Synthesis and characterization of the 6α- and 6β-hydroxylated derivatives of corticosterone, 11-dehydrocorticosterone, and 11-deoxycortisol

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This report describes the synthesis of 6α,17,21- and 6β,17,21-trihydroxypregn-4-ene-3,20-dione, 6α,7,21- and 6β,11β,21-trihydroxypregn-4-ene-3,20-dione, and—for the first time—that of 6α,21- and 6β,21-dihydroxypregn-4-ene-3,11,20-trione. The former four compounds were prepared by 6-hydroxylation of 17,21-trihydroxypregn-4-ene-3,20-dione and 11β,21-dihydroxypregn-4-ene-3,20-dione, respectively. This was achieved by autoxidation or by oxidation with 3-chloroperbenzoic acid, of the 3-methoxypregna-3,5-dienes of the latter two steroids. The yield of the 6β-hydroxylated steroids, but not of their corresponding 6α-epimers, was higher using autoxidation than the peracid. The two 6-hydroxylated pregnenetriones were prepared from 6α,21-diacetoxy-11β-hydroxypregn-4-ene-3,20-dione and 6β,21-diacetoxy-11β-hydroxypregn-4-ene-3,20-dione, respectively, by oxidation with pyridinium chlorochromate. The above-mentioned six steroids were identified and characterized by nuclear magnetic resonance, infrared, ultraviolet, high performance liquid chromatography, gas chromatography, and mass spectrometry. (Steroids **58:**495–503, 1993)

Keywords: synthesis; corticosteroids; 6-hydroxylation; NMR; HPLC; GC/MS

Introduction

The urinary steroid profiles of patients with congenital adrenal hyperplasia (CAH) due to deficiencies of 17α-hydroxylase (17OHD)[1,2] or 11β-hydroxylase (11OHD)[3] have been well described.[2–8]

Although new metabolites of corticosterone (B) and 11-deoxycortisol (S) have been discovered during the last 15 years, some were not completely identified.[2,5–8] Amongst these are the metabolites of B: 3α,6α,11β,21-tetrahydroxy-5β-pregnan-20-one (6αOH-5βTHB) and 3α,6α,11β,20α,21-pentahydroxy-5β-pregnane (6αOH-20αHHB), the metabolites of 11-dehydrocorticosterone (A): 3α,6α,21-trihydroxy-5β-pregnane-11,20-dione (6αOH-5βTHA) and 3α,6α,20α,21-tetrahydroxy-5β-pregnan-11-one (6αOH-20αHHA) and those of S: 3α,6α,17,21-tetrahydroxy-5β-pregnan-20-one (6αOH-5βTHS) and 3α,6α,17,20α,21-pentahydroxy-5β-pregnane (6αOH-20αHHS).

In urine of an 11OHD human neonate the presence of 6αOH-5βTHS has been demonstrated, but this steroid was not fully identified.[6] Also the steroid 6αOH-5βTHB was only tentatively identified in the urines of 17OHD patients.[2,8]

Because the 6α-hydroxylated derivatives of 3α, 11β, 21 - trihydroxy - 5α(and 5β) - pregnan - 20 - one (THB), 3α,21-dihydroxy-5α(and 5β)-pregnane-11,20-dione (THA), and 3α17,21-trihydroxy-5α(and 5β)-pregnan-20-one (THS) are not commercially available we decided to synthesize these 6-hydroxylated steroids.

This report describes the synthesis and the physicochemical properties of 6α,17,21- (IVα) and 6β,17,21-

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trihydroxypregn-4-ene-3,20-dione (IVβ), 6α,11β,21-(IXα) and 6β,11β,21-trihydroxypregn-4-ene-3,20-dione (IXβ), and 6α,21- (XIIα) and 6β,21-dihydroxypregn-4-ene-3,11,20-trione (XIIβ) as intermediates for the synthesis of the corresponding tetrahydro metabolites.

It should also be noted that the synthesis of IVα, IVβ, and IXβ has been reported previously,[9,10] and that these three steroids are commercially available, but they have not been analyzed by ^{1}H NMR and the 6α-hydroxylated steroids IVα and IXα were insufficiently characterized.

Experimental

Chemicals

The compounds I (17,21-dihydroxypregn-4-ene-3,20-dione), V (11β-21-dihydroxypregn-4-ene-3,20-dione), and XIII (21-hydroxypregn-4-ene-3,11,20-trione) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All chemically pure solvents and inorganic compounds were purchased from Janssen Chimica (Tilburg, The Netherlands). Chemically pure pyridine and ethyl acetate were obtained from Merck (Darmstadt, Germany). 3-Chloroperbenzoic acid (mCPBA), technical grade, was purchased from Aldrich Chemie (Brussels, Belgium). Analytically pure chemicals were purchased from Janssen Chimica: acetic anhydride, N,N-dimethylformamide, KOH, NH_4Cl, 2,2-dimethoxypropane, and trimethyl orthoformate, or from Merck: $CDCl_3$, methanol and perchloric acid (70%). Dichloromethane (DCM, HPLC grade) was purchased from Rathburn Chemicals Ltd (Walkerburn, UK). Methoxamine · HCl and N-trimethylsilyl imidazole were obtained from Pierce (Rockford, IL, USA). TLC aluminum sheets silica gel 60 F_{254} or aluminum oxide F_{254} were purchased from Merck. Aluminum oxide H (10–40 μm), aluminum oxide 90 (pH 9, ≥ 60 μm), and silica gel H (10–40 μm) for preparative chromatography were obtained from Merck. Florisil was purchased from Fluka Chemie AG (Buchs, Switzerland).

Chromatography

(1) Thin-layer chromatography (TLC). Preliminary analyses were peformed using TLC aluminum sheets with 0.2 mm layer of aluminum oxide F_{254} (system TLC-A) or 0.2 mm silica gel F_{254} (system TLC-B). The plates were developed in DCM or DCM mixed with 1 to 10% methanol, denoted by the suffixes 1–10.

(2) Centrifugal liquid chromatography (CLC). For liquid chromatographic separations on a semipreparative scale a Hitachi CLC-5 was used. The instrument contained a rotating disk of 20 cm diameter and 3 mm height filled with aluminum oxide H or aluminum oxide 90 (system CLC-A), or silicagel H (system CLC-B). The steroids monitored at 254 nm were eluted with one of the solvents mentioned above.

(3) High performance liquid chromatography (HPLC). The equipment (Millipore, Waters Chromatography Division, Milford, Mass, USA) consisted of two 6000 A pumps, a U6K injector, a two-channel 440 absorbance detector monitoring at 254 and 280 nm, and a 730 data module, managed by a 720 system controller. The retention times of the compounds, IV, IX, and XII were measured by 2 systems. System 1 consisted of a normal-phase column (150 × 3.9 mm) packed with 5 μm Hypersil spheres (Shandon Southern Products Ltd. Astmour, Cheshire UK). The steroids were eluted with dichloromethane/methanol/water (97.25 : 2.5 : 0.25 v/v) at a flow rate of 1.5 ml/minute. System 2 consisted of a reversed-phase column (150 × 3.9 mm) packed with 5 μm spheres of octadecylsilyl Hypersil (Shandon). The steroids were eluted with methanol/water (35 : 65 v/v) at a flow rate of 1.5 ml/minute.

(4) Gas chromatography/mass spectrometry (GC and GC/MS). For the determination of the methylene unit (MU) values and mass spectra of the steroids IV, IX, and XII the steroids were derivatized to the 3,20-dimethoxime pertrimethylsilyl ethers (MO-TMS) as described previously.[11] GC. An HP 5890 GC (Hewlett-Packard Nederland B.V., Amstelveen, The Netherlands) was equipped with an HP 7373 injector, an HP fused silica column (type 549-1-07A [cross-linked methyl silicone(ultra)] of 37.5 m, 0.20 mm diameter and a film thickness of 0.11 μm), and a flame ionization detector. The helium gas flow was set at 0.5 ml/minute. The temperature of the injector and detector was kept at 300 C. The oven temperature started at 215 C (2 minutes), was increased at a rate of 1.5 C/minute to 270 C followed by a rate of 30 C/minute to and kept at 300 C during 10 minutes. Retention times and peak areas were recorded using a Nelson Analytical Data system. The MU values of the steroids were calculated by linear interpolation of their retention times between those of the co-injected mixture of the 4 *n*-alkanes $C_{31}H_{64}$ to $C_{34}H_{70}$. GC/MS. For the recording of the mass spectra of the title compounds IV, IX, and XII a HP 5890 GC was equipped with a CP Sil 5 CB capillary column (25 m × 0.25 mm; Chrompack, Middelburg, The Netherlands) and coupled to a VG 70-250S mass spectrometer (VG Instruments, Manchester, UK). The column was heated from 80–200 C in 6 minutes (20 C/minute) and to 234 C in 34 minutes (1 C/minute). Electron impact (EI) mass spectra were recorded by repetitive scanning from 100–800 amu at 70 eV and a resolving power m/Δm = 1000.

Mass spectrometry (MS)

Mass spectra were recorded on AEI-MS-902 spectrometer at a resolving power m/Δm = 10,000 to determine the exact molecular mass of the underivatized steroids.

Infrared spectroscopy (IR)

The infrared spectra of all steroids were recorded in KBr from 2500 to 17000 nm (4000–600 cm^{-1}) on a Pye Unicam SP3-200 or a Perkin-Elmer 257 spectrometer.

Ultraviolet spectroscopy (UV)

The UV-absorbance maxima (λ_{max}) and the half bandwidths (λ_{hb}) at the half-maximal absorbances ($\lambda_{0.5max} > \lambda_{max}$) were measured in dry ethanol on a Zeiss spectrophotometer, equipped with an M4 QII monochromator.

Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were recorded in $CDCl_3$ on a Hitachi Perkin-Elmer high resolution NMR spectrometer at 60 MHz or on a Varian-VXR-300 apparatus at 300 MHz. The chemical shifts are given in δ units (ppm) relative to the internal standard Me_4Si. The used abbreviations are: ABq = AB quartet; b = broad; br d = broad doublet; bm = broad multiplet; d = doublet; nd = narrow doublet; nm = narrow multiplet; vb = very broad; and – = unknown. The δ values noted without an abbreviation refer to singlet resonance peaks.

Synthesis of 6α,17,21-trihydroxypregn-4-ene-3,20-dione (IVα) and 6β,17,21-trihydroxypregn-4-ene-3,20-dione (IVβ)

The compounds IVα and IVβ were obtained from compound I in 3 steps (see Figure 1).

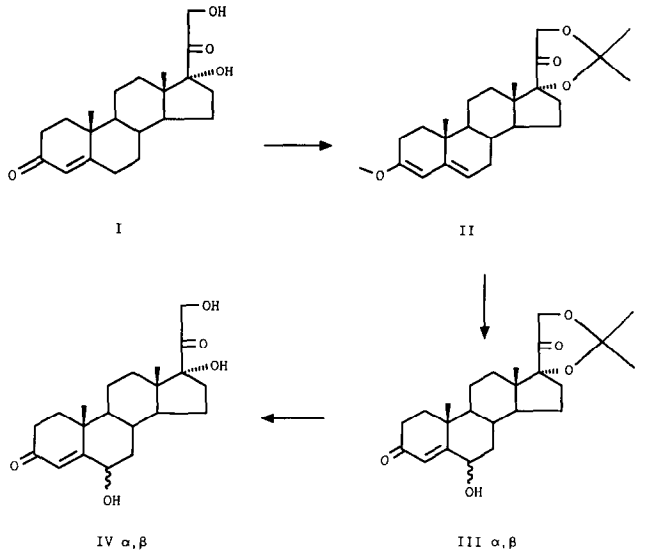

Figure 1 Synthetic pathway from 17,21-dihydroxypregn-4-ene-3,20-dione (I) to 6α,17,21-trihydroxypregn-4-ene,3,20-dione (IVα) and 6β,17,21-trihydroxypregn-4-ene-3,20-dione (IVβ).

Step (1) compound II. A mixture of compound I (5.00 g, 14.4 mmol), 2,2-dimethoxypropane (75 ml), N,N-dimethylformamide (30 ml), and *p*-toluenesulfonic acid (0.125 g) was refluxed for 6 hours as described previously.[12-14] The mixture was cooled to 20 C and an aqueous saturated solution of $NaHCO_3$ was added. The steroid was extracted with DCM (2 × 200 ml). The combined organic layers were dried (Na_2SO_4) and concentrated to give 6 g of II (purity: 90% by NMR). Pure II (8.8 mmol; 61%) was obtained after crystallization from ethanol (EtOH). NMR (60 MHz): δ 0.67 (s, 18-CH_3), 1.00 (s, 19-CH_3), 1.43 and 1.50 (17,21-$C(CH_3)_2$), 3.50 (s, 3-O-CH_3)), 4.10 and 4.20 (d, 21-CH_2), 5.10 (s, 4-H), 5.23 (s, 6-H). IR: 2950, 1710 (C=O), 1650 and 1630 (diene) cm^{-1}.

Step (2) IIIα and IIIβ. IIIα and IIIβ were obtained from II by oxidation with mCPBA as previously described for monoperphtalic acid[9] or by autoxidation[12,15,16] (O_2/hν). mCPBA. A solution of mCPBA (0.40 g, 1.86 mmol) in DCM (20 ml) was slowly added under nitrogen at 0 C to a cold solution of II (0.550 g, 1.37 mmol) and trimethyl orthoformate (0.2 ml) in DCM (20 ml). After 5 minutes the organic phase was washed with aqueous $NaHCO_3$ (2 × 20 ml), water (2 × 20 ml), brine (20 ml), dried (Na_2SO_4), and concentrated. The resulting steroids (0.670 g) were purified by the system CLC-A using DCM at a flow of 6 ml/minute. After discarding a first fraction (0.200 g) a mixture of 0.140 g IIIβ and IIIα was obtained (β : α ~ 3 : 1). O_2/hν. A suspension of II (4.0 g, 10.0 mmol) in EtOH (400 ml) was stirred vigorously in contact with the air at 20 C and illuminated by a 300 W (UV) light source placed at a distance of 45 cm from the reaction vessel. The reaction was monitored by the system TLC-A_4. After one day compound II (R_f = 0.8) had disappeared. TLC-A_4: 3 spots: R_f 0.34 (IIIα), 0.42 (IIIβ), and 0.90 (unknown compound). The yield was approximately 4.0 g IIIα + IIIβ. The reaction products together were purified by the system CLC-A using DCM at a flow of 6.2 ml/minute during 5.5 hours. Using mCPBA 0.290 mmol (15%) of IIIβ and 0.097 mmol (5%) of IIIα (3 : 1) was obtained from 1.86 mmol of II. Using O_2/hν 7.46 mmol (50%) of IIIβ and 0.75 mmol (5%) of IIIα was obtained from 15 mmol of compound II. NMR (60 MHz): IIIα: δ 0.68 (s, 18-CH_3), 1.17 (s, 19-CH_3), 1.41 and 1.48 (17,21-$C(CH_3)_2$), 4.08 and 4.16 (d, 21-CH_2), 4.32 (vb 6-H), 6.16 (s, 4-H); IIIβ: δ 0.68 (s, 18-CH_3), 1.33 (s, 19-CH_3), 1.38 and 1.45 (17,21-$C(CH_3)_2$), 4.05 and 4.12 (d, 21-CH_2), 4.29 (b 6-H), 5.72 (s, 4-H).

Step (3) IVα and IVβ. A solution of IIIβ (0.900 g, 2.25 mmol) in MeOH (25 ml) was ultrasonically degassed, one molar HCl (1.0 ml) was added and, after standing for 2 hours at 20 C under nitrogen, the steroids were extracted with DCM (250 ml). The organic solution was washed with a saturated aqueous solution of $NaHCO_3$ (12.5 ml), dried (Na_2SO_4), and concentrated. 0.83 Mmol of IIIα was similarly processed. The products were separately purified by the system CLC-B_4 at a flow-rate of 6 ml/minute and yielded 1.30 mmol (52%) of IVβ (6βOH-S) and 0.44 mmol (53%) of IVα (6αOH-S). The exact mass for IVβ ($C_{21}H_{30}O_5$, calculated 362.209) was found to be 362.208. Aliquots of IVα and IVβ were further purified by HPLC system 1 before final analysis. IVα: IR: IVα 3450 (OH), 2950, 1730 (20-oxo), 1660 (3-oxo), 1660 (4-ene); UV λ_{max} 241 and λ_{hb} 15.2 nm; IVβ: IR: 3470 (OH), 2980, 1710 (20-oxo), 1670 (3-oxo) 1640 (4-ene) cm^{-1}; UV: λ_{max} 237 and λ_{hb} 17.4 nm. Other characteristic data which identify the steroids as 6αOH-S and 6βOH-S are given in Tables 1 (NMR), 2 (HPLC and GC), and 3 (GC/MS).

Synthesis of 6α,11β,21-trihydroxypregn-4-ene-3,20-dione (IXα) and 6β,11β,21-trihydroxypregn-4-ene-3,20-dione (IXβ)

IXβ was obtained in 4 (see Figure 2) and IXα in 7 steps (see Figures 2 and 3).

Step (1) compound VI. To a solution of corticosterone (V, 5.9 g, 17.0 mmol) in pyridine (27 ml) acetic anhydride (12 ml) was added. After one day at 20 C the solvent was removed. The residue was dissolved in DCM (150 ml) and the solution was washed with saturated aqueous $NaHCO_3$ (60 ml), saturated aqueous NH_4Cl (60 ml), water (60 ml), and saturated NaCl (60 ml). The steroid solution was dried (Na_2SO_4) and concentrated to yield 16.8 mmol (99%) VI, the 21-acetoxy derivative of V. NMR (60 MHz): δ 0.93 (s, 18-CH_3), 1.43 (s, 19-CH_3), 2.10 (s, 21-OC=OCH_3), 4.34 (b, 11-H), 4.53 and 4.60 (d, 21-CH_2), 5.63 (s, 4-H); IR: 3440 (OH), 2940, 1725 (20-oxo), 1650 (3-oxo) cm^{-1}.

Step (2) compound VII. To a solution of crude VI (5.4 g, 13 mmol), in dioxane (55 ml), trimethyl orthoformate (5.5 ml, 53 mmol), MeOH (0.55 ml), and 40% aqueous $HClO_4$ (0.75 ml) were added. The mixture was stirred for 30 minutes at 20 C. Pyridine (1.5 ml) was added to the dark reaction mixture, followed by addition of water (60 ml). The yellow precipitate was collected, washed with water, and dried in vacuum above KOH. The solid (5.2 g) was crystallized from DCM/MeOH to give 2.85 g (54%) of pure VII. NMR (60 MHz): δ 0.93 (s, 18-CH_3), 1.20 (s, 19-CH_3), 2.13 (s, 21-OCOCH_3), 3.55 (s, 3-OCH_3), 4.40 (b, 11-H), 4.53 and 4.59 (d, 21-CH_2), 5.05 and 5.13 (s, 4-H and 6-H or reversed); IR: 3580 (OH), 2920, 1730 (20-oxo), 1643 and 1630 (diene) cm^{-1}.

Step (3) compounds VIIIα and VIIIβ. mCPBA. A solution of mCPBA (0.30 mmol) in DCM (10 ml) was slowly added under nitrogen at 0 C to a cold solution of VII (0.100 g, 0.25 mmol) in DCM (15 ml). After the workup as described for III, the steroids were separated by using the systems CLC-B_1 and CLC-B_4 at a flow-rate of 5 ml/minute. The yield of VIIIα and VIIIβ was 2.5 and 7.5%. O_2/hν. A suspension of VII (1.0 g, 2.48 mmol) in EtOH (300 ml) was illuminated as described for III. After 1 day the clear solution was concentrated. By chromatography (system

Table 1 ^{1}H NMR (300 MHz) spectra of the original steroids[a] I, V, and XIII, and the final 6-hydroxylated steroids IVα, IVβ, IXα, IXβ, XIIα, and XIIβ.

	C-4 H	C-6 H	C-11 H	C-21 H_2	C-19 H_3	C-18 H_3
S (I)[a]	5.68	—	—	4.50;4.35 ABq (J = 2Hz)	1.18	0.69
6αOH-S (IVα)	6.02 nd[b] (J = 1.8Hz)	4.13 vb	—	4.51;4.13 ABq (J = 18Hz)	1.04	0.52
6βOH-S (IVβ)	5.75	4.25 b	—	4.62;4.23 ABq (J = 18Hz)	1.34	0.65
B (V)[a]	5.63	—	4.38 b	4.11;4.17 d	1.42	0.90
6αOH-B (IXα)	6.06 nd (J = 1.2Hz)	4.44 vb	4.39 b	4.17;4.15 ABq (J = 1.2Hz)	1.40	0.90
6βOH-B (IXβ)	5.68	4.28 b	4.20 b	4.12;4.09 d	1.57	0.85
A (XIII)[a]	5.64	—	—	4.11	1.35	0.65
6αOH-A (XIIα)	6.16 nd (J = 1.2Hz)	4.35 vb	—	4.17;4.15 ABq (J = 2.8Hz)	1.40	0.67
6βOH-A (XIIβ)	5.81	4.36 b	—	4.17;4.16 br d	1.58	0.69

[a] The spectra of the original compounds were measured at 60 MHz.
[b] The abbreviations used are given in **Experimental**.

Table 2 HPLC and GC. Capacity factors (k')[a] and the MU values[b] of the final 6-hydroxylated compounds IVα, IVβ, IXα, IXβ, XIIα, and XIIβ.

	HPLC[a]		GC[b]			
	NP[c]	RP[d]	1	2	3	4
6βOH-S (IVβ)	4.9	6.3	31.38 (26)	31.60 (100)	—	—
6αOH-S (IVα)	7.9	9.1	31.77 (45)	32.14 (100)	—	—
6βOH-B (IXβ)	4.8	8.6	32.43 (32)	32.57 (100)	32.74 (6)	32.86 (17)
6αOH-B (IXα)	5.0	3.8	33.15 (53)	33.34 (100)	33.60 (8)	33.79 (17)
6βOH-A (XIIβ)	2.6	4.2	31.62 (9)	31.85 (8)	32.00 (22)	32.16 (100)
6αOH-A (XIIα)	3.8	4.7	32.47 (41)	32.77 (100)	32.97 (8)	33.24 (22)

[a] The numbers refer to the capacity factor $k' = (t_R - t_m)/t_m$, where t_R and t_m are the retention times of the steroid and the mobile phase, respectively.
[b] Methylene unit values of the methoxime trimethylsilyl ethers are given with the percentage of the peaks relative to that of the main peak within parentheses.
[c] NP: normal phase (system 1).
[d] RP: reversed-phase (system 2). Details are given under **Experimental**.

CLC-B_4) 1.9 mmol (77%) of pure VIIIβ and 0.15 mmol ($\approx$6%) of crude VIIIα were obtained. The total yield from 5.0 mmol of VII was 2.7 mmol (54%) of VIIIβ and 0.3 mmol (6%) of VIIIα, a ratio of 9 : 1. The data of VIIIβ: NMR (60 MHz): δ 0.92 (s, 18-CH_3), 1.60 (s, 19-CH_3), 2.11 (s, 21-OCOCH_3), 4.35 (b, 11-H and 6-H), 4.50 and 4.60 (d, 21-CH_2), 5.76 (s, 4-H); IR: 3465 (OH), 2950, 1730 (20-oxo), 1680 (3-oxo) cm^{-1}.

Step (4) compound IXβ. To a solution of VIIIβ (0.210 g, 0.52 mmol) in MeOH (15 ml) was added KOH (0.048 g) dissolved in MeOH (0.40 ml). The solution was kept under N_2 at 20 C and after 1 hour neutralized with acetic acid/MeOH (1 : 9). Upon evaporation of the solvent the solid was suspended ultrasonically in 2 ml saturated aqueous NH_4Cl and filtered yielding 100 mg crude IXβ. After purification on the system CLC-B_4 0.041 mmol (8%) of (IXβ) was obtained. An aliquot of IXβ was further purified by HPLC system 2. The exact mass of IXβ ($C_{21}H_{30}O_5$) was found to be 362.208 (calculated 362.209); IR: 3460 (OH), 2950, 1720 (20-oxo), 1670 (3-oxo) cm^{-1}. UV: λ_{max} 236 and λ_{hb} 17.6 nm. Other characteristic data of IXβ which identify the steroid as 6βOH-B are shown in Tables 1 (NMR), 2 (HPLC and GC), and 3 (GC/MS). Due to the low yield of IXα this compound was obtained from Xβ (see below).

Step (5) compound Xβ. To obtain more IXα and IXβ, and also to synthesize XIIα and XIIβ, compound VIIIβ was used. To a solution of VIIIβ (1.0 g, 2.5 mmol) in pyridine (4.0 ml) acetic anhydride (1.7 ml) was added. The mixture was kept at room temperature for 1 day and processed as described for compound VI (1st step). The yield of the diacetate Xβ was 2.24 mmol (91%). NMR: (60 MHz): δ 0.95 (s, 18-CH_3), 1.49 (s, 19-CH_3), 2.00 (s, 6-OCOCH_3), 2.11 (s, 21-OCOCH_3), 4.34 (b, 11-H), 4.50 and 4.57 (d, 21-CH_2), 5.30 (b, 6-H), 5.80 (s, 4-H).

Step (6) compound Xα. Dry HCl gas was passed through a solution of Xβ (1.0 g, 2.24 mmol) in a mixture of dry DCM (145 ml) and EtOH (1 ml) at 0 C as described in the literature.[12,17] After 5 minutes the temperature was raised to 20 C and the gas was passed through for 1.5 hours followed by N_2 gas for 10 minutes. After removal of the solvent, 0.85 g (85%) of the epimer Xα was obained by chromatography (system CLC-B_2). NMR: (60 MHz): δ 0.92 (s, 18-CH_3), 1.46 (s, 19-CH_3), 2.09 (s, 6-OCOCH_3), 2.11 (s, 21-OCOCH_3), 4.34 (b, 11-H), 4.51 and 4.59 (d, 21-CH_2), 5.36 and 5.44 (bd, 6-H), 5.78 (s, 4-H).

Step (7) compound IXα. One millimole of the diacetate Xα was hydrolyzed and the steroid product was purified as described

Table 3. GC/MS. Mass fragments of the final 6-hydroxylated compounds[a] IVα, IVβ, IXα, IXβ, XIIα, and XIIβ.

	M+	−15[b]	−31	−47	−90	−103	−15 −90	−31 −90	−90 −90	−15 −90 −90	−31 −90 −90				
m/z	636[c]	621	605	589	546	533	531	515	456	441	425	276	246	244	103
6βOH-S (IVβ)	55[d]	9	100	2	13	26	3	47	8	2	38	8	13	15	27
6αOH-S (IVα)	60	10	100	2	5	26	4	37	3	2	22	6	10	7	7
m/z	636	621	605	589	546	533	531	515	456	441	425	188	158	143	103
6βOH-B (IXβ)	91	100	17	7	8	7	8	14	3	3	7	18	8	10	40
6αOH-B (IXα)	100	86	21	8	8	8	8	16	3	2	8	18	8	9	37
m/z	650	635	619	603	560	547	545	529	470	—	439	276	246	244	103
6αOH-E[e]	56	15	100	3	5	26	9	61	4	—	64	14	17	9	82

	M+	−15	−31	−47	−90	−103	−15 −90	−31 −90	−90 −90				
m/z	562	547	531	515	472	459	457	441	382	188	158	143	103
6βOH-A (XIIβ)	43	100	15	47	2	1	1	4	1	3	6	2	17
6αOH-A (XIIα)	47	100	15	42	1	1	1	3	3	4	5	2	14
m/z	446	431	415	399	356								
6βOH-Adr[e]	17	100	23	84	2								

[a] The compounds measured as the methoxime trimethylsilyl ethers.
[b] The losses from the parent ion.
[c] The characteristic ions over m/z 100.
[d] The relative intensities of the fragment ions (given as percentage of the leading ion) were taken from the mass spectrum of the main peak from the GC column (see Table 2).
[e] For purposes of comparison the data of 6α,17,21-trihydroxypregn-4-ene-3,11,20-trione (6αOH-E) and 6β-hydroxyandrost-4-ene-3,11,17-trione (6βOH-Adr) have been added.

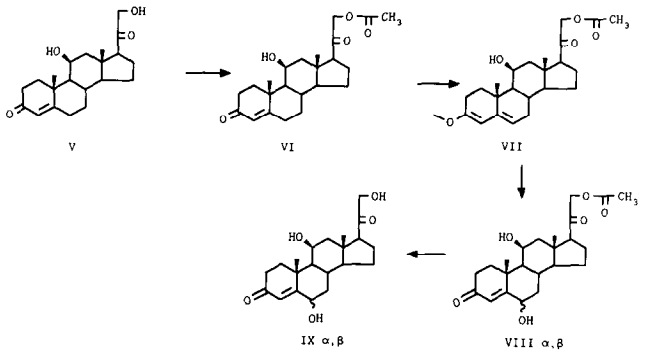

Figure 2 Synthetic pathway from 11β,21-dihydroxypregn-4-ene-3,20-dione (V) to 6β,11β,21-trihydroxypregn-4-ene-3,20-dione (IXβ) and 6α,11β,21-trihydroxypregn-4-ene-3,20-dione (IXα).

for IXβ. Compound IXα was obtained in a yield of 0.36 mmol (35%). An aliquot of IXα was further purified by HPLC system 2. IR: 3450 (OH), 2950, 1720 (20-oxo), 1660 (3-oxo) cm^{-1}. UV: λ_{max} 241 and λ_{hb} 15.2 nm. Other characteristic data of IXα which identify the steroid as 6αOH-B are shown in Tables 1 (NMR), 2 (HPLC and GC), and 3 (GC/MS).

Synthesis of 6α,21-dihydroxypregn-4-ene-3,11,20-trione (XIIα) and 6β,21-dihydroxypregn-4-ene-3,11,20-trione (XIIβ)

XIIα and XIIβ were synthesized in 2 steps (see Figure 3).

Step (1) compounds XIα and XIβ. To a suspension of pyridinium chlorochromate[18] (0.30 g, 1.4 mmol) in DCM (1 ml) was added a solution of Xα (0.400 g, 0.90 mmol) in DCM (1 ml). The mixture was stirred vigorously for 1.5 hours at 20 C, then filtered through Florisil. The Florisil was washed with DCM (2 ml). The filtrate was evaporated under reduced pressure to give 0.79 mmol (88%) of crude XIα. NMR: (60 MHz): δ 0.70 (s, 18-CH_3), 1.44 (s, 19-CH_3), 2.10 (s, 6-$OCOCH_3$), 2.10 (s, 21-$OCOCH_3$), 4.50 (s, 21-CH_2), 5.41 (vb, 6-H), 5.86 (s, 4-H). Compound XIβ was similarly prepared. From 0.45 mmol of Xβ a yield of 0.38 mmol (84%) of XIβ was obtained. NMR: (60 MHz): δ 0.71 (s, 18-CH_3), 1.48 (s, 19-CH_3), 2.03 (s, 6-$OCOCH_3$), 2.15 (s, 21-$OCOCH_3$), 4.51 (s, 21-CH_2), 5.37 (b, 6-H), 5.88 (s, 4-H).

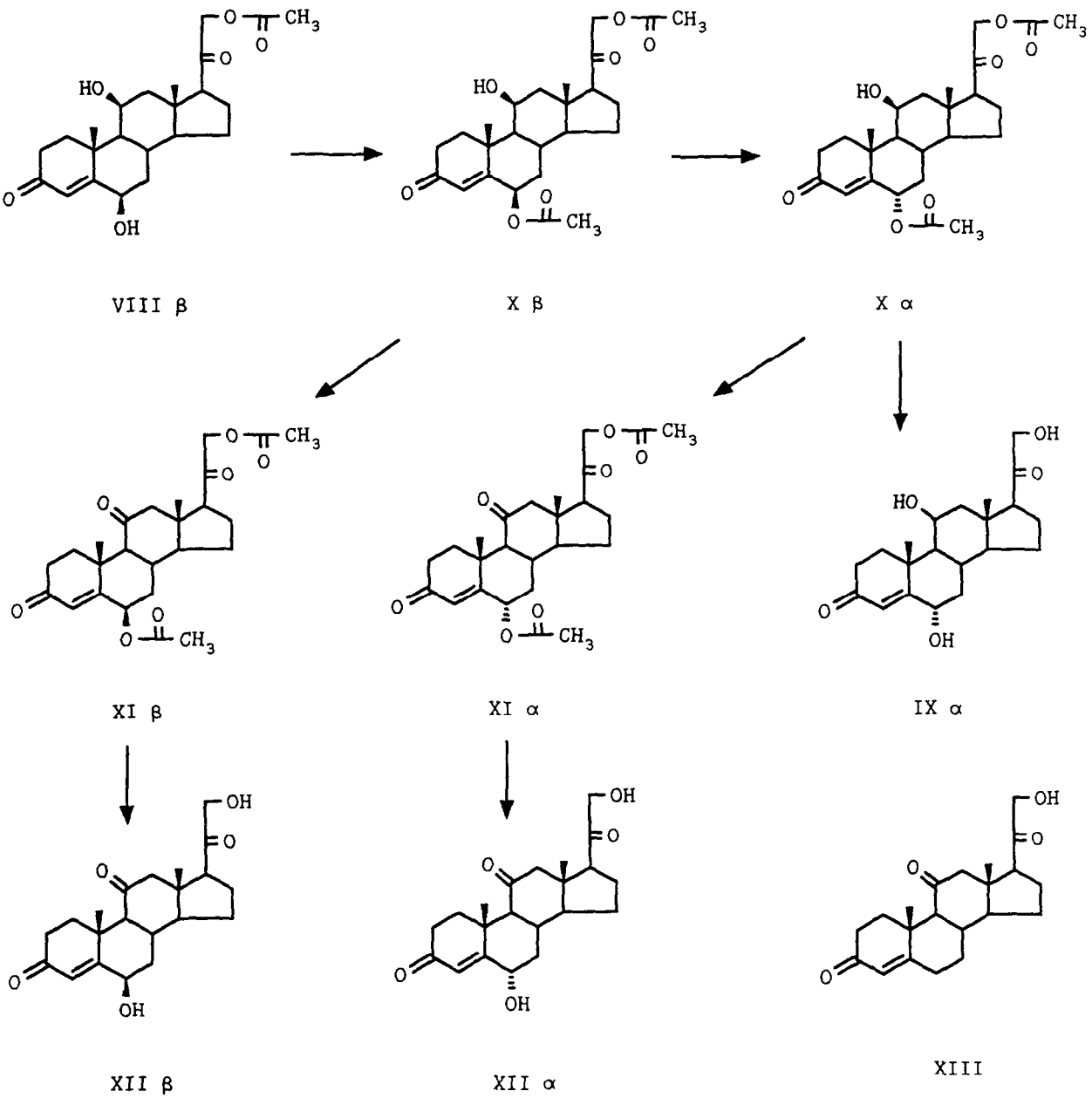

Figure 3 Synthetic pathway from 21-acetoxy-6β,11β-dihydroxypregn-4-ene,3,20-dione (VIIIβ) to 6α,11β,21-trihydroxypregn-4-ene-3,20-dione (IXα), and to 6α,21-dihydroxypregn-4-ene-3,11,20-trione (XIIα) and 6β,21-dihydroxypregn-4-ene-3,11,20-trione (XIIβ).

Step (2) compounds XIIα and XIIβ. XIα and XIβ were hydrolyzed as described for Xα and VIIIβ and the resulting steroids XIIα and XIIβ were purified by the system CLC-B_4 at a flow-rate of 6 ml/minute. From 0.90 mmol of XIα 0.50 mmol (67%) of 6αOH-A (XIIα) was obtained and hydrolysis of 170 mg crude (0.38 mmol) of XIβ yielded 0.10 mmol (32%) of 6βOH-B (XIIβ) and 0.11 mmol of XIIα (36%). Aliquots of XIIα and XIIβ were further purified by HPLC system 1. The exact masses of XIIα and XIIβ ($C_{21}H_{28}O_5$) were found to be 360.192 (calculated 360.194).

XIIα: IR: 3400 (OH), 2950, 1710 (20- and 11-oxo), 1670 (3-oxo) 1630 (ene) cm^{-1}. UV: λ_{max} 238 and λ_{hb} 15.8 nm. XIIβ: IR: 3350 (OH), 2950, 1660 (oxo and ene) cm^{-1}. UV: λ_{max} 232 and λ_{hb} 21.2 nm. Other characteristic data of XIIα and XIIβ which identify the steroids as 6αOH-A and 6βOH-A, respectively, are shown in Tables 1 (NMR), 2 (HPLC and GC), and 3 (GC/MS).

Results and discussion

We have successfully synthesized and identified the 6α- and 6β-hydroxylated derivatives of the compounds 11-deoxycortisol, corticosterone, and 11-dehydrocorticosterone. The structures of these six hydroxylated steroids were assigned on the basis of analyses made by MS, IR, NMR, *UV*, HPLC, GC, and GC/MS.

MS

As shown above the steroid products have the right molecular mass, pointing to the presence of an extra oxygen. This also follows from the mass spectra of the

MO-TMS ethers of the 6-hydroxy steroids IV, IX and XII. Table 3 shows that the m/z of the parent ion of IV and IX is 636, equal to that of cortisol, and that of XII is 562, equal to that of cortisone.

IR

From the IR spectral data it was observed (not shown) that the absorbance maxima of the C-OH bands at ~3400 cm^{-1} were smaller than those of the C-H bands at ~2950 cm^{-1} for each of the steroids S (I), B (V), and A (XIII), but for each of the 6-hydroxylated derivatives of S (IV), B (IX), and A (XII), the absorbance maxima of the C-OH bands were larger than (for XIIβ equal to) those of the C-H bands, especially for the 6α-hydroxy steroids. This means that the number of hydroxyl groups is increased.

Hydroxylation

The oxidation of the 3-methoxy-pregna-3,5-dienes II and VII in this study led to their hydroxylation at C-6: III and VIII, as reported in the literature.[9,12,15,16] In all these studies the yield of the 6β-hydroxylated steroids was larger than that of the 6α-hydroxylated epimers. Therefore it is concluded that in this study the 6β-hydroxylated steroids are the main compounds found after oxidation.

NMR

The direct proof of the stereochemistry at C-6 is derived from ^{1}H NMR measurements as shown in Table 1.

On the one hand the ^{1}H NMR spectral data show an increase of the chemical shift of the C-19 protons from δ 1.18 in compound S (I) to δ 1.34 in IVβ, from δ 1.42 in compound B (V) to δ 1.57 in IXβ, and from δ 1.35 in compound A (XIII) to 1.58 in XIIβ. This increase of δ is due to the axial position of the 6-hydroxy group in IVβ, IXβ, and in XIIβ, which thus are 6βOH-S, 6βOH-B, and 6βOH-A, respectively. A similar downfield shift of the C-19 protons from δ 1.33 in aldosterone to δ 1.47 in 6β-hydroxy-aldosterone has been reported previously.[19]

On the other hand the increase of the chemical shift of the C-4 proton from δ 5.68 in compound I to 6.02 in IVα, from δ 5.63 in compound V to 6.06 in IXα, and from δ 5.64 in compound XIII to 6.16 in XIIα, is due to the equatorial position of the 6-hydroxyl group in IVα, IXα, and in XIIα, which thus are 6αOH-S, 6αOH-B, and 6αOH-A, respectively. From these 2 findings it can be stated that (1) the chemical shift of the C_4-H signal in the 6α-hydroxylated steroids is higher than that in the 6β-hydroxylated isomers and the original compounds, as has also been observed for the 17,21-acetonides of 6α-hydroxy- and 6β-hydroxy-cortisol, and of 6α-hydroxy- and 6β-hydroxy-cortisone (unpublished results) and for 6α-hydroxy-aldosterone;[19] and that (2) the chemical shift of the C-19-H_3 signal of 6β-hydroxylated steroids is higher than that in the 6α-hydroxylated isomers and the original compounds. This latter conclusion is also in accordance with analogous observations for the A-ring reduced derivatives of 6β- and 6α-hydroxycortisol, and of 6β- and 6α-hydroxycortisone.[16]

HPLC

The 6α and 6β isomers of the synthesized steroids IV, IX, and XII show a different retention by normal phase chromatography. As can be observed from Table 2 (HPLC/NP) the 6α-hydroxylated compounds IVα, IXα, and XIIα are more polar than their respective 6β-hydroxylated epimers IVβ, IXβ, and XIIβ. This is in accordance with analog observations in the literature.[9,12,15,16] The capacity factors of 6βOH-B (IXβ) k' = 4.8 and of 6αOH-B (IXα) k' = 5.0 did not differ sufficiently to finally separate and purify these 2 compounds. This was achieved by using reversed-phase chromatography (HPLC/RP). With respect to the data obtained using the normal phase column, the retention of the 3 pairs of 6α and 6β epimers by the reversed-phase column was reversed only in case of the 6-hydroxy derivatives of corticosterone. For the other 4 steroids the 6α-hydroxylated compounds eluted after the corresponding 6β-hydroxylated isomers in both HPLC systems. This latter observation was also made for the 6-hydroxylated derivatives of cortisol and cortisone (unpublished results).

GC

Table 2 also shows the methylene unit values (normalized retention times) of the MO-TMS ethers of the synthesized compounds IV, IX, and XII. As expected[20,21] 2 isomers 3-E and 3-Z were detected for both 6-hydroxylated compounds IVβ and IVα, and 4 isomers 3-E,20-E; 3-E,20-Z; 3-Z,20-E; and 3-Z,20-Z in case of the compounds IXβ, IXα, XIIβ, and XIIα. The following 2 facts confirm previously described observations.[21] 1) The MU value of the last eluting peak of each of the 6β-hydroxylated steroids is smaller than that of the first eluting peak of the corresponding 6α-hydroxylated isomer. 2) For these ethers of IV, IX, and XII it is also observed that the difference(s) between the MU values of the 2 or 4 peaks of each of the 6β-hydroxylated steroids is (are) smaller than the (matching) difference(s) for the corresponding 6α-hydroxylated isomers.

GC/MS

Table 3 shows that mass spectra of IVα and IVβ are the same and do not qualitatively differ from that of cortisol (11β,17,21-trihydroxypregn-4-ene-3,20-dione). Therefore the only way to discriminate between 6β-hydroxy-S (IVβ) and cortisol in a urinary steroid profile of an 11OHD patient, is to measure the MU pair values of the compound: the MU pair of 6β-hydroxy-S is 31.38/31.60 and that of cortisol is 32.61/32.69.

The mass spectra of IXα and IXβ do not differ significantly, but are strikingly different from those of IVα

and IVβ. Apart from the known mass fragment ions m/z 276, 246, 244, and 103 in case of a dihydroxyacetone side chain,[22,23] as in IV, and m/z 188, 175 (absent in IX), and 103 in case of a 21-hydroxy-20-oxo side chain,[21–23] as in IX, Table 3 also indicates that the relative intensities of the ions m/z 636 (M^+) and 621 $(M\text{-}15)^+$ are much higher in IX than in IV, but that the relative intensity of the ion m/z 605 $(M\text{-}31)^+$ is much lower in IX than IV. The high intensity of the fragment $(M\text{-}15)^+$ has also been noted in the mass spectrum of 6α,21-dihydroxypregn-4-ene-3,20-dione.[21]

The relatively high intensity of the fragment $(M\text{-}15)^+$ and low intensity of the fragment $(M\text{-}31)^+$ were also observed in the mass spectra of 6-hydroxy-A (XII), where m/z 188 was very low despite the 21-hydroxy-20-oxo side chain. We also noted a relatively high intensity of the fragment m/z 431 $(M\text{-}15)^+$ in the spectrum of 6β-hydroxyandrost-4-ene-3,11,17-trione (6βOH-Adr, see Table 3). Because the intensity of the fragment ion $(M\text{-}15)^+$ in the published mass spectrum of 6α,20β,21-trihydroxypregn-4-en-3-one was also larger than that of the molecular ion,[21] the relatively high intensity of the ion $(M\text{-}15)^+$ might be a common feature of 6-hydroxy-3-oxo-4-ene steroids with a 21-hydroxy-20-oxo side chain or 17-oxo group.

It is stated that the mass fragment ion $(M\text{-}47)^+$ characterizes the 6-hydroxy-3-oxo-4-ene structure of MO-TMS derivatives of 6-hydroxylated steroids, with the exception of 6β-hydroxycortisol and 6β-hydroxycortisone.[21] Table 3 shows that the $(M\text{-}47)^+$ fragment is almost absent in the spectra of 6-hydroxy-S (IVα and IVβ) and 6α-hydroxycortisone (unpublished spectrum), weakly present in the spectra of 6-hydroxy-B (IXα and IXβ), but clearly present in the mass spectra of 6α-hydroxy-A (XIIα), 6β-hydroxy-A (XIIβ), and 6β-hydroxyandrost-4-ene-3,11,17-trione. Thus we confirm the above mentioned statement[21] and conclude that the fragment ion $(M\text{-}47)^+$ is actually present in 6-hydroxy-3-oxo-4-ene steroids with the exception of 6-hydroxy-cortisol, 6-hydroxy-cortisone, and 6-hydroxy-corticosterone.

UV

It was observed that in accordance with the published data[9,10,24,25] the wavelengths of maximal absorbance (λ_{max}) of the synthesized 6β-hydroxylated steroids IVβ, IXβ, and XIIβ are about 5 nm lower than the λ_{max} of the corresponding epimers IVα, IXα, and XIIα, and than the λ_{max} of the corresponding 6-deoxy compounds, I, V, and XIII. The absorption band of each of the 6β-hydroxylated steroids was slightly broader than that of the corresponding 6α-hydroxylated compound. The ratios of the half bandwidths λ_{hb} (see Experimental) of IVβ and IVα was 1.15, that of IXβ and IXα 1.16, and that of XIIβ and XIIα 1.34.

Yield

The yield of the 6-hydroxylated steroids from the precursor 3-methoxy-3,5-dienes was dependent on the method of oxidation. The pathway via autoxidation by O_2 in light, finally gave more acetonide of 6β-hydroxy-S (IIIβ) than that via oxidation with mCPBA. The yields were 52% and 15%, respectively. For the 21-acetate of 6βOH-B (VIIIβ) the yields were 77% (O_2/hν) and < 10% (mCPBA). Regarding the directly obtained 6α-hydroxylated compounds IIIα and VIIIα the yields were almost the same with both methods. We obtained 5% IIIα via O_2/hν and 5% IIIα with mCPBA. For VIIIα the yields were < 6% (O_2/hν) and about 2.5% (mCPBA). In general we may conclude that the autoxidative pathway can be better controlled and leads to higher yields of the 6-hydroxylated steroids than that with mCPBA. The preferable route to a higher yield of 6α-hydroxylated compounds is the combination of autoxidation (O_2/hν) and conversion of the 6β- to the 6α-hydroxy epimers, as described previously.[12] However, if the reaction time of the mCPBA reaction is decreased to seconds instead of the 5 minutes (in this report), it could be possible to get a higher yield of the 6-hydroxylated compounds, although in contrast to the used reaction time (4 hours) for the synthesis of 6α- and 6β-hydroxyaldosterone.[19] Preliminary experiments with 3-methoxy-4-androsta-3,5-dien-17-one indicated that with increasing reaction time more products were obtained without intact A-ring and less of the wanted steroids (unpublished results).

Although in accordance with the literature[9,12,15,16] the yield of the directly obtained 6α-hydroxylated compounds is much lower than that of the 6β-hydroxylated isomers, it is likely to assume that each of the former compounds is thermodynamically more stable than the corresponding steroid of the latter group, where the axially positioned 6β group is sterically hindered by the C_{19}-methyl group. Xβ was epimerized to Xα by HCl in anhydrous DCM only if ethanol was present, because without ethanol Xα was not obtained. The epimerization possibly occurred via the intermediate 6β,21-diacetoxy-3-ethoxy pregna-3,5-dien-20-one, as suggested earlier.[17] From the latter report it is known that without ethanol 6β-acetoxy-5-hydroxy-5α-pregnane-3,20-dione was dehydrated to 6β-acetoxypregn-4-ene-3,20-dione instead of being epimerized to 6α-acetoxypregn-4-ene-3,20-dione in the presence of 0.7% EtOH.[17]

Melting points of the synthesized steroids have not been taken. However, from the data in Table 1 (NMR) and Table 2 (HPLC and GC) it is clear that the final steroid products were pure and that the 6α-hydroxylated compounds were completely separated from their 6β-hydroxylated isomers. After identification, these steroids were used for the synthesis of the corresponding A-ring-reduced metabolites, mentioned in the introduction. The synthesis of these compounds will be reported separately.

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Abbreviations

I = 17,21-dihydroxypregn-4-ene-3,20-dione (S);
II = 17,21-dihydroxy-3-methoxypregna-3,5-dien-20-one 17,21-acetonide;
IIIα = 6α,17,21-trihydroxypregn-4-ene-3,20-dione 17,21-acetonide;
IIIβ = 6β,17,21-trihydroxypregn-4-ene-3,20-dione 17,21-acetonide;
IVα = 6α,17,21-trihydroxypregn-4-ene-3,20-dione (6αOH-S);
IVβ = 6β,17,21-trihydroxypregn-4-ene-3,20-dione (6βOH-S);
V = 11β,21-dihydroxypregn-4-ene-3,20-dione (B);
VI = 21-acetoxy-11β-hydroxypregn-4-ene-3,20-dione;
VII = 21-acetoxy-11β-hydroxy-3-methoxypregna-3,5-dien-20-one;
VIIIα = 21-acetoxy-6α,11β-dihydroxypregn-4-ene-3,20-dione;
VIIIβ = 21-acetoxy-6β,11β-dihydroxypregn-4-ene-3,20-dione;
IXα = 6α,11β,21-trihydroxypregn-4-ene-3,20-dione (6αOH-B);
IXβ = 6β,11β,21-trihydroxypregn-4-ene-3,20-dione (6βOH-B);
Xα = 6α,21-diacetoxy-11β-hydroxypregn-4-ene-3,20-dione;
Xβ = 6β,21-diacetoxy-11β-hydroxypregn-4-ene-3,20-dione;
XIα = 6α,21-diacetoxypregn-4-ene-3,11,20-trione;
XIβ = 6β,21-diacetoxypregn-4-ene-3,11,20-trione;
XIIα = 6α,21-dihydroxypregn-4-ene-3,11,20-trione (6αOH-A);
XIIβ = 6β,21-dihydroxypregn-4-ene-3,11,20-trione (6βOH-A);
XIII = 21-hydroxypregn-4-ene-3,11,20-trione (A).
11OHD, 17OHD = congenital adrenal hyperplasia due to 11β- or 17α-hydroxylase deficiency;

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