peaks.

ether was: M^+ 448 (8), 433 (12), 392 (15), 211 (15), 129 (31), 73 (100). NMR data are given in Table 2 and Table 3.

6β-Hydroxy-17α-methyltestosterone (IIβ)

17α-Methyltestosterone bis-TMS ether. 17α-Methyltestosterone **II** (3 g, 9.9 mmol) was dissolved in 30 mL ethyl acetate to which 5.5 mL (29.7 mmol) MSTFA and 75 mg ammonium iodide was added. The reaction mixture was refluxed for 30 min, diluted with 400 mL *n*-pentane, washed with 50 mL 2% aqueous potassium carbonate, and evaporated to dryness. The reaction yielded 10% of the mono-TMS ether and 90% of the bis-TMS 3,5-dienol ether as analyzed by GC/MS (preparation of dienol ether, see Experimental). Partial EI mass spectrum of the **II** bis-TMS 3,5-dienol ether is presented in Table 1.

6β-Hydroxy-17α-methyltestosterone (IIβ). The dry residue containing the 17α-methyltestosterone bis-TMS was suspended with 100 mL isopropanol into a 300 mL beaker, and while stirring it was exposed to sunlight from a glass window for 8 h. The reaction mixture was stored for one week in the dark at room temperature and then evaporated to dryness. Within this time the

Table 3 ^{13}C NMR (300 MHz) spectra of steroids **I–V** and their 6-hydroxylated structures

	Testosterone			A[a]	17α-Methyltestosterone		Metandienone			4-Chloro-1,2-dehydro-17α-methyltestosterone				Fluoxymesterone	
C	I[b]	6β-Hydroxy	6α-Hydroxy	6β-Hydroxy	II	6β-Hydroxy	III	6β-Hydroxy	6α-Hydroxy	IV	6β-Hydroxy	6β-Hydroxy[c]	6α-Hydroxy	V[c]	6β-Hydroxy[c]
1	36.1	36.4	36.3	37.1	35.7	37.2	155.9	157.3	155.7	155.5	156.6	157.7	155.8	25.5	28.8
2	34.1	34.2	33.8	34.2	34.0	34.3	127.5	126.7	127.5	126.2	125.2	124.2	125.5	33.4	33.5
3	198.0	200.3	199.3	200.1	199.6	200.3	186.4	186.7	186.3	178.3	178.6	177.6	178.3	197.5	198.7
4	124.2	126.4	119.8	126.0	123.7	126.4	123.8	125.8	119.6	128.2	130.3	128.1	126.5	123.8	125.1
5	170.4	168.2	171.1	168.5	171.3	168.2	169.1	166.1	170.3	162.6	158.9	160.2	160.8	169.4	166.1
6	32.8	73.1	68.5	73.1	32.8	73.1	33.3	73.8	68.2	28.9	68.5	67.0	71.9	30.1	69.7
7	32.2	38.0	40.9	38.6	(31.7)	38.2	32.8	39.8	39.7	32.2	38.8	39.7	42.8	27.6	33.1
8	36.1	29.8	34.2	29.8	36.5	30.6	36.4	30.9	34.8	36.3	30.5	30.0	35.6	34.8	30.8
9	54.6	53.7	53.6	53.7	53.8	53.6	52.2	52.0	52.2	53.1	51.6	51.8	54.9	100.6	100.7
10	39.0	38.1	39.1	37.9	38.7	38.1	43.6	43.7	43.7	46.3	46.4	45.1	47.9	(43.3)	43.0
11	21.2	20.6	20.7	21.0	20.7	20.6	22.6	22.3	22.5	22.9	22.5	22.1	23.2	69.0	69.0
12	37.1	37.1	36.3	39.4	(31.4)	31.4	31.4	31.4	31.2	31.3	31.3	31.0	31.3	36.2	36.3
13	43.2	42.9	42.9	42.5	45.6	45.5	45.7	45.7	45.7	45.6	45.6	44.3	45.7	(43.5)	43.9
14	51.1	50.5	50.2	55.9	50.1	50.1	49.8	49.8	49.6	49.7	49.8	48.9	49.4	44.6	44.6
15	23.8	23.3	23.3	24.1	23.2	23.2	23.4	23.4	23.4	23.3	23.3	23.1	23.3	22.8	22.9
16	30.7	30.5	30.4	28.1	38.9	38.9	38.8	38.8	38.7	38.8	38.8	39.0	38.7	38.0	38.0
17	81.3	81.7	81.5	56.1	81.5	81.6	81.4	81.5	81.5	81.4	81.5	79.5	81.3	79.9	80.0
18	11.3	11.1	11.0	12.0	13.7	14.0	14.0	14.1	14.0	14.0	14.8	14.1	14.0	15.9	15.8
19	17.3	19.6	18.3	19.4	17.4	19.5	18.7	20.4	19.1	19.2	20.8	20.1	19.4	21.2	22.9
20		25.8		35.7	25.6		25.8	25.8	25.8	25.8	25.8	26.0	25.8	26.2	26.2

[a]A = 6β-Hydroxycholest-4-en-3-one (only C-1–C-20), lit.[23] (in deuterochloroform).
[b]Lit.[23] value for testosterone (in deuterochloroform).
[c]Substance dissolved in dimethylsulfoxide-d_6.
Shielding data are given in ppm downfield from TMS. Values in parentheses represent shielding assignments that may be interchanges.

17β-O-TMS ether was completely hydrolyzed, and **IIβ** was purified on silica gel 60 (bed 3 × 40 cm) using ethyl acetate/*n*-pentane (80:20, v/v). The fractions containing the **IIβ** were combined and crystallized from ethyl acetate yielding 490 mg of 98% pure compound. The percent yield was 15.7% and the m.p. was 250–252°C (lit.[25] 252–253°C), HRMS 318.218 amu (calculated 318.219 amu). The EI mass spectrum of the **IIβ** bis-TMS ether is shown in Figure 5A, and NMR data are shown in Table 2 and Table 3.

6β-Hydroxymetandienone (IIIβ)

Metandienone bis-TMS ether. Metandienone (5 g, 16.7 mmol) was dissolved in 50 mL ethyl acetate to which 10 mL (54.0 mmol) MSTFA and 100 mg of ammonium iodide were added. The reaction mixture was refluxed for 60 min. Crystallization was allowed to proceed over night at −20°C. Upon filtering and drying **III,** bis-TMS 3,5-dienol ether (97% GC/flame ionization dectector (FID) result) was obtained with EI-mass spectrum: M^+ 444 (29), 429 (5). 339 (20), 299 (15), 206 (100), 191 (2), 143 (20), 73 (91).

6β-Hydroxymetandienone *(IIIβ)* and 6α-Hydroxymetandienone *(IIIα).* The dried residue with **III** bis-TMS was suspended with 200 mL isopropanol in a 500 mL beaker, and while stirring it was exposed to light (60 W spotline lamp, Phillips) at a distance of 20 cm from the solution for 5 h. To hydrolyze the 17β-O-TMS ether of the autooxidation product, 1 mL of 1N hydrochloric acid was added to the mixture, and after 30 min it was diluted with 500 mL of diethyl ether and washed with 50 mL 2% aqueous potassium carbonate. The organic phase was evaporated to dryness and separated twice on silica gel 60 (bed 3 × 40 cm) using *n*-pentane/ethyl acetate (80:20, v/v). The fractions containing the 6β-hydroxymetandienone were combined, dried, crystallized from ethyl acetate, and yielded 1.1 g of 98% pure compound. The percent yield was 20.9%, and the m.p. was 229–230°C, HRMS 316.205 amu (calculated 316.204 amu). Figure 6A shows the EI-MS of **IIIβ** bis-TMS ether. NMR: 7.06 (d, $J_{1,2}$ = 10.1 Hz, C-1H), 6.21 (dd, $J_{1,2}$ = 10.2 and $J_{2,4}$ = 1.8 Hz, C-2H); for other characteristic NMR data see Table 2 and Table 3.

Part of the fractions containing **IIIα** were further purified by preparative HPLC (column: Nucleosil [R] RP-18, 7 μm, 25 × 1 cm, Macherey-Nagel, Düren, Germany) using water/acetonitrile as the solvent. This yielded 25 mg of pure **IIIα** with a m.p. of 198–199°C, HRMS 316.203 amu (calculated 316.204 amu). The EI-mass spectrum of **IIIα** bis-TMS ether was: M^+ 460 (3), 445 (2), 370 (6), 355 (4), 313 (7), 281 (73), 209 (61), 191 (20), 143 (83), 73 (100). NMR: 7.05 (d, $J_{1,2}$ = 10.2 Hz, C-1H), 6.25 (dd, $J_{1,2}$ = 10.2 and $J_{2,4}$ = 1.9 Hz, C-2H); other characteristic NMR data are presented in Table 2 and Table 3.

6β-Hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone (IVβ)

4-Chloro-1,2-dehydro-17α-methyltestosterone *IV.* 4-Chloro-17α-methyltestosterone (5 g, 15.8 mmol) was dissolved in 240 mL dioxane (distilled over lithium hydride) to which 4 g (17.6 mmol) DDQ was added. The reaction mixture was refluxed for 2 days, cooled, filtered, diluted with 1,000 mL diethyl ether, washed three times with 200 mL aqueous 1% potassium carbonate and two times with 200 mL twice distilled water, and concentrated to dryness under reduced pressure. The reaction yielded about 61% of **IV,** 33% of unchanged starting material, and 6% of side products (GC/FID result). The 4-chloro-1,2-dehydro-17α-methyltestosterone **IV** was further separated on silica gel 60 (Merck, particle size 200–300 μm, bed 3 × 40 cm) using ethyl acetate/*n*-pentane (35:65, v/v) and yielded 2.8 g of **IV,** 95% pure (GC/FID and GC/MS result) with a percent yield of 53%.

The reaction product was analyzed by GC/MS underivatized and after trimethylsilylation (see Derivatization for GC/MS) as its TMS ether and bis-TMS 3,5-dienol ether. The gas chromatographic retention time and EI-MS were identical with reference substance **IV** from Jenapharm. For NMR data see Table 2 and Table 3.

4-Chloro-1,2-dehydro-17α-methyltestosterone bis-TMS ether. 4-Chloro-1,2-dehydro-17α-methyltestosterone **IV** (2.3 g, 6.9 mmol) was dissolved in 30 mL of ethyl acetate to which 5 mL (27.0 mmol) MSTFA and 75 mg ammonium iodide was added. After 50 min of refluxing, the mixture was diluted with 500 mL *n*-pentane and washed with 50 mL 2% potassium carbonate solution. The organic layer was dried over sodium sulfate, filtered, and evaporated to dryness. The reaction yielded **IV** bis-TMS enol ether with 95% of the product as the main reaction product. The EI-MS was: M^+ 478 (100), 480 (44), 373 (41), 375 (17), 240 (100), 242 (37), 225 (49), 143 (49), and 73 (71).

6β-Hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone *(IVβ)* and 6α-hydroxy-4-chloro-1,2-dehydro17α-methyltestosterone *(IVα).* The **IV** bis-TMS enol ether residue was suspended with 200 mL of isopropanol in a 500 mL beaker, and while stirring, it was exposed to light (60 W spotline lamp, Phillips) with a distance of 30 cm from the solution of 3.5 h. The reaction yielded 42% of **IV,** 29% of **IVβ,** 3% of **IVα,** and 26% of further reaction products. All TMS ethers were hydrolyzed at that moment. The solution was concentrated to dryness and the reaction products were separated on silica gel 60 (Merck, particle size 200–300 μm, bed 1 × 45 cm) using ethyl acetate/*n*-pentane (1:1, v/v).

The isolated fractions containing the 6β-hydroxy and 6α-hydroxy products were combined and further purified via preparative HPLC (column: Nucleosil (R) RP-18, 7 μm, 25 × 1 cm, Macherey-Nagel, Düren, Germany) using water/acetonitrile as the solvent. The isolated 6β- and 6α-hydroxy fractions were dried and crystallized from methanol yielding 110 mg of **IVβ** with a percent yield 4.6% and a m.p. of 249–252°C, HRMS 350.163 amu (calculated 350.165 amu). Figure 7A shows the EI-spectrum of **IVβ** bis-TMS ether. NMR: 7.11 (d, $J_{1,2}$ = 10.1 Hz, C-1H), 6.33 (d, $J_{1,2}$ = 10.1 Hz, C-2H) and other characteristic NMR data are presented in Table 2 and Table 3. **IVα** (38 mg) was also isolated with a percent yield of 1.6% and a m.p. 109–111°C, HRMS 350.164 amu (calculated 350.165 amu). The EI spectrum of **IVα** bis-TMS ether was: M^+ 494 (4), 479 (6), 459 (8), 404 (10), 315 (51), 317 (17), 279 (54), 243 (30), 245 (12), 143 (100), and 73 (67). NMR: 7.05 (d, $J_{1,2}$ = 10.1 Hz, C-1H), 6.37 (d, $J_{1,2}$ = 10.0 Hz, C-2H); for other characteristic NMR data see Table 2 and Table 3.

6β-Hydroxyfluoxymesterone (Vβ)

Fluoxymesterone 17-O-TMS ether 3-O-TMS 3,5-dienol ether. Fluoxymesterone (2 g, 5.9 mmol) was dissolved in 20 mL ethyl acetate to which 4 mL (21.6 mmol) MSTFA and 50 mg ammonium iodide were added. The reaction mixture was refluxed for 5 min, diluted with 200 mL *n*-pentane, washed with 20 mL 5% aqueous carbonate solution, dried over sodium sulfate, and evaporated to dryness under reduced pressure. The reaction was analyzed by GC/MS and yielded 10% tris-TMS product and 90% of the desired 17-O-TMS ether 3-O-TMS 3,5-dienol ether (preparation of enol ether, see Experimental). The EI-MS of the 17-O-TMS ether 3-O-TMS 3,5-dienol ether was: M^+ 480 (53), 390 (36), 375 (11), 335 (59), 319 (10), 208 (8), 193 (9), 143 (13), 73 (100).

6β-Hydroxyfluoxymesterone *(Vβ).* The fluoxymesterone bis-TMS was suspended with 100 ml ethanol in a 200 mL beaker, and while stirring exposed to sunlight through a glass window for 4.5 h. The reaction mixture was stored in darkness for 1 week at room temperature, then evaporated to dryness, and separated via silica

gel 60 (1 × 50 cm) using ethyl acetate/*n*-pentane (80:20, v/v). The fractions containing the **Vβ** were combined and crystallized from ethyl acetate/methanol yielding 460 mg of pure compound with a percent yield of 21.9% and crystal sublimation at 300–302°C, HRMS 352.204 amu (calculated 352.205 amu). EI mass spectrum of **Vβ** bis-TMS ether, see Figure 8A. NMR: 4.12 (br, 11α-H) in DMSO-d_6 and other characteristic data are presented in Table 2 and Table 3.

[6,6-$^2H_2$]-4-Chloro-1,2-dehydro-17α-methyltestosterone ([6,6-$^2H_2$]*IV*). [6,6-$^2H_2$]**IV** was synthesized to assign the ^{13}C NMR signal for C-6 and C-7 in **IV.** IV (50 mg) was dissolved in 2 mL methyl alcohol-d, 0.2 mL deuterium oxide, and 20 μL sodium deuteroxide (40% solution in D_2O), and heated for 4 days at 60°C. The solution was concentrated to a volume of 0.2 mL, diluted with 5 mL deuterium oxide, and extracted with 50 mL of tert.butyl methyl ether. The organic layer was concentrated to dryness and the [6,6-$^2H_2$]**IV** was isolated by HPLC, yielding 11 mg of pure substance. The deuteration of **IV** was not complete and yielded the following incorporation of deuterium: d_0 0%, d_1 29%, d_2 50%, and d_3 21% (MS analysis). Interestingly, that in addition to the enolizable C-6 hydrogens, the C-1 hydrogen was also partially exchanged. The latter was confirmed by ^1H and ^{13}C NMR. ^1H NMR: 7.11 (t, J = 5.0 Hz, C-1H), 6.36 (d, $J_{1,2}$ = 10.1 Hz, C-2H), 1.32 (s, 3H, C-19), 1.19 (s, 3H, C-20), 0.94 (s, 3H, C-18). The ^{13}C NMR spectra of [6,6-$^2H_2$]**IV** was identical with the spectra of **IV** (Table 3) with the following exceptions: a) the 28.9 ppm signal (C-6) in **IV** was not present in the methylene trace of the DEPT 135° experiment, b) the 32.2 ppm signal (C-7) was split into 32.1 and 32.0 ppm, c) a weak triplet with 28.3, 28.6, and 28.8 ppm was detected in the methyl/methine trace of the DEPT 135° experiment, and d) the 155.5 ppm signal (C-1) was split into 155.5 and 155.4 ppm.

Preparation of silyl dienol ethers of I, II, and V for GC/MS characterization

The intermediate silyl dienol ethers were characterized by GC/MS. The reaction with MSTFA/NH_4I favors the formation of the TMS 3,5-dienol ether more than 99%. TMS 2,4-dienol ethers were obtained when 20 μg of **I,** [1α,2α-$^2H_2$]**I, II,** and **V** were derivatized with 100 μL MSTFA (or MSTFA/Imi) to which 2 mg potassium acetate (KAc) was added, and heated for 3 h at 60°C. EI-MS spectra and GC retention indices of TMS 2,4-dienol and 3,5-dienol ethers are listed in Table 1 and Table 4.

Oxidation of 6α-hydroxy-(IVα) and 6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone (IVβ) with chromium trioxide for GC/MS analysis

IVα and **IVβ** were oxidized, isolated, and derivatized separately. **IVα (IVβ)** (1 mg) was treated with 5 mg of chromium trioxide dissolved in 0.5 mL of 96% of acetic acid. After 5 min at room temperature, 2.5 mL of 20% potassium hydroxide solution was added and the reaction products were extracted with 5 mL of tert.butyl methyl ether. An aliquot (0.5 mL) of the organic layer was concentrated to dryness under reduced pressure and analyzed underivatized and as its silyl ether (see Derivatization for GC/MS). Oxidation of **IVβ** yielded 4-chloro-17α-methyl-17β-hydroxy-androsta-1,4-diene-3,6-dione **IVc** (97%) and 3% of unchanged starting material. Oxidation of **IVα** yielded the same oxidation product **IVc** (80%) and 20% of unchanged starting material. The molecular mass of **IVc** as determined by GC/MS analysis of underivatized **IVc** is M^+ 348 and trimethylsilylated **IVc** (bis-TMS product) M^+ 492. The molecular mass of **IVα** and **IVβ** is 350.

Table 4 Temperature-programmed Kovats indices of steroids (**I–V**) and their 6β-hydroxy and 6α-hydroxy isomers as TMS ether and TMS enol ether

Steroid	Kovats index	D[a]
TMS ether		
Testosterone (**I**) TMS	2607	
6β-Hydroxy-**I** bis-TMS	2701	94
6α-Hydroxy-**I** bis-TMS	2814	207
TMS ether TMS enol ether		
Testosterone (**I**) bis-TMS (2,4-diene)[b]	2606	
Testosterone (**I**) bis-TMS (3,5-diene)	2652	
6α-Hydroxy-**I** tris-TMS (2,4-diene)[b]	2763	
6β-Hydroxy-**I** tris-TMS (2,4-diene)[b]	2703	
6β/6α-Hydroxy-**I** tris-TMS (3,5-diene)	2823	171
TMS ether		
17α-Methyltestosterone (**II**) TMS	2699	
6β-Hydroxy-**II** bis-TMS	2798	99
TMS ether TMS enol ether		
17α-Methyltestosterone (**II**) bis-TMS (2,4-diene)[b]	2695	
17α-Methyltestosterone (**II**) bis-TMS (3,5-diene)	2743	
6β-Hydroxy-**II** tris-TMS (2,4-diene)[b]	2790	
6β-Hydroxy-**II** tris-TMS (3,5-diene)	2905	162
TMS ether		
Metandienone (**III**) TMS	2732	
6β-Hydroxy-**III** bis-TMS	2840	108
6α-Hydroxy-**III** bis-TMS	2945	213
TMS ether TMS enol ether		
Metandienone (**III**) bis-TMS	2733	
6β/6α-Hydroxy-**III** tris-TMS	2888	155
TMS ether		
4-Chloro-1,2,dehydro-17α-methyltestosterone (**IV**) TMS	2956	
6β-Hydroxy-**IV** bis-TMS	3001	45
6α-Hydroxy-**IV** bis-TMS	3139	183
TMS ether TMS enol ether		
4-Chloro-1,2-dehydro-17α-methyltestosterone (**IV**) bis-TMS	2931	
6α-Hydroxy-**IV** tris-TMS	3018	87
TMS ether		
Fluoxymesterone (**V**) TMS	2921	
6β-Hydroxy-**V** bis-TMS	2995	74
TMS ether TMS enol ether		
Fluoxymesterone (**V**) bis-TMS (2,4-diene)[b]	2912	
Fluoxymesterone (**V**) bis-TMS (3,5-diene)	2947	
Fluoxymesterone (**V**) tris-TMS (2,4-diene)[b]	2890	
Fluoxymesterone (**V**) tris-TMS (3,5-diene)	2935	
6β-Hydroxy-**V** tetra-TMS (2,4-diene)	2946	11
6β-Hydroxy-**V** tetra-TMS (3,5-diene)	3061	126

Column (HP 1) see Experimental, GC/FID, temperature program (180°C — 5°C/min — 320°C).
[a]D = Difference of indices of 6-hydroxy isomer and parent steroid.
[b]2,4-Dien isomer was obtained by derivatization with KAc/MSTFA.

Results and discussion

6β-Hydroxylation via autooxidation of the TMS 3,5-dienol ether

The reaction scheme for 6β-hydroxylation is shown in Figure 2 for the synthesis of 6β-hydroxyfluoxymesterone **(Vβ).** Autooxidation of the TMS 3,5-dienol ethers of **I–V** led to hydroxylation at C-6. The yield of 6β-hydroxy product was 10-fold larger than that of the 6α-hydroxy products. The isolation of reaction products was sufficient to obtain pure substances, and reaction yields in the synthesis of **Iβ, IIβ,** and **IVβ** were not optimized. The analytical data of synthesized 6β-hydroxytestosterone **(Iβ)** were identical with data obtained from purchased **Iβ.** The exact structures of the hydroxylated products were assigned on analytical data obtained from GC, MS, HPLC, ^{1}H NMR, and ^{13}C NMR. From the GC, HPLC, and NMR data it was concluded that the 6-hydroxy steroids were pure and the 6α-hydroxy isomers were completely separated from the 6β-hydroxy isomers.

Synthesis of TMS enol ether intermediates

The advantage of this method compared to the method of Gardi and Lusignani,[17] who used *n*-alkyl enol ethers for autooxidation by light, is that TMS enol ethers of 17β-hydroxy-17α-methyl and androsta-1,4-dien-3-one steroids are quantitatively formed using MSTFA/TMIS. The synthesis of *n*-alkyl enol ethers of 17β-hydroxy-17α-methyl and androsta-1,4-dien-3-one steroids using acidic conditions was poor and yielded high amounts of decomposition products.

The conversion of 3-keto-4-ene steroids with MSTFA/TMIS to TMS 3,5-dienol ethers was easily performed using ethylacetate as a solvent and heating to reflux. **I** and **II** were completely derivatized within 5–10 min, whereas enolization of the 3-keto-1,4-dienes **III** and **IV** was slower and longer refluxing was necessary (30 min for **III** and 50 min for **IV**). The reaction with fluoxymesterone was stopped after 10 min to avoid trimethylsilylation of the 11β-hydroxy group. This was necessary since hydrolysis of the 11β-hydroxy TMS ether was very difficult, and even under weak acidic conditions (30% acetic acid) was very slow and yielded a high percentage of decomposition products.

For **I, II,** and **V** the reaction with MSTFA/TMIS largely favors the enolization to form the TMS 3,5-dienol ether (less than 1–2% of the TMS 2,4-dienol ether was produced). Characterization of the silyl dienol ethers of **I, II,** and **V** was performed by GC/MS. The TMS 2,4-dienol and 3,5-dienol ether isomers can be distinguished by their EI mass spectra (Table 1) and GC retention times (Table 4). TMS 2,4-dienol ethers of **I, II,** and **V** were obtained by derivatization with MSTFA/potassium acetate as described by Zimmermann.[26] NMR data of the TMS 2,4-dienol and 3,5 dienol ethers were not measured since they were not end products (these data should give precise criteria to distinguish between them).

The formation of the two TMS dienol structures as a function of the catalyst employed was examined for the derivatization of **Iα** and **Iβ.** Trimethylsilylation of **Iα** and **Iβ** with MSTFA/TMIS yielded the same tris-TMS product, **I** tris-TMS 3,5-dienol ether. Trimethylsilylation of **Iα** and **Iβ** with MSTFA/potassium acetate yielded two different reaction products, **Iβ** tris-TMS 2,4-dienol ether and **Iα** tris-TMS 2,4-dienol ether (Table 4). The TMS 2,4-dienol ethers of **I, II,** and **V** elute from the GC column approximately 40 methylene units before the TMS 3,5-dienol ethers (Table 4).

The EI-MS of the TMS 2,4-dienol ethers of **I, II,** and **V** display a characteristic and abundant A/B-ring fragmentation with m/z 194 and 193 (Table 1) compared to the TMS 3,5-dienol ethers, which show a very strong molecular ion. The 2,4-dienol ether of [1α,2α-$^{2}H_2$]testosterone shows corresponding fragment ions at m/z 196 and 195. These fragment ions can be explained by an A/B-ring fragmentation with fissions of the C-9–C-10 and C-6–C-7 bonds and mi-

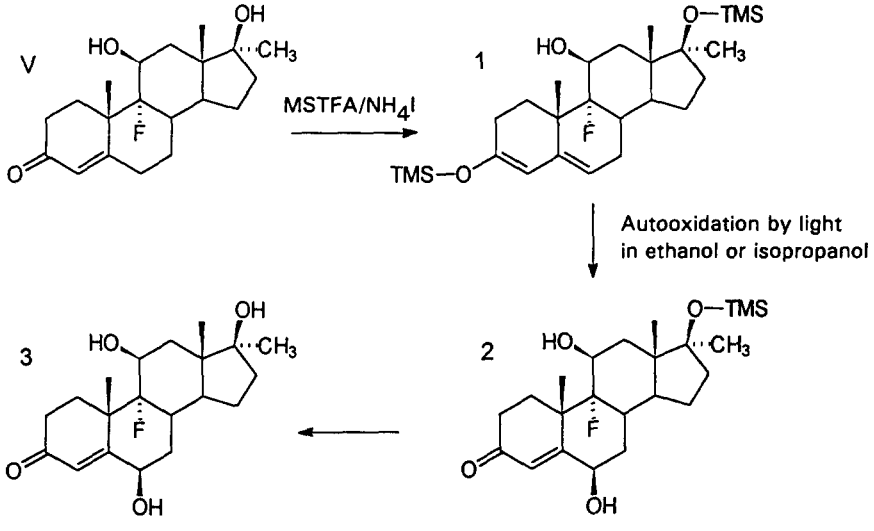

Figure 2 Reaction scheme for synthesis of 6β-hydroxyfluoxymesterone (3). **V,** fluoxymesterone; 1, fluoxymesterone 17β-TMS ether 3-TMS enol ether; 2, 6β-hydroxyfluoxymesterone 17β-TMS ether.

gration of a hydrogen. The 2,4-dienol ether of [1α, 2α-$^2H_2$]testosterone bis-TMS 2,4-dienol ether displays two molecular ions m/z 433 (30%) and m/z 434 (70%), the ion m/z 433 resulting from enolization to C-2 with elimination of the deuterium at C-2α. The ion m/z 434 indicates loss of the hydrogen at C-2β.

Solvent for autooxidation

The solvents ethanol and isopropanol were compared for the autooxidation of **III** bis-TMS enol ether and showed no differences in the yield of reaction products when 600 mg of TMS dienol ether was suspended in 50 ml solvent. Lower concentrations of substance (for example 5 mg of **III** dissolved in 5 mL solvent) yielded low amounts of 6β-hydroxy metabolite when ethanol was used compared to isopropanol. This was mainly a result of the stability of the TMS enol ether, which, in lower concentrations, was more rapidly hydrolyzed in ethanol than in isopropanol.

Light for autooxidation

The autooxidation reaction was investigated using direct sunlight, sunlight broken by a glass window, and lamp light. The first synthesis was performed using direct sunlight. The change to lamp light allowed for control of the reaction conditions. Four different kinds of lamps (40 W spotline lamp, 60 W halogen lamp, 80 W flower halogen lamp, and 1250 W halogen) were tested for the autooxidation of **III** TMS enol ether. The reaction was monitored by GC/FID and showed for all of the lamps the same distribution of reaction products with about 64% of 6β-hydroxy product, 6% of 6α-hydroxy isomer, 5% of 6-one product, 5% of bis-TMS enol ether (starting substance), and 20% of further reaction products (Figure 3). The reaction was stopped when more than 95% of the starting material (TMS enol ether) had reacted. For the 40, 60, and 80 W lamps, the reaction time was between 6 and 8 h, whereas for the 1250 W lamp, the reaction time was shortened to 2.5 h. For this lamp, the distance from the solvent was doubled compared to the other lamps because the solvent otherwise heated to a boil.

Hydrolysis of the 17β-O-TMS ether

When the autooxidation reaction was finished, the samples were stored in the dark. In the ethanol solvent the 17β-O-TMS ether fully hydrolyzed within 7 days, whereas in isopropanol, the products were more stable and complete hydrolysis was achieved only after 4 weeks. Immediate hydrolysis can be carried out by acidification of the reaction mixture with hydrochloric acid (to a 0.065 M concentration) directly after the photolysis.

*6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone (**IV**β)*

The autooxidation of **IV** in isopropanol was accompanied by the generation of acidic side products (including perhaps hydrochloric acid), which hydrolyzed the bis-TMS enol ether to such an extent that the reaction was finished after 3.5 h and yielded 29% of **IV**β, 42% of the starting material **IV**, and 29% of further reaction products including **IV**α (GC/FID results). The 17β-O-TMS ether was completely hydrolyzed under these conditions. A further synthesis using alkaline reaction conditions was not performed.

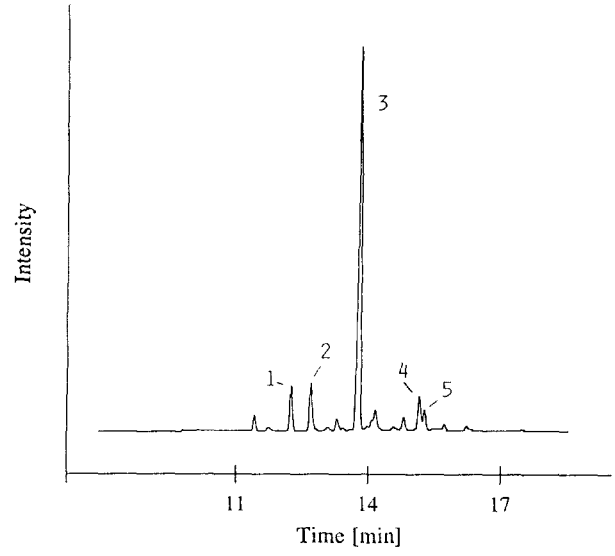

Figure 3 GC/FID chromatogram of 6β-hydroxylation reaction of metandienone: metandienone, bis-TMS dissolved in isopropanol 6 h after exposure to room light (60 W lamp); column: Chrompack WCOT fused silica CP-SIL 8CB, cross-linked 5% phenyl methyl silicone (SE 54), 17 m × 0.25 mm I.D., 0.25 μm film thickness; initial temperature 180°C, program rate 5°C/min, final temperature 320°C; sample preparation: an aliquot of the reaction mixture was dried and derivatized with MSTFA/Imi (100:2, v:w); 1, metandienone TMS, 2, metandienone bis-TMS, 3, 6β-hydroxymetandienone bis-TMS, 4, 6α-hydroxymetandienone bis-TMS, and 5, 17β-hydroxy-17α-methylandrosta-1,4-diene-3,6-dione tris-TMS.

Oxidation of the isolated reaction products **IV**β and **IV**α with chromium trioxide yielded the same product with a molecular ion m/z 348, compared to the molecular ion m/z 350 for **IV**α and **IV**β. This indicates that the hydroxy group of the two isolated autooxidation products are located at the same carbon position, which was confirmed by NMR as C-6.

NMR spectroscopy

^{1}H NMR. Structural elucidation of the stereochemistry at C-6 was derived from ^{1}H NMR chemical shift values and proton coupling constants as presented in Table 2. The 4-H signal is shifted slightly downfield by the 6β-hydroxy group, and measured chemical shift values were between 0.06 and 0.09 ppm. In contrast, the 6α-hydroxy group in **I**α and **III**α shifted the C-4 proton strongly downfield, 0.45 ppm in **I**α and 0.41 ppm in **III**α. No other hydroxylation has this effect on 4-H.[27] The assigned values for **I**α and **I**β are in agreement with published data.[28] The stereochemistry of the 4-chloro steroids **IV**α and **IV**β could not be assigned by a 4-H shift. A further assignment of the C-6

stereochemistry is the resonance signal of the 6α and 6β protons. The 6α proton gives a narrow signal with J = 2.8-2.9 Hz for **Iβ, IIβ, IVβ,** and a doublet for **Vβ** with J = 2.9 Hz. **IIIβ** shows a broad singlet (unresolved triplet) with $w_{1/2} \cong 6.7$ Hz. The axial 6β proton in **Iα** and **IVα** gives a multiplet of poorly defined profile ($w_{1/2} \cong 23$ Hz), and the 6β proton in **IIIα** shows a double doublet with J = 5.4 and 11.3 Hz. This coupling is only possible through an axial-axial coupling between the 6β-H and 7α-H. The 6α-7α and 6α-7β coupling constants are smaller, between 2.5 and 3 Hz, as reported for 6β-hydroxyaldosterone by Kirk et al.[27]

A classical proof of the location of hydroxy groups is the examination of the resonance signals of the 18- and 19-protons as summarized in Zürcher's data collection[29] and in the reference tables of Bridgeman et al.[30] As shown in Table 2 the chemical shift for the C-19 protons in deuterochloroform was between 0.19 and 0.20 ppm for the 6β-isomer compared to the parent steroid (Table 2) and in agreement with the additional shift of 0.19 ppm for the C-19 signal for 6β-hydroxy groups in 5β and Δ^4 steroids, which was reported.[29] The C-19 protons were less influenced by the equatorial 6α-hydroxy group: 0.0 ppm shift for **Iα,** −0.02 ppm for **IIIα,** and 0.04 ppm for **IVα.** The additional shift for the C-18 protons by a 6β-hydroxy group was calculated by Zürcher as 0.04 ppm and is near the measured values of 0.03 ppm for **Iβ-IVβ** (Table 2).[29]

^{13}C NMR. The shielding data (Table 3) for testosterone, the 17α-methyl steroids, and their 6β-hydroxy and 6α-hydroxy isomers were assigned by comparison with spectra of 17β-hydroxy-17α-methyl and 17α-hydroxy-17β-methyl steroids, published by Schänzer et al.[31] and with spectra of closely related compounds, published by Blunt and Stothers.[32] Methylene, methine/methyl, and quaternary carbons were confirmed by comparison to DEPT 135° experiments. The C-6 and C-7 shielding data for 4-chloro-1,2-dehydro-17α-methyltestosterone (**IV**) were elucidated using [6,6-$^2H_2$]**IV.**

Table 5 ^{13}C NMR shielding data, substitution effects of a 6-hydroxy group at testosterone **I,** 17α-methyltestosterone **II,** metandienone **III,** 4-chloro-1,2-dehydro-17α-methyltestosterone **IV,** and fluoxymesterone **V** (difference between 6-hydroxy steroid and parent steroid)

6β-Hydroxy	Iβ	IIβ	IIIβ	IVβ	Vβ[a]	Lit.[b]
C-1	+0.3	+1.5	+1.4	+1.1	+3.3	+1.7
C-4	+2.2	+2.7	+2.0	+2.1	+1.3	−3.1
C-5	−2.2	−3.1	−3.0	−3.7	−3.3	+2.7
C-6	+40.3	+40.3	+40.5	+39.6	+39.6	+43.3
C-7	+5.8	+6.5	+7.0	+6.6	+5.5	+7.4
C-8	−6.3	−5.9	−5.9	−5.8	−4.0	−5.3
C-19	+2.3	+2.1	+1.7	+1.6	+1.7	+3.5
6α-Hydroxy	Iα		IIIα	IVα		Lit.[b]
C-1	+0.2		−0.2	+0.3		+0.1
C-4	−4.4		−4.2	−1.7		−6.4
C-5	+0.7		+1.2	−1.8		+6.7
C-6	+35.7		+34.9	+43.0		+40.8
C-7	+8.7		+9.4	+10.6		+9.5
C-8	−1.9		−1.6	−0.7		−1.3
C-19	+1.0		+0.4	+0.2		+1.1

[a]Substance dissolved in dimethylsulfoxide-d_6.
[b]Subsitution effects of a 6-hydroxy group at saturated 5α-androstane, published by Blunt and Stothers.[32]

Table 6 HPLC retention times of steroids (**I–V**) and their 6β-hydroxy and 6α-hydroxy isomers

Substance	Retention time (min)	λ_{max}	R_1	R_2
Testosterone (I)	15.8	244		
6β-Hydroxy-I (Iβ)	10.1	240	0.64	1.07
6α-Hydroxy-I (Iα)	9.4	244	0.59	
17α-Methyltestosterone (II)	16.8	242		
6β-Hydroxy-II (IIβ)	11.2	239	0.67	
Metandienone (III)	14.9	246		
6β-Hydroxy-III (IIIβ)	9.9	250	0.66	1.10
6α-Hydroxy-III (IIIα)	9.0	248	0.60	
4-Chloro-1,2-dehydro-17α-methyltestosterone (IV)	17.5	247		
6β-Hydroxy-IV (IVβ)	12.2	252	0.70	1.26
6α-Hydroxy-IV (IVα)	9.7	248	0.55	
Fluoxymesterone (V)	12.6	240		
6β-Hydroxy-V (Vβ)	8.6	234	0.68	

For HPLC conditions, see Experimental.
R_1 = Relative retention time of 6-hydroxy steroid to parent steroid.
R_2 = Relative retention time of 6β-hydroxy to 6α-hydroxy steroid.

Substitution effects of the 6α- and 6β-hydroxy group were calculated for neighboring carbons and compared with substitution effects of a 6α- and 6β-hydroxy group to 5α-androstanes (Table 5).[32] The substitution effects of the 6-hydroxy group for carbons 6, 7, 8, and 19 at androst-4-en-3-ones are similar to the reported values at saturated 5α-androstanes. The C-6 signal at the 6β-hydroxy steroids **Iβ–Vβ** is shifted downfield between 39.6 and 40.5 ppm, compared to the parent steroids, and varied to a small degree. For the 6α-hydroxy isomers the variation is larger. **IVα** shows a shift for C-6 of +43.0 ppm as compared to +35.7 ppm for **Iα** and +34.9 ppm for **IIIα.** The reason for this difference can be explained by orientation of the equatorial 6α-hydroxy group in **IVα,** which is sterically influenced by the neighboring chloro atom in position C-4.

HPLC

In HPLC, the elution order on a reverse-phase RP-18 column (LiChrosorb Merck) was 6α-hydroxy isomer < 6β-hydroxy isomer < parent steroid (Table 6), showing the higher polarity of the 6α-hydroxy isomer (equatorial 6-hydroxy group) as compared to the 6β-hydroxy isomer (axial 6-hydroxy group). The relative retention time as compared to the parent steroid was between 0.64 and 0.70 for the 6β-hydroxy isomers **Iβ–Vβ** and between 0.55 and 0.60 for the 6α-hydroxy isomers **Iα, IIIα,** and **IVα.**

Derivatization for GC/MS

For GC/MS analysis the polar 6-hydroxy steroids must be trimethylsilylated to obtain stable GC derivatives. Deriva-

tization with MSTFA/Imi (100:2, v/w) yielded quantitatively bis-TMS ethers with trimethysilylation of the hydroxy groups at C-6 (secondary) and C-17β (tertiary). Imidazole was used as a catalyst to derivatize the sterically hindered tertiary 17β-hydroxy group in 17α-methyl-17β-hydroxy steroids.

TMS enol ethers were obtained when TMIS was used as a catalyst (prepared with a mixture of MSTFA/ammonium iodide; see Experimental). 6β-Hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone **IVβ** could not be enolized, whereas the 6α-hydroxy isomer **IVα** formed a TMS enol ether with the TMIS catalyst. The 6-hydroxy isomers of the 3-keto-4-ene steroids testosterone and 17α-methyltestosterone were enolized to the 3,5-diene structure (yield > 99%) with MSTFA/TMIS. The 2,4-dien isomer was quantitatively formed using MSTFA/Imi/potassium acetate. The retention indices of the TMS 3,5-dienol ethers and TMS 2,4-dienol ethers are summarized in Table 4. 6β-Hydroxyfluoxymesterone reacted with MSTFA/TMIS to form tris-TMS 2,4-dienol ether (30%) and tris-TMS 3,5-dienol ether (70%). Reaction of the 11β-hydroxy group in fluoxymesterone with MSTFA/TMIS was very slow, and the samples were heated overnight at 60°C.

Gas chromatography

Temperature programmed Kovats indices for the steroid TMS ethers were estimated using a OV 1 capillary column (Table 4). The elution order of the TMS ethers is parent steroid < 6β-hydroxy isomer < 6α-hydroxy isomer and inverse to the HPLC elution order of the underivatized steroids (Table 6). The TMS ether derivative of the 6β-hydroxy (6α-hydroxy) isomer of **Iβ–Vβ (Iα, IIIα, IVα)** eluted between 45 and 108 (183 and 213) methylene units after the TMS ether derivative of the parent steroid. The difference between the 6β-hydroxy and 6α-hydroxy isomer was 105–138 methylene units.

The per-TMS 3,5-dienol ethers were eluted between 87 and 171 methylene units later than the per-TMS 3,5 dienol ethers of the parent steroids.

Mass spectrometry

The EI mass spectra of the TMS ethers of the 6β-hydroxy steroids and the per-TMS 3,5-dienol ethers are shown in Figures 4–8. The TMS ethers of the 17α-methyl-17β-hydroxy steroids **IIβ–Vβ** (Figures 5A, 6A, 7A, and 8A), which were obtained via derivatization with MSTFA/Imi, display similar fragmentation with a base peak m/z 143 (D-ring fragmentation). The intensity of the m/z 143 ion varied between 17.8% and 23.5% of the total ion current, whereas the intensity of the molecular ion was low, between 0.7 and 1.6% of the total ion current.

The fragmentation pattern changed for the 3-keto-4-ene steroids, when they were enolized with MSTFA/TMIS to the 3,5-dienol ethers. The TMS 3,5-dienol ethers of **Iβ, Iα, IIβ,** and **Vβ** show abundant molecular ions with m/z 520 for **Iβ/Iα** tris-TMS (Figure 4B), m/z 534 for **IIβ** tris-TMS (Figure 5B), and m/z 640 for **Vβ** tetra-TMS (Figure 8B). The TMS dienol ethers of the androsta-1,4-dien-3-ones show an abundant $M^+ - 15$ ion (loss of a methyl group of the molecular ion) with m/z 517 for **IIIβ/IIIα** tris-TMS (Figure 6B) and m/z 551 for the **IVα** tris-TMS (Figure 7B). The intensity of the D-ring fragment m/z 143 for the TMS 3,5-dienol ethers **IIβ–Vβ** are only 0.4–1.4% of the total ion current, whereas the intensity of M^+ or $M^+ - 15$ ion is between 20.2% and 29.0% of the total ion current. The TMS 3,5-dienol ether structure seems to be a prerequisite for stabilizing the M^+ or $M^+ - 15$ ion. The corresponding TMS 2,4-dienol ethers of **Iβ, Iα, IIβ,** and **Vβ** do not show such a stable molecular ion.

Metabolism

6β-Hydroxy metabolism in humans was investigated for testosterone, 17α-methyltestosterone, boldenone, metandi-

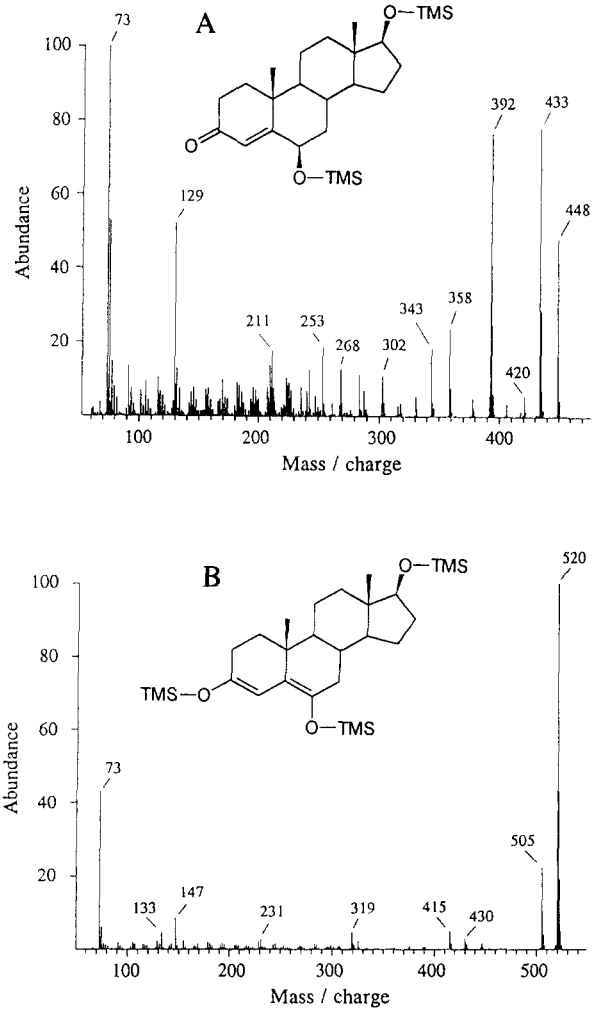

Figure 4 A: EI spectrum of 6β-hydroxytestosterone bis-TMS. B: EI spectrum of 6β-hydroxytestosterone tris-TMS.

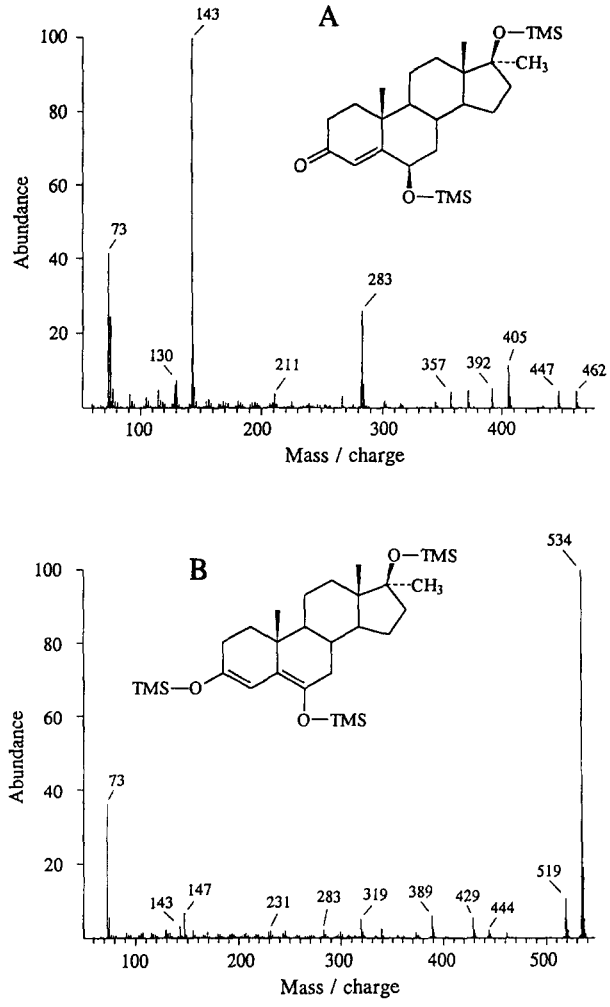

Figure 5 A: EI spectrum of 6β-hydroxy-17α-methyltestosterone bis-TMS. B: EI spectrum of 6β-hydroxy-17α-methyltestosterone tris-TMS.

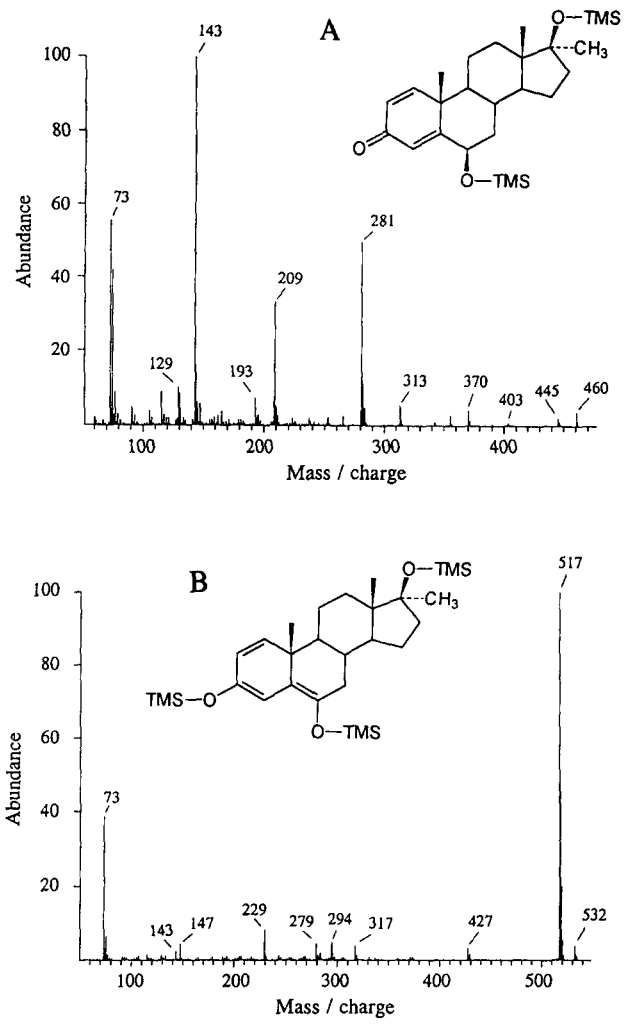

Figure 6 A: EI spectrum of 6β-hydroxymetandienone bis-TMS. B: EI spectrum of 6β-hydroxymetandienone tris-TMS.

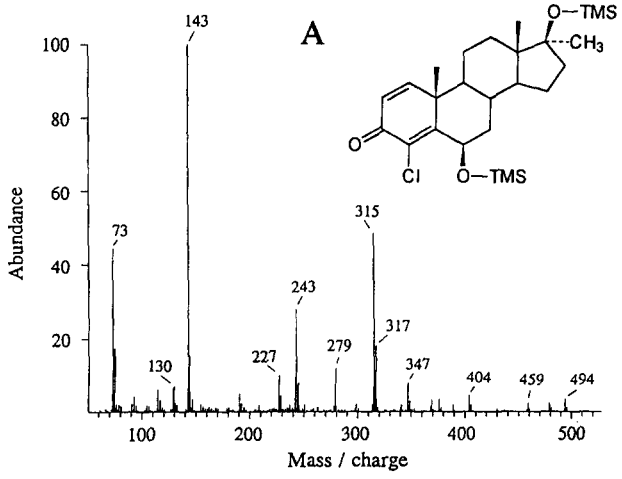

Figure 7 A: EI spectrum of 6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone bis-TMS. B: EI spectrum of 6α-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone tris-TMS.

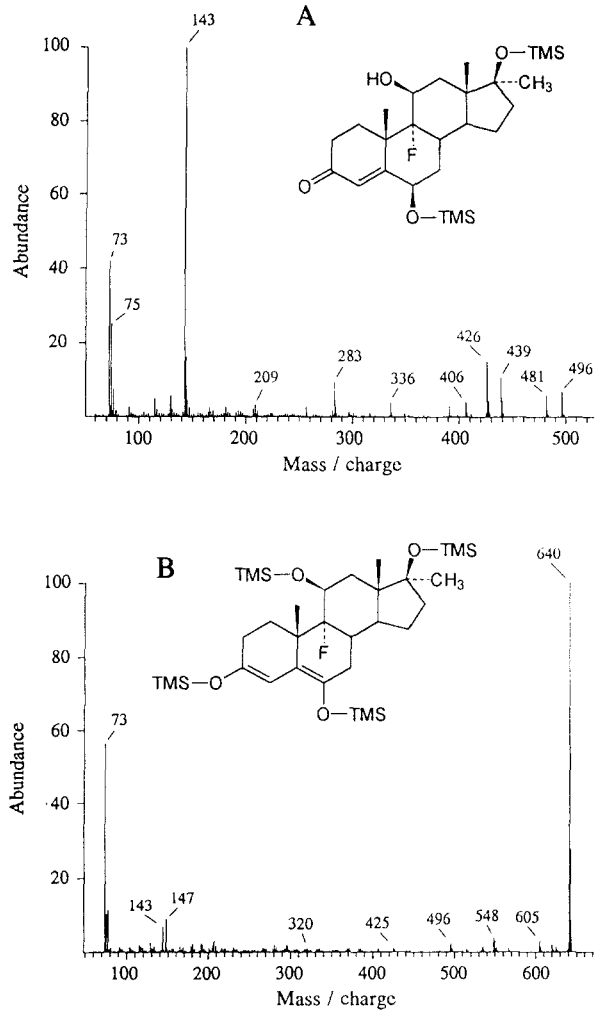

Figure 8 A: EI spectrum of 6β-hydroxyfluoxymesterone bis-TMS. B: EI spectrum of 6β-hydroxyfluoxymesterone tetra-TMS.

enone, 4-chloro-1,2-dehydro-17α-methyltestosterone, and fluoxymesterone.

Metabolism studies with boldenone[22] and metandienone[21] with quantification of 6β-hydroxy metabolites have already been published. The main metabolites of **IV** were identified by GC/MS and in accordance with published studies of Dürbeck et al.[33] The main metabolites of fluoxymesterone have been synthesized and will be reported separately. Quantification of metabolites was performed by GC/MS (selected ion monitoring) and GC/FID. The results are summarized in Table 7 and show the extent of the metabolism to 6β-hydroxy steroids after administration of a single dose. In the metabolism of metandienone **III**, 4-chloro-1,2-dehydro-17α-methyltestosterone **IV**, and fluoxymesterone **V**, 6β-hydroxylation is a major metabolic pathway. All 6β-hydroxy-metabolites of **III**, **IV**, and **V** were excreted unconjugated. The excretion of 6β-hydroxy metabolites calculated from the sum of all detected metabolites was between 17% and 46%. 6β-Hydroxy metabolites are not metabolic end-products. In metandienone and 4-chloro-1,2-dehydro-17α-methyltestosterone metabolism, the 6β-hydroxy product is further hydroxylated at C-12 and C-16. 12-Hydroxylated metabolites of 17β-hydroxy-17α-methyl steroids can be identified by their EI mass spectrum, their TMS-deriviatives showing intense C/D-ring fragments at m/z 143 and 170.[21] The 16-hydroxylated metabolites display abundant D-ring fragments at m/z 218 and 231.[21,34] In fluoxymesterone metabolism the 3-keto group of 6β-hydroxy-fluoxymesterone is further reduced to the 3α-hydroxy isomer which is excreted in comparable amount to the 6β-hydroxy metabolite.

The metabolism studies included boldenone (17β-hydroxyandrosta-1,4-dien-3-one), which is an anabolic steroid and can be compared to its 17α-methylated product, which is metandienone **III** (17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one). In boldenone metabolism the total excretion of 6β-hydroxyboldenone was lower than 0.5% compared to the sum of excreted boldenone and other metabolites. A further 6β-hydroxy metabolite of boldenone, the 17β-hydroxy oxidized product 6β-hydroxyandrosta-1,4-diene-3,17-dione, was identified and excreted at levels seven times higher than 6β-hydroxyboldenone.

Excretion studies with deuterated testosterone and 17α-methyltestosterone show that 6β-hydroxy metabolism plays a minor role when the metabolism of the A-ring is rapid and not inhibited by an additional double bond at C-1–C-2, as it is in **III**, **IV**, and boldenone.

Acknowledgments

We thank Dr. B. Wolf and O. Aulenbacher (Grünenthal GMBH, Aachen, Germany) for the registration of NMR data, Dr. H. Geyer (Deutsche Sporthochschule Köln) for assisting in excretion studies, and the Bundesinstitut für Sportwissenschaft, Köln and the International Athletic Foundation for their financial support.

Names and abbreviations

I testosterone (17β-hydroxyandrost-4-en-3-one)
Iα 6α-hydroxytestosterone
Iβ 6β-hydroxytestosterone
II 17α-methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one)
IIβ 6β-hydroxy-17α-methyltestosterone
III metandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one)
IIIα 6α-hydroxymetandienone
IIIβ 6β-hydroxymetandienone
IV 4-chloro-1,2-dehydro-17α-methyltestosterone (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-one)
IVα 6α-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone
IVβ 6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone
V fluoxymesterone (9-fluoro-11β,17β-dihydroxy-17α-methylandrost-4-en-3-one)
Vβ 6β-hydroxyfluoxymesterone

Table 7 Urinary excretion of 6β-hydroxy metabolites after oral administration of a single dose of deuterated testosterone and the anabolic steroids boldenone, 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, metandienone, and 17α-methyltestosterone

Steroid	Amount administered (mg)	Sum of all metabolites (%)	6β-Hydroxy metabolite (%)	Further 6β-hydroxy metabolite (%)
[16,16,17-$^2H_3$]Testosterone	20	55.9	0.10	NE[a]
17α-Methyltestosterone	100	18.0	0.01	NE
	10	16.5	0.01	NE
Boldenone	20	29.0	0.11	0.80[b]
	40	75.0	0.14	0.89[b]
	80	74.4	0.36	2.77[b]
Metandienone	12	8.2	1.78	0.66[c]
	24	11.4	2.04	0.58[c]
	40	18.6	8.50	0.65[c]
4-Chloro-1,2-dehydro-17α-methyltestosterone	20	2.7	0.72	0.93[d]
				0.80[e]
Fluoxymesterone	22	15.2	4.05	3.06[f]
	40	34.0	6.60	10.90[f]

Percent values are calculated as excreted amount of metabolite relative to the applied amount of steroid.
[a]NE = Not estimated.
[b]6β-Hydroxyandrosta-1,4-diene-3,17-dione (boldenone metabolite).
[c]6β-Hydroxy-17-epimetandienone.
[d]6β,12-Dihydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone.
[e]6β,16-Dihydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone.
[f]9-Fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetol.

References

1. Schänzer W, Donike M (1993). Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal Chim Acta* **275**:23–48.
2. Rongone EL, Segaloff A (1963). In vivo metabolism of Δ^1, 17α-methyltestosterone in man. *Steroids* **1**:170–184.
3. Galletti F, Gardi R (1971). Metabolism of 1-dehydroandrostanes in man. I. metabolism of 17β-hydroxyandrosta-1,4-dien-3-one, 17β-cyclopent-1'-enyloxyandrosta-1,4-dien-3-one (quinbolone) and androsta-1,4-diene-3,17-dione. *Steroids* **18**:39–50.
4. Schubert K, Wehrbeger K (1970). Stoffwechsel von Steroidpharmaka. II. Isolierung und Charakterisierung der Metaboliten von 4-Chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-on. *Endokrinologie* **55**:257–269.
5. Schumann G, Schubert K (1970). Stoffwechsel von Steroidpharmaka. IV. Isolierung und Identifizierung eines dihydroxylierten Metaboliten von 4-Chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-one. *Endokrinologie* **65**:172–177.
6. Schlegel J, Schubert K (1975). Die Verwendung von mikrobiellen Enzymen bei Stoffwechselstudien der Steroide. *Acta microbiol Acad Sci Hung* **22**:397–402.
7. Burstein S, Dorfman RI, Nadel EM (1954). 6β-Hydroxcortisol—A new steroid in human urine. *Arch Biochem Biophys* **53**:307–308.
8. Nadel EM, Burstein S, Dorfman RI (1956). Isolation of polar reducing corticosteroids from human urine. *Arch Biochem Biophys* **53**:144–149.
9. Ehrenstein M (1941). Investigations on steroids. V. Acetolysis of the stereoisomeric 5,6-oxides and preparation of the acetates of 4-androstene-3,17-dione-6(α)-ol and 6(α)-hydroxy-11-desoxycorticosterone. *J Org Chem* **6**:626–646.
10. Ehrenstein M (1948). Investigations on steroids. X. Revision of nomenclature of previously described compounds. *J Org Chem* **13**:214–222.
11. Plattner PA, Lang W (1944). Über Steroide und Sexualhormone. Über die Konfiguration der beiden Cholesterin-oxyde und des "trans"-Cholestantriols-(3β,5,6). *Helv Chim Acta* **27**:1872–1880.
12. Herzig PTH, Ehrenstein M (1951). Investigations on steroids. XIV. Studies in the series of the adrenal cortical hormones: Dehydration of 6β,21-diacetoxyallopregane-5-ol-3,20-dione. *J Org Chem* **16**:1050–1063.
13. Balant CP, Ehrenstein M (1952). Investigations on steroids, XX. 6β- and 6α-acetoxy- and hydroxy-derivatives of progesterone and androstendione. *J Org Chem* **17**:1587–1596.
14. Sondheimer F, Mancera O, Rosenkranz G (1954). Steroids. LVI. C-6 oxygenated derivatives of cortical hormones. *J Am Chem Soc* **76**:5020–5023.
15. Romo J, Rosenkranz G, Djerassi C, Sondheimer F (1954). Steroids. LV. Steroidal sapogenins. XXXV. Chemical introduction of the 6β-hydroxy group into Δ^4-3-ketones by a two step sequence. *J Org Chem* **19**:1509–1515.
16. Dusza JP, Joseph JP, Bernstein S (1962). C-6 Hydroxylated steroids. III. A new preparative method. *J Org Chem* **27**:4046–4047.
17. Gardi R and Lusignani A (1967). Autooxidation of steroid $\Delta^{3,5}$-dien-3-ol ethers. A simple route to 6β-hydroxy Δ^4-3-ketones. *J Org Chem* **32**:2647–2649.
18. Dehennin L, Reiffsteck A, Scholler R (1980). Simple methods for the synthesis of twenty different highly enriched deuterium labelled steroids, suitable as internal standards for isotope dilution mass spectrometry. *Biomed Mass Spectrum* **7**:493–499.
19. Ringold HJ, Batres E, Mancera O, Rosenkranz G (1956). Steroids. LXXXII. Synthesis of 4-halo hormone analogs. *J Org Chem* **21**:1432–1435.
20. Schänzer W, Donike M (1995). Synthesis of deuterated steroids for GC/MS quantification of endogeous steroids. In Donike (Ed): *Recent Advances in Doping Analysis (2). Proceedings of the 12th Cologne Workshop on Dope Analysis* 10.-15.1994 Köln, 93-112. Verlag Sport und Buch Strauß, 1995, Köhn.
21. Schänzer W, Geyer H, and Donike M (1991). Metabolism of metandienone in man: identification and synthesis of conjugated excreted urinary metabolites, determination of excretion rates and gas chromatographic-mass spectrometric identification of bis-hydroxylated metabolites. *J Steroid Biochem Mol Biol* **38**:441–464.
22. Schänzer W, Donike M (1992). Metabolism of boldenone in man: gas chromatographic-mass spectrometric identification of urinary excreted metabolites and determination of excretion rates. *Biol Mass Spectrom* **21**:3–16.
23. Donike M, Zimmerman J, Bärwald K-R, Schänzer W, Christ V,

Klostermann K, Operfmann G (1984). Routine Bestimmung von Anabolika im Harn. *D Z Sportmed* **35**:14–23.

24. Donike M, Zimmermmann J (1980). Zur Darstellung von Trimethylsilyl-Triethylsily- und tert.-Butyl-dimethylsily-enolethern von Ketosteroiden für gas chromatographische und massenspektrometrische Untersuchungen. *J Chromatog* **202**:483–486.
25. Eppstein SH, Meister PD, Leigh HM, Peterson DH, Murray HC, Reinker LM, Wettstein A (1954). Microbiological transformation of steroids. X. The oxygenation of androgens by Rhizopus. *J Am Chem Soc* **76**:3174–3179.
26. Zimmermann J. (1980). Untersuchung über die Testosteron- und Nortestosteron- Elimination nach oraler, rektaler und percutaner Gabe. Diplom thesis, German Sports University, Cologne.
27. Kirk DN, Toms HC, Douglas C, White KA (1990). A survey of the high-field ^{1}H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds. *J Chem Perkin Trans* **2**:1567–1594.
28. Kirk DN, Miller BW, Cooley G (1989). 18-Substituted steroids. Part 16. Synthesis of 6β-hydroxy- and 6α-hydroxyaldosterone and their 17α-isomers. J Chem Res M:1274–1289.
29. Zürcher RF (1963). Protonenresonanzspektroskopie und Steroidstruktur II. Die Lage der C-18 und C-19-Methylsignale in Abhängigkeit von den Substituenten am Steroidgerüst. *Helv Chim Acta* **46**:2054–2088.
30. Bridgeman JE, Cherry PC, Clegg AS, Evans JM, Jones ERH, Kasal A, Kumar V, Meakins GD, Morisawa Y, Richards EE, Woodgate PD (1970). Microbiological Hydroxylation of steroids. Part I. Proton magnetic resonance spectra of ketones, alcohols, and acetates in the androstane, pregnane, and estrane series. *J Chem Soc* C:250–257.
31. Schänzer W, Opfermann G, Donike M (1992). 17-Epimerization of 17α-methyl anabolic steroids in humans: metabolism and synthesis of 17α-hydroxy-17β-methyl steroids. *Steroids* **57**:537–550.
32. Blunt JW, Stothers JF (1977). ^{13}C NMR spectra of steroids—a survey and commentary. *Org Magn Reson* **9**:439–464.
33. Dürbeck HW, Büker I, Scheulen B, Telin B (1983). GC and capillary column GC/MS determination of synthetic anabolic steroids. II. 4-Chlorometandienone (oral turinabol) and its metabolites. *J Chromatogr Sci* **21**:405–410.
34. Schänzer W, Opfermann G, Donike M (1990). Metabolism of stanozolol: identification and synthesis of urinary metabolites. *J Steroid Biochem* **36**:253–174.