POLAR CORTICOSTEROIDS IN HUMAN NEONATAL URINE; SYNTHESIS AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF RING A REDUCED 6-HYDROXYLATED CORTICOSTEROIDS.

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This report describes the synthesis of 3α,6β,11β,17α,21-pentahydroxy-5β-pregnane-20-one, 3α,6β,11β,17α,21-pentahydroxy-5α-pregnane-20-one, 3α,6α,11β,17α,21-pentahydroxy-5β-pregnane-20-one, 3α,6α,11β,17α,21-pentahydroxy-5α-pregnane-20-one, 3α,6β,17α,21-tetrahydroxy-5β-pregnane-11,20-dione, 3α,6β,17α,21-tetrahydroxy-5α-pregnane-11,20-dione, 3α,6α,17α,21-tetrahydroxy-5β-pregnane-11,20-dione and 3α,6α,17α,21-tetrahydroxy-5α-pregnane-11,20-dione.

The gas chromatographic-mass spectrometric properties of these compounds are given. Proof of structure was accomplished using gas chromatography-mass spectrometry, microchemical reactions, optical rotatory dispersion and nuclear magnetic resonance spectroscopy.

Introduction.

In 1960 Ulstrom et al. [1] reported the presence of an extremely polar saturated metabolite of cortisol in the urine of newborn infants. Daniilescu-Goldinberg et al. [2,3] agreed with the postulation of Ulstrom that this compound might be 6β-hydroxy-tetrahydrocortisol (3α,6β,11β,17α,21-pentahydroxy-5β-pregnane-20-one). The same authors found in neonatal urine evidence for the presence of an even more polar cortisol metabolite [17] of unknown structure.

As part of our attempts to identify the polar neonatal cortisol metabolites positively, we have undertaken the synthesis of ring A reduced, 6-hydroxylated derivatives of cortisol.

This report describes the synthesis, proof of structure, and the gas chromatographic-mass spectrometric properties of the compounds mentioned above. As the main goal of this work was to obtain qualitative data on these compounds, the quantitative aspects of these syntheses were not considered, except in one case.

To our knowledge none of these compounds have been synthesized or fully characterized before.

Materials and Methods.

All solvents (analytical grade), reagents, ready made thin layer chromatography plates and silica gel chromatography columns (Merck Fertigsäule A and B) were purchased from Merck, Darmstadt, Germany, and 10% palladium on charcoal from Koch Light, Colnbrook, Bucks., England. 3α-hydroxysteroid-oxidoreductase was obtained from Nyegaard & Co., Oslo, Norway, and other biochemicals from Boehringer, Mannheim, Germany. Reference steroids were from Steraloids, Pawling, N.J., U.S.A., and stationary phases for gas chromatography from Chrompack, Middelburg, The Netherlands.

Thin layer chromatography (TLC). System I: 0.25 mm silica gel plates developed in chloroform-methanol-water 865-125-10 (v/v/v). System II: 0.25 mm kieselguhr plates impregnated with 10% ethylene glycol in acetone and developed in ethyl acetate saturated with ethylene glycol.

High Performance Liquid Chromatography (HPLC). Instrumentation: A Waters Associates pump model 6000, a model U6K injector and a model 440 absorbance detector operated at 254 or 280 nm (254 nm was used only for the detection of Δ-4,3-ketones). Column: A Waters Associates μ-Porasil 30x0.4 cm silica gel column. Flow rate 2 ml/min. Systems: I: 3.5% methanol and 0.35% water in chloroform; system II: 7.5% methanol and 0.7% water in chloroform.

Preparative Column Chromatography (PCC). Columns: Type A, 20x1 cm silica gel (Fertigsäule A) operated at a flow rate of 100 ml/hr. The eluate was collected in fractions of 10 ml.
Type B, 30x2.5 cm silica gel (Fertigsäule B) operated at a flow rate of 250 ml/hr; fraction size 25 ml.
Systems: I: 2% methanol in chloroform; system II: 5% methanol in chloroform.

Gas Chromatography (GC). Instrument: A Packard-Becker model 409 gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen. The following columns and operating conditions were used:
A: 2m x 2mm (i.d.) glass column packed with 3% OV-1 on Chromosorb W-HP, 80-100 mesh; temperature program from 210-270°C at 1°C/min; flow rate 18 ml/min.
B: 2m x 2mm (i.d.) glass column packed with 3% Silar 10C on Gas-chrom Q, 100-120 mesh; isothermal at 220°C; flow rate 10 ml/min.

Derivatives for gas chromatography. O-methoxime-trimethylsilyl ethers (MO-TMS ethers) were prepared as described previously [4]. Under the conditions used all hydroxy groups were silylated.

Retention data. These data are given as relative retention times (t_R) or as M.U. (methylene units) values. M.U. values were

calculated by linear intrapolation of the retention time of a compound between the retention times of the two bracketing even numbered n-alkanes. All retention times were determined on the Packard-Becker gas chromatograph.

Retention data for HPLC are given as uncorrected retention times.

Gas Chromatography-Mass Spectrometry (GC-MS). The instrument used was a Varian Aerograph 1400 gas chromatograph coupled to a Varian MAT-112 double focussing mass spectrometer by a variable slit separator. Gas chromatographic columns of 1.2 mm (i.d.) were used. These columns were otherwise identical to the columns mentioned above. Carrier gas, helium; flow rate 6 ml/min. Separator- and ion source temperature, 260°C; ionization energy, 70 eV; scan speed, 100 a.m.u./sec.

Optical Rotatory Dispersion and Nuclear Magnetic Resonance spectra. ORD spectra were recorded on a Varian Cary 90 instrument using methanol as the solvent. The NMR spectra were recorded on a Varian XL-100 at 100 megacycles. Tetradeutero-methanol was used as the solvent.

Experimental.

The synthetic pathways and the numbers assigned to the different steroid structures are presented in Fig. 1.

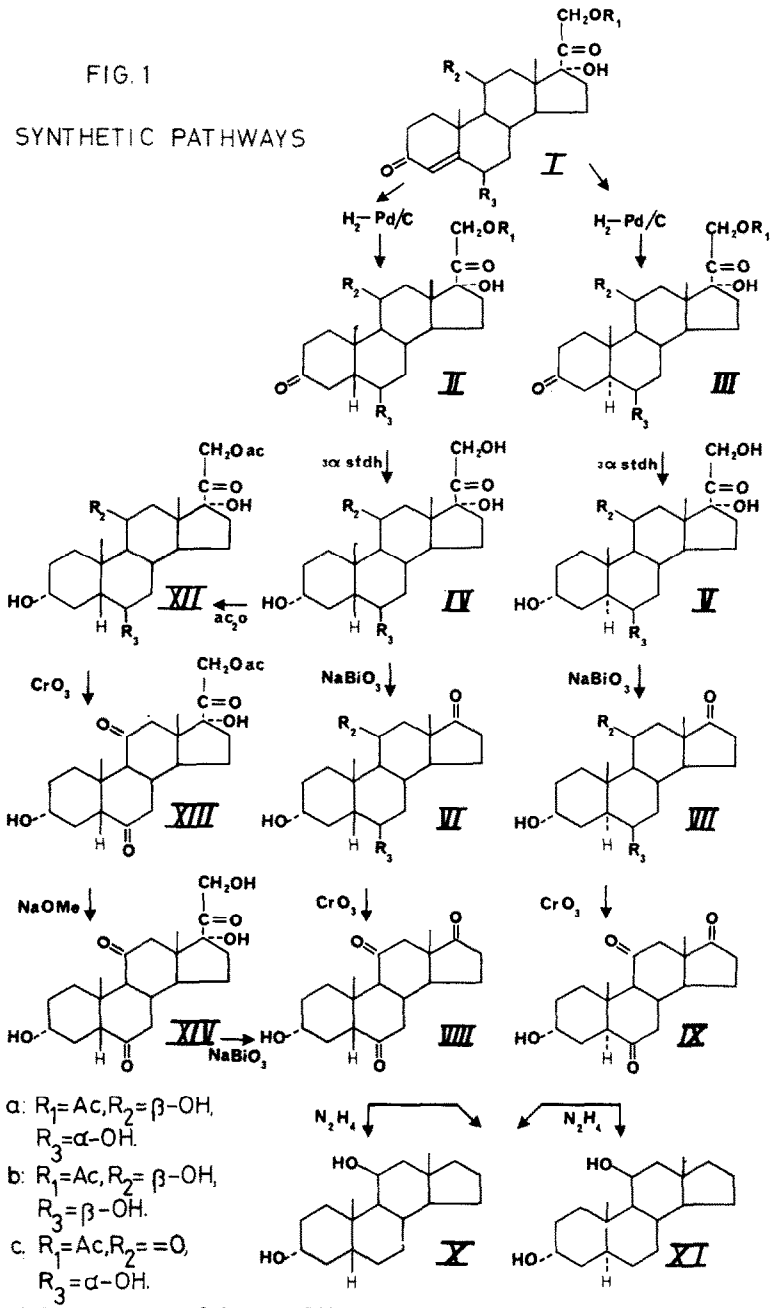
1. The preparation of the 21-acetates of 6α-hydroxy-cortisol, 6β-hydroxy-cortisol, 6α-hydroxy-cortisone and 6β-hydroxy-cortisone (Ia, Ib, Ic and Id).

Ia, Ib, Ic and Id were prepared according to the method of Gardi and Lusignani [5] from cortisol- and cortisone-21-acetates, respectively, via the 3-ethyl-dienol ethers [6]. In this preparation cortisol-21-acetate yielded Ib as the main product accompanied by about 25% Ia, as was estimated from TLC analysis (system I, detection by UV absorption at 254 nm). The yields from cortisone-21-acetate were 85% Id and 15% Ic. Ia and Ib as well as Ic and Id could be separated preparatively by PCC, column type B, system I.

To confirm the identity of the products Ia, Ib, Ic and Id, approximately 100 µg of each of these compounds were deacetylated by adding 0.5 ml of an 0.5% solution of sodium methoxide in methanol (w/v) to the dry sample and leaving the solution for 30 min at room temperature. The solutions were neutralized with acetic acid, evaporated under a stream of nitrogen and the dry steroids were converted into MO-TMS ethers. GC-MS analysis (column A) showed that Ib_1, Ic_1 and Id_1 were identical to authentic reference compounds (Steraloids), all compounds exhibiting two gas chromatographic peaks (syn- and anti isomers of the 3-methoxime function) with M.U. values 32.84/33.12, 32.58/33.00 and 32.45/32.72,

FIG. 1

SYNTHETIC PATHWAYS



a: R_1=Ac, R_2=β-OH, R_3=α-OH.

b: R_1=Ac, R_2=β-OH, R_3=β-OH.

c. R_1=Ac, R_2==O, R_3=α-OH.

d: R_1=Ac, R_2==O, R_3=β-OH.

a_1, b_1, c_1, d_1,: R_1=H, otherwise as a, b, c, and d respectively.

respectively. No authentic 6α-hydroxy-cortisol was available to check the identity of Ia_1 (M.U. values 33.24/33.63), but the mass spectrum of its MO-TMS derivative was virtually identical to that of Ib_1 MO-TMS ether. Further proof for the identity of Ia_1 was obtained by oxidizing 100 µg of this compound with a mixture of 0.35 ml 2 % chromium trioxide solution in water (w/v) and 0.5 ml of acetic acid at room temperature for 60 min. The reaction mixture was diluted with 5 ml of water and extracted with 2 x 10 ml of ethyl acetate. After evaporation of the combined extracts the product obtained was identical by HPLC (system I) to the products obtained from Ib, Ic and Id by the same procedure (21-acetoxy-17α-hydroxy-pregn-4-ene-3,6,11,20-tetrone, retention time 110 sec, detection at 254 nm).

2. The synthesis of 6β-hydroxy-tetrahydrocortisol (IVb) and 6β-hydroxy-allotetrahydrocortisol (Vb).

100 mg of 6β-hydroxy-cortisol-21-acetate (Ib) were dissolved in 10 ml of dioxane (freshly distilled from sodium wire) and 200 mg of 10% palladium on charcoal (w/w) were added. Hydrogenation was carried out at room temperature by passing a gentle stream of hydrogen through the solution, until TLC (system I, detection at 254 nm) revealed that Ib was no longer present in the reaction mixture. Analysis of the reaction mixture by TLC system II (detection with blue tetrazolium [7]) revealed two spots (R_F 0.36 and R_F 0.40) slightly less polar than Ib (R_F 0.35) and one strong apolar spot (R_F 0.72), presumably a hydrogenolysis product.

The reaction mixture was filtered over a Celite pad, evaporated in vacuo and redissolved in 2 ml of methanol-chloroform 2 : 98 (v/v). This solution was applied to a PCC column type A and eluted with the same solvent (system I). The eluate was monitored by analyzing aliquots of each fraction of 10 ml by TLC (system I, detection with blue tetrazolium). The products slightly less polar than Ib were collected in fractions 19-21 and 27-34, respectively. Fractions 22-26 contained a mixture of both compounds and these fractions were pooled and rechromatographed to obtain additional amounts of the products.

Both compounds were deacetylated and purified by HPLC (system II). Approximately 100 µg of the purified C-21 alcohols were converted into their MO-TMS derivatives. GC-MS analysis (column A) showed for each compound two gas chromatographic peaks. The apolar compound had M.U. values 33.11/33.47 and the more polar one 32.10/32.31. Both compounds exhibited very similar mass spectra consistent with the structure 6β,11β,17α,21-tetrahydroxy-5α/β-pregnane-3,20-dione MO-TMS ether (see tables 1 and 2, compounds IIb_1 and $IIIb_1$).

The optical rotatory dispersion curves of both C-21 alcohols showed strong positive Cotton effects due to the C-3 and C-20 carbonyl groups. In table 3, the molecular amplitudes calculated from these spectra are compared to those recorded from authentic 11β,17α,21-trihydroxy-5α-pregnane-3,20-dione and 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione (Steraloids).

Table 1. Gas chromatographic data of the different steroids

Steroid number[a]	Compounds[b] hydroxy groups	ketones	Trivial name	Retention data OV-1	Silar 10C
IIb_1	6β,11β,17α,21	-5β-P-3,20		32.10 32.31	
$IIIb_1$	6β,11β,17α,21	-5α-P-3,20		33.11 33.47	
IId_1	6β,17α,21	-5β-P-3,11,20		31.67 31.81	
$IIId_1$	6β,17α,21	-5α-P-3,11,20		32.89 33.33	
IVb	3α,6β,11β,17α,21	-5β-P-20	6β-OH-THF	30.96	
Vb	3α,6β,11β,17α,21	-5α-P-20	6β-OH-ATHF	31.04	
IVa	3α,6α,11β,17α,21	-5β-P-20	6α-OH-THF	31.12	
Va	3α,6α,11β,17α,21	-5α-P-20	6α-OH-ATHF	31.39	
IVd	3α,6β,17α,21	-5β-P-11,20	6β-OH-THE	30.80	
Vd	3α,6β,17α,21	-5α-P-11,20	6β-OH-ATHE	31.42	
IVc	3α,6α,17α,21	-5β-P-11,20	6α-OH-THE	30.55	
Vc	3α,6α,17α,21	-5α-P-11,20	6α-OH-ATHE	31.30	
XIV	3α,17α,21	-5β-P-6,11,20		30.99	
VIII	3α	-5β-A-6,11,17		1.36[c]	2.14[c] 2.21
IX	3α	-5α-A-6,11,17		1.40[c]	1.72[c]
X	3α,11β	-5β-A		24.91	
XI	3α,11β	-5α-A		24.68	

a, See Fig. 1; b, Steroids of the pregnane series are abbreviated to P, and steroids of the androstane series to A. c, Retention data are given as M.U. values, except the numbers marked with "c", which are retention times relative to 3α-hydroxy-5α-androstane-11,17-dione MO-TMS ether.

On the basis of these data, the less polar epimer was identified as 6β,11β,17α,21-tetrahydroxy-5α-pregnane-3,20-dione ($IIIb_1$) and the more polar epimer as 6β,11β,17α,21-tetrahydroxy-5β-pregnane-3,20-dione (IIb_1). These assignments are in perfect agreement with theoretical predictions based on the Octant Rule [8, 9].

10 mg of IIb_1 as well as of $IIIb_1$ were converted into the 3α-hydroxy compounds by incubation with 1.75 U of 3α-hydroxysteroid-oxido-

Table 2. Mass spectrometric data of intermediate compounds and compounds used in the confirmation of structures.

Steroid number[a]	Characteristic ions and their relative intensities[b]													
	M^+	fragment ions												
	726	695	605	515	425	276	246	244	202	172	168	143	114	103
IIb_1	*5*	*54*	*31*	*28*	*22*	*37*	*63*	*40*	*39*	*45*	*52*	*55*	*82*	*100*
	3	*35*	*23*	*28*	*25*	*31*	*60*	*40*	*39*	*44*	*52*	*57*	*75*	*100*
$IIIb_1$	*6*	*83*	*41*	*30*	*23*	*38*	*79*	*44*	*34*	*48*	*48*	*34*	*98*	*100*
	3	*46*	*26*	*20*	*16*	*27*	*70*	*36*	*31*	*42*	*47*	*30*	*79*	*100*
	652	621	531	441	288	280	276	258	246	198	168	143	114	103
IId_1	*14*	*75*	*57*	*41*	*13*	*15*	*14*	*27*	*27*	*51*	*84*	*51*	*51*	*100*
	7	*41*	*38*	*32*	*12*	*12*	*14*	*24*	*25*	*46*	*74*	*49*	*72*	*100*
$IIId_1$	*12*	*69*	*65*	*34*	*13*	*19*	*11*	*22*	*27*	*50*	*68*	*50*	*51*	*100*
	15	*85*	*73*	*33*	*14*	*12*	*12*	*25*	*24*	*49*	*76*	*48*	*46*	*100*
	652	621	531	441	288	276	258	246	198	168	143	129	114	103
XIV	*15*	*93*	*45*	*25*	*18*	*20*	*23*	*25*	*60*	*80*	*40*	*48*	*58*	*100*
	448	433	417	358	343	327	297							
VIII	*94*	*14*	*39*	*47*	*100*	*79*	*45*							
IX	*39*	*5*	*5*	*15*	*100*	*27*	*11*							
	436	421	346	339	331	320	256	241	197	184	169	156	107	
X	*4*	*4*	*31*	*.5*	*9*	*3*	*100*	*60*	*27*	*27*	*20*	*28*	*19*	
XI	*18*	*6*	*54*	*24*	*40*	*20*	*100*	*98*	*88*	*100*	*64*	*100*	*67*	

a, See table 1 and Fig. 1; b, Only characteristic ions over m/e 100 are shown. The relative intensities (in italics) of the ions are given as percentage of the most abundant ion.

Table 3. Optical Rotatory Dispersion data.

Compound	Molecular Amplitude
11β,17α,21-trihydroxy-5α-pregnane-3,20-dione	+182
11β,17α,21-trihydroxy-5β-pregnane-3,20-dione	+130
6β,11β,17α,21-tetrahydroxy-5α-pregnane-3,20-dione (less polar epimer)	+180
6β,11β,17α,21-tetrahydroxy-5β-pregnane-3,20-dione (more polar epimer)	+101

reductase (3α-STDH, E.C. 1.1.1.50) and 50 mg of NADH in 20 ml of 0.1 M acetate buffer of pH 6.0 according to the equation:

$$\text{3-keto-steroid} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{3}\alpha\text{-hydroxy-steroid} + \text{NAD}^+$$

Only if NAD^+ was removed from the reaction mixture, could acceptable amounts of the 3α-hydroxy-steroid be obtained (50-70% conversion). This was achieved by adding a solution of 85 mg of sodium L-malate in 5 ml of water adjusted at pH 6.0 and 1100 U of malate-dehydrogenase (MDH, E.C. 1.1.1.37) to the reaction mixture.

After 24 hours of incubation at 37°C the reaction mixtures were extracted three times with five volumes of ethyl acetate, the extracts were combined, dried over anhydrous sodium sulfate and taken to dryness in vacuo. TLC (system I, detection with blue tetrazolium) showed that the starting compounds were for 50-70% converted into the products IVb and Vb. The crude products were purified by PCC (column type A, system II). The yield was 3.6 mg IVb (m.p. 218-220°C) and 2.9 mg Vb (m.p. 206-208°C).

100 µg of both compounds were converted into the MO-TMS derivatives and analyzed by GC-MS (column A). The mass spectra (fig. 2 and 3) were consistent with the structure 3α,6β,11β,17α,21-pentahydroxy-5α/β-pregnane-20-one MO-TMS ether. These mass spectra show the molecular ion (m/e 771), the M-31 ion (m/e 740) caused by the loss of the methoxy group from the 20-methoxime function and several ions originating from the consecutive loss of trimethylsiloxyl groups (m/e 650, 560, 470 and 380). The ions at m/e 244, 246 and 276 are typical for the MO-TMS ethers of tetrahydro-corticosteroids bearing a dihydroxy-acetone side chain.

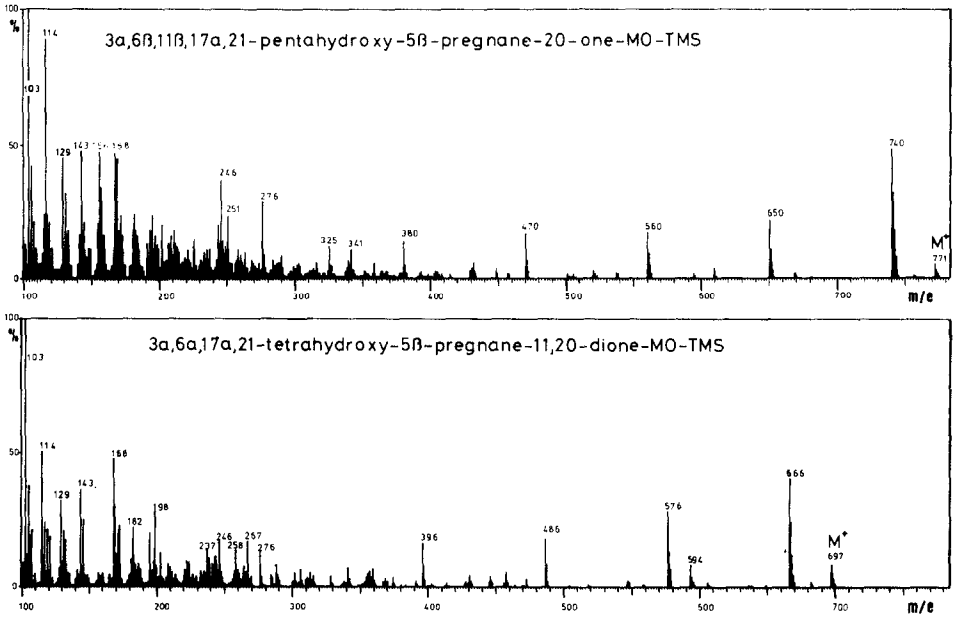


Fig. 2. Mass spectra of 6β-hydroxy-tetrahydrocortisol and 6α-hydroxy-tetrahydrocortisone MO-TMS ethers.

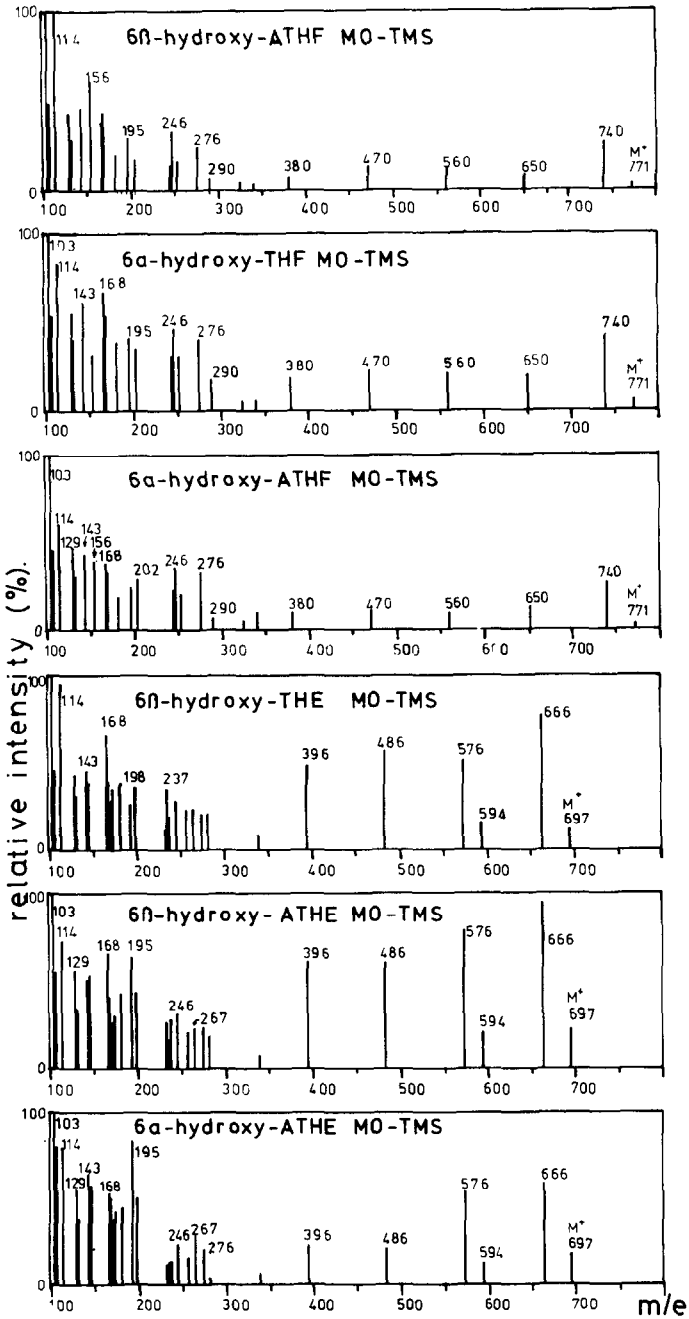


Fig. 3.

Mass spectra of the synthesized steroid MO-TMS ethers. Only the most abundant ions are shown. Isotope peaks are deleted.

The configuration of the 3α-hydroxy group was confirmed by the conversion of 200 µg of both IVb and Vb into their 17-keto analoques by sodium bismuthate oxidation [10]. These 17-keto analoques (VIb and VIIb) were subjected to chromium trioxide oxidation for 20 min as described under 1 to yield VIII and IX, respectively. The 6- and 17 carbonyl groups were subsequently removed by Wolff-Kishner reduction according to the method of Vandenheuvel [11]. Both VIII and IX yielded a 50/50 mixture of X and XI as was shown by GC-MS. The latter compounds were also obtained by Wolff-Kishner reduction of authentic 3α-hydroxy-5α-androstane-11,17-dione and 3α-hydroxy-5β-androstane-11,17-dione. The GC-MS data of compounds VIII-XI are presented in tables 1 and 2.

In the reactions described above the 6β-hydroxy group is almost as easily oxidized as the 11β-hydroxy group. This is in agreement with the results of Schreiber and Eschenmoser [12]. The isomerization of the A-B ring junction at C-5 is a known "risk" of the Wolff-Kishner reduction [13] as well as the side reaction leading to an 11β-hydroxy group [11]. Compounds VIII and IX were analyzed on both an OV-1 column and a Silar 10C column. An interesting phenomenon was that VIII in contrast to IX gave rise to two gas chromatographic peaks on Silar 10C.

3. The synthesis of 6β-hydroxy-tetrahydrocortisone (IVd) and 6β-hydroxy-allotetrahydrocortisone (Vd).

IVd and Vd were prepared from 6β-hydroxy-cortisone-21-acetate (Id) in exactly the same way as IVb and Vb were prepared from Ib. Hydrogenation of Id yielded IId and IIId in a ratio of approximately 3 : 1, easily separable by PCC (column type A, system I).

The conversion factor in the enzymatic 3α-hydroxylation was again 50-70% being somewhat better for IVd than for Vd. The melting points were 199-200°C and 180-183°, respectively. The mass spectra of the MO-TMS ethers of IVd and Vd (fig. 3) show the molecular ion (m/e 697), the M-31 ion (m/e 666) and the ions caused by the loss of the trimethylsiloxyl groups (m/e 576, 486 and 396). The ion at m/e 306 (M-31-4x90) is weak but visible. The ion at m/e 594 is due to the loss of the C-21 CH_2OTMS function. In the lower part of the mass spectra many peaks also observed in the mass spectra of the MO-TMS ethers of IVb and Vb are present. Proof of structure was accomplished by converting IVd and Vd into their 17-keto analoques (VId and VIId, respectively) and oxidizing these compounds with chromium trioxide for 20 min to give VIII and IX, respectively, identical by GC-MS of their derivatives to the compounds obtained from IVb and Vb previously.

4. The synthesis of 6α-hydroxy-tetrahydrocortisol (IVa) and 6α-hydroxy-allotetrahydrocortisol (Va).

IVa and Va were prepared from 6α-hydroxy-cortisol-21-acetate (Ia) as outlined for IVb and Vb from Ib. It was, however, not

possible to separate the intermediates IIa and IIIa preparatively. Therefore, the mixture of these epimers was subjected to deacetylation and 3α-hydroxylation. The products (IVa and Va) were separated by HPLC system II. The retention times were 818 and 721 sec, respectively. The concentration of IVa in the mixture was about three times as high as the concentration of Va. The melting points were 152-154°C and 231-232°C, respectively. The GC-MS data of the MO-TMS ethers of IVa and Va are presented in table 1 and fig. 3.

To confirm the structure of IVa, 200 µg of this compound were acetylated under mild conditions [10] to give the C-21 monoacetate. This acetate (XIIa) was purified by HPLC (system II, retention time 341 sec) and subjected to chromium trioxide oxidation for 30 min. The oxidation product (XIII) was purified by HPLC (system II, retention time 78 sec), deacetylated, and a part of it was converted into the MO-TMS ether. This compound (XIV MO-TMS) was identical by GC-MS to the product obtained by the same procedure from IVb (tables 1 and 2). The remainder of XIV was converted into its 17-keto analoque by sodium bismuthate degradation and the product was identical to VIII (tables 1 and 2).

The HPLC purification of XIII showed also that a considerable amount of the 21-acetate of IVc (see below) was present in the reaction mixture. This undoubtedly is due to the lower oxidation rate of a 6α-hydroxy group as compared to 6β- and 11β-hydroxy groups [12].

5. The synthesis of 6α-hydroxy-tetrahydrocortisone (IVc) and 6α-hydroxy-allotetrahydrocortisone (Vc).

The same synthetic procedure as described above was applied to 6α-hydroxy-cortisone-21-acetate. The mixture of IIc and IIIc was subjected to deacetylation and enzymatic 3α-hydroxylation to yield IVc and Vc. These compounds were separated by HPLC (system II, retention times 579 and 492 sec, respectively). The melting points of the purified compounds were 144-146°C and 149-151°C, respectively. GC-MS data of the MO-TMS ethers of IVc and Vc are presented in table 1 and fig. 2 and 3.

The structure of IVc was confirmed by converting 200 µg of this compound into XIV in the same way as described above for the conversion of IVa into XIV. The final product was identical (by GC-MS) to the product obtained from IVa. Also the 17-keto analoque of this product was identical to VIII.

6. Nuclear Magnetic Resonance analysis.

The NMR spectra of the synthesized steroids were recorded using the less common CD_3OD as the solvent due to the low solubility of these compounds in $CDCl_3$. In table 4 the chemical shifts of the C-18 and C-19 methyl resonances are compared to values computed from Zürcher's tables [16] for the corresponding C-21 acetates. Especially the chemical shift of the C-19 methyl resonance is extremely useful in the determination of the stereochemistry at C-5 and C-6. The observed chemical shifts correlate very well with the calculated values. The observed chemical shifts of the

C-18 methyl resonance are consistently 0.08-0.09 ppm at higher field when compared to the calculated values. This undoubtedly is due to the fact that the calculated values refer to C-21 acetates and that these values are based on observations made in deutero-chloroform.

Table 4. NMR data of the synthesized compounds.

COMPOUND	OBSERVED VALUES (a)		CALCULATED VALUES (a,b)	
	C-18	C-19	C-18	C-19
6β-hydroxy-tetrahydrocortisone	0.58	1.30	0.67	1.33
6β-hydroxy-allotetrahydrocortisone	0.58	1.21	0.67	1.23
6α-hydroxy-tetrahydrocortisone	0.54	1.14	0.63	1.13
6α-hydroxy-allotetrahydrocortisone	0.54	1.03	0.63	1.01
6β-hydroxy-tetrahydrocortisol	0.86	1.33	0.94	1.37
6β-hydroxy-allotetrahydrocortisol	0.86	1.23	0.94	1.27
6α-hydroxy-tetrahydrocortisol	0.83	1.15	0.91	1.17
6α-hydroxy-allotetrahydrocortisol	0.83	1.04	0.91	1.05

a. All values in ppm downfield from tetramethylsilane (δ).
b. Calculated from the tables of Zürcher [16] for the corresponding C-21 acetates.

7. Analysis of the synthesized compounds.

As can be seen in fig. 2 and 3, the mass spectra of the different 6-hydroxylated pregnane-pentolone MO-TMS ethers are very similar. Only minor variations in relative ion intensities are observed. The same is true for the mass spectra of the MO-TMS ethers of the different 6-hydroxylated pregnane-tetrol-diones. It is questionable whether such minor differences could provide a reliable tool for discriminating between the different isomers of each group of compounds. On the basis of retention times in gas chromatography a discrimination between the different 6-hydroxylated pregnane-tetrol-dione MO-TMS ethers is very well possible. However, the small differences between the retention times of the different pregnane-pentolone MO-TMS ethers (except 6α-hydroxy-allotetrahydrocortisol MO-TMS ether) do not allow an adequate identification on the basis of retention times. In this case high resolution gas chromatography on open-tubular columns could provide the solution.

A different solution to this analytical problem is to use a combination of high performance liquid chromatography and gas chromatography-mass spectrometry. Fig. 4 shows that by HPLC a mixture of all eight synthesized 6-hydroxylated steroids can be separated into five fractions. Further separation of the MO-TMS ethers prepared from these fractions by gas chromatography-mass spectrometry enables the identification and quantitation of all eight steroids separately (cf. table 1).

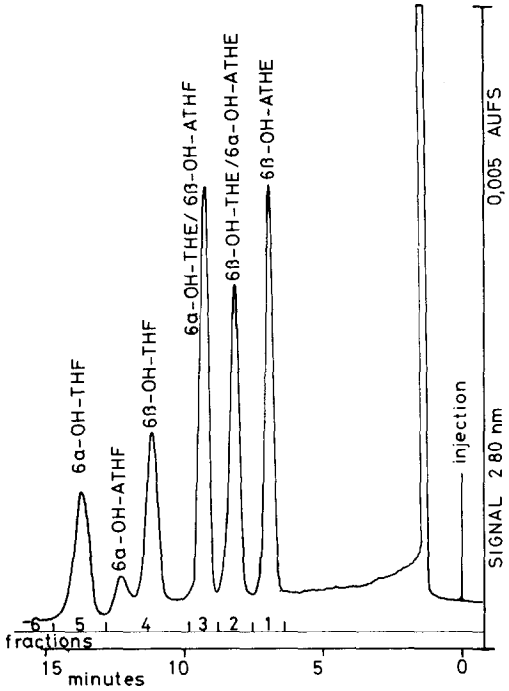


Fig. 4 HPLC separation of the synthesized steroids. Column: μ-Porasil 30x0.4 cm System: 7.5% methanol and 0.7% water in chloroform Flow rate: 2 ml/min

Discussion.

During the preparation of this paper Setchell et al. [14 and 15] reported the identification of 6β-hydroxy-tetrahydrocortisol in the urine of the baboon. Also several other saturated corticosteroids having six oxygen functions were found. However, these authors could not provide definite evidence for the stereochemistry of the 6β-hydroxy group, because reference steroids were not available. A comparison between the mass spectra in fig. 2 and 3 and the mass spectra reported by Setchell et al. shows that except for variations in relative ion intensities there is good agreement between these spectra.

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17. The following trivial names were used:
cortisol, 11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione
cortisone, 17α,21-dihydroxy-pregn-4-ene-3,11,20-trione
6α(β)-hydroxy-cortisol, 6α(β),11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione.
6α(β)-hydroxy-cortisone, 6α(β),17α,21-trihydroxy-pregn-4-ene-3,11,20-trione.
6α(β)-hydroxy-tetrahydrocortisone, 3α,6α(β),17α,21-tetrahydroxy-5β-pregnane-11,20-dione.
6α(β)-hydroxy-allotetrahydrocortisone, 3α,6α(β),17α,21-tetrahydroxy-5α-pregnane-11,20-dione.
6α(β)-hydroxy-tetrahydrocortisol, 3α,6α(β),11β,17α,21-pentahydroxy-5β-pregnane-20-one
6α(β)-hydroxy-allotetrahydrocortisol, 3α,6α(β),11β,17α,21-pentahydroxy-5α-pregnane-20-one.