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Synthesis and Identification of Twelve A-Ring Reduced 6α- and 6β-Hydroxylated Compounds Derived from 11-Deoxycortisol, Corticosterone and 11-Dehydrocorticosterone

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The synthesis and identification of 12 A-ring reduced 6α-(and 6β-)hydroxylated compounds derived from 11-deoxycortisol (S), corticosterone (B) and 11-dehydrocorticosterone (A) are reported here. These steroids were prepared in two steps from the corresponding 6 6α-(and 6β-)hydroxy-4-pregnene-3-ones. Selective reduction of the 4,5 double bond yielded 12 6α-(and 6β)hydroxy-5α-(and 5β)pregnane-3,20-diones. Enzymatic reduction of these compounds with NADH and 3α-hydroxysteroid dehydrogenase yielded the corresponding tetrahydro steroids. The steroids were characterized by high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC and GC/MS) and in part by ^{1}H-NMR. 6βOH-THS and 6βOH-5αTHS were identified by ^{1}H-NMR. The structures of the two precursors, i.e. 6βOH-5βDHS and 6βOH-5αDHS were confirmed by ^{1}H-NMR using two-dimensional spectra. 6αOH-THS was identified by comparing its HPLC, GC and MS data with those of the steroid obtained by enzymatic oxidation of the standard reference steroid 6αOH-20βHHS to the corresponding 20-ketosteroid. The other steroids, e.g. 6αOH-THB and 6αOH-5αTHB were identified by using the proved sequence of elution of each of the epimer pairs on the normal phase HPLC column (5α < 5β), and by the reversed order of elution of the same epimer pair as the methoxime-trimethylsilyl ethers on the GC column (5α > 5β) and by the mass spectra, with the exception of 6βOH-THA.

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Abbreviations: CAH, congenital adrenal hyperplasia; 11OHD, 11β-hydroxylase deficiency; 17OHD, 17α-hydroxylase deficiency; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; 6αOH-S, 6α,17,21-trihydroxy-4-pregnene-3,20-dione; 6βOH-S, 6β,17,21-trihydroxy-4-pregnene-3,20-dione; 6αOH-5αDHS, 6α,17,21-trihydroxy-5α-pregnane-3,20- dione; 6αOH-5βDHS, 6α,17,21-trihydroxy-5β-pregnane-3,20-dione; 6βOH-5αDHS, 6β,17,21-trihydroxy-5α-pregnane-3,20-dione; 6βOH-5βDHS, 6β,17,21-trihydroxy-5β-pregnane-3,20- dione; 6αOH-5αTHS, 3α,6α,17,21-tetrahydroxy-5α-pregnan-20-one; 6αOH-THS, 3α,6α,17,21-tetrahydroxy-5β-pregnan-20-one; 6βOH-5αTHS, 3α,6β,17,21-tetrahydroxy-5α-pregnan-20-one; 6βOH-THS, 3α,6β,17,21-tetrahydroxy-5β-pregnan-20-one; 6αOH-20αHHS, 3α,6α,17,20α,21-pentahydroxy-5β-pregnane; 6αOH-20βHHS, 3α,6α,17,20β,21-pentahydroxy-5β-pregnane; 6αOH-B, 6α,11β,21-trihydroxy-4-pregnene-3,20-dione; 6βOH-B, 6β,11β,21-trihydroxy-4-pregnene-3,20-dione; 6αOH-5αDHB, 6α,11β,21-trihydroxy-5α-pregnane-3,20-dione; 6αOH-5βDHB, 6α,11β,21-trihydroxy-5β-pregnane-3,20-dione; 6βOH-5αDHB, 6β,11β,21-trihydroxy-5α-pregnane-3,20-dione; 6βOH-5βDHB, 6β,11β,21-trihydroxy-5β-pregnane-3,20-dione; 6αOH-5αTHB, 3α,6α,11β,21-tetrahydroxy-5α-pregnan-20-one; 6αOH-THB, 3α,6α,11β,21-tetrahydroxy-5β-pregnan-20-one; 6βOH-5αTHB, 3α,6β,11β,21-tetrahydroxy-5α-pregnan-20-one; 6βOH-THB, 3α,6β,11β,21-tetrahydroxy-5β-pregnan-20-one; 6αOH-A, 6α,21-dihydroxy-4-pregnene-3,11,20-trione; 6βOH-A, 6β,21-dihydroxy-4-pregnene-3,11,20-trione; 6αOH-5αDHA, 6α,21-dihydroxy-5α-pregnane-3,11,20-trione; 6αOH-5βDHA, 6α,21- dihydroxy-5β-pregnane-3,11,20-trione; 6βOH-5αDHA, 6β,21-dihydroxy-5α-pregnane-3,11,20-trione; 6βOH-5βDHA, 6β,21-dihydroxy-5β-pregnane-3,11,20-trione; 6αOH-5αTHA, 3α,6α,21-trihydroxy-5α-pregnane-11,20-dione; 6αOH-THA, 3α,6α,21-trihydroxy-5β-pregnane-11,20-dione; 6βOH-5αTHA, 3α,6β,21-trihydroxy-5α-pregnane-11,20-dione; 6βOH-THA, 3α,6β,21-trihydroxy-5β-pregnane-11,20-dione.

INTRODUCTION

In the urinary steroid profiles of children and adults with congenital adrenal hyperplasia (CAH) due to 11β-hydroxylase (11OHD) or 17α-hydroxylase deficiency (17OHD) a small number of peaks occur, which were likely to be 6α-hydroxy metabolites of 3α,17,21-trihydroxy-5β(and 5α)-pregnan-20-one (THS and 5αTHS), or 3α,11β,21-trihydroxy-5β(and 5α)-pregnan-20-one (THB and 5αTHB), respectively.

To our knowledge these steroids were not sufficiently identified or characterized. In urine of an 11OHD human neonate the presence of 6αOH-THS has been demonstrated but this steroid was not fully described [1]. Also the steroid 6αOH-THB was only tentatively identified in the urines of 17OHD patients [2–4]. Finally, the urinary steroid profile of a 3-week-old baby with 18-oxygenation deficiency was dominated by a hydroxylated metabolite of 3α,21-dihydroxy-5β-pregnane-11,20-dione (THA). This metabolite was reported as 6ξ-hydroxytetrahydrocompound A (3α,6ξ,21-trihydroxy-5β-pregnane-11,20-dione) [5].

The mass spectrum of 6αOH-THS [6] resembles that of 3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one (THF). This means that the presence of the former compound in urine can be defined only by using the GC methylene unit (MU) value combined with the proper mass spectrum.

In order to identify the above mentioned steroids in the urines of 11- and 17OHD patients, it was decided to synthesize the commercially unavailable title compounds and to measure their physico-chemical parameters using high performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS) and, in part, nuclear magnetic resonance (^{1}H-NMR). For this purpose we started with the corresponding 6α-hydroxylated and 6β-hydroxylated 4-pregnen-3-oxosteroids, the synthesis and characterization of which has been described recently [7].

EXPERIMENTAL

Chemicals

6αOH-20βHHS and -20αHHS were obtained from the former M.C.R. steroid reference collection (London, England; Curator, the late Professor D. N. Kirk). 3α-Hydroxysteroid dehydrogenase (3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase, EC 1.1.1.50; *Pseudomonas testosteroni*), 20β-hydroxysteroid dehydrogenase [(20R)-17,20,21-trihydroxysteroid:NAD$^+$ oxidoreductase, EC 1.1.1.53; *Streptomyces hydrogenans*], NADH, NAD$^+$, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.49; *Leuconostoc mesenteroides*) and glucose-6-phosphate were purchased from Boehringer Mannheim (Mannheim, Germany). Dichloromethane (HPLC grade) was purchased from Rathburn Chemicals Ltd (Walkerburn, England). Derivatization reagents were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany).

HPLC

The equipment (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.) and the normal phase conditions have been described previously [8]. The eluent composition is given in the Tables 1 and 2.

Synthesis of the title compounds

A-ring reduction. After presaturation of 0.4 mg 10% palladium on charcoal in 1 ml ethanol–water (90:10, v/v) by H_2 gas for 5 min, small amounts of the 6α-hydroxy and/or 6β-hydroxy 3-oxo-4-pregnenes in 0.2 ml 90% ethanol were added and reduced by H_2 for 10 min. The reaction mixture was passed through a 0.45 μm membrane filter (4.3 cm^2 area, Bio-Rad Labs, Richmond, CA, U.S.A.) with use of methanol and taken to dryness with N_2 gas at 60°C. The two 5-dihydro derivatives of each starting compound were purified by HPLC according to the conditions in Table 1. Aliquots of (each of) the two steroids were dissolved in 20 μl ethanol and enzymatically reduced at C-3 with 3 μmol NADH in 0.75 ml 0.1 M phosphate, 3 mM $MgCl_2$ and 1 mM EDTA at pH 7.0 during 18 h at 37°C with the use of 0.1 U of 3α-hydroxysteroid dehydrogenase, 10 μmol glucose-6-phosphate and 2 U of glucose-6-phosphate dehydrogenase. Upon addition of water the reaction mixture was extracted on a Sep-pak C_{18} cartridge (Millipore, Waters Chromatography Division) and the two tetrahydro derivatives of each starting compound were purified and separated by HPLC as shown in Table 2.

Enzymatic oxidation. An aliquot of 6αOH-20βHHS was dissolved in 1.0 ml 0.35 M ethanol, 10 mM Tris chloride pH 8.0 and 1 mM NAD. After addition of 0.1 U of 20β-hydroxysteroid dehydrogenase the compound was oxidized at room temperature for 2 h in the dark. The steroids in the mixture were then extracted on a Sep-pak C_{18} cartridge and the eluted compounds were taken to dryness with N_2. The reaction products were separated by HPLC.

Derivatization

Reference and synthesized steroids were derivatized to the methoxime-trimethylsilyl ethers as described previously [9].

GC and GC/MS

HP 5890 GC (Hewlett Packard Nederland B.V., Amstelveen, The Netherlands) was equipped with a HP 7373 injector, a 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. A HP 5890

GC was equipped with a Chrompack (The Netherlands) CP Sil 5 CB capillary column (25 m × 0.25 mm) and coupled to a VG 70-250S mass spectrometer (VG Instruments, Manchester, England). The conditions for use of these equipments have been described previously in detail [8].

Methylene units [10]. By co-injection or separate runs of a series of 7 *n*-alkanes from C_{28} to C_{34} the GC retention index of a steroid was calculated as the methylene unit (MU) value, by linear or non-linear (parabolic) interpolation between the retention times of the two alkanes eluting directly before and the two alkanes eluting directly after the considered steroid.

Nuclear magnetic resonance (NMR). 300 MHz ^{1}H-NMR spectra were recorded in CD_3OD and or $CDCl_3$ on a Varian VXR300 spectrometer. Two-dimensional ^{1}H-homonuclear shift-correlated spectra (COSY) and nuclear-Overhauser-effect spectra (NOESY) were obtained with 2048 data points in the f_2 dimension and zero-filling to 2048 data points in the f_1 dimension (512 or fewer actual experiments were usually acquired) to achieve a symmetrical data matrix on transformation using sinusoidal (COSY) or shifted sinusoidal (NOESY) multiplication in each dimension followed by symmetrization of the final data matrices [11, 12].

Molecular mechanics calculations. The α and β configurations of the A-ring in the 6βOH-DHS and 6βOH-THS isomers were visualized by use of molecular mechanics calculations (MM2). The structures of these steroids were built up with the CAChe WorkSystem (Tektronix) and the geometries were optimized using the MM2 force field [13, 14]. The obtained minimal energy conformations of the steroids were used to interpret the 2D-NMR spectra of the 6β-hydroxylated derivatives of compound S, by determining the interatomic distances between the relevant protons in the A and B rings of the 5α and 5β steroids.

RESULTS

Synthesized Hydroxylated Steroids

HPLC

5-Dihydrosteroids. Table 1 shows that the 5-dihydro derivatives of 6βOH-S: 6βOH-5αDHS and -5βDHS, and those of 6αOH-S: 6αOH-5αDHS and -5βDHS, could be separated from each other by normal-phase HPLC. For both epimer pairs it is shown that the 5αDH steroid eluted earlier than the 5βDH compound. However, the 5-dihydro derivatives of 6βOH-B: 6βOH-5αDHB and -5βDHB, and those of 6αOH-B: 6αOH-5βDHB and -5βDHB, 6βOH-A: 6βOH-5αDHA and -5βDHA, and 6αOH-A: 6αOH-5αDHA and -5βDHA, could not be completely separated. For each of the latter 4 pairs of epimers the two 5-dihydro steroids were purified and collected in one fraction.

Table 1. Chromatographic (HPLC) date of the 5α-(and) 5β)-dihydro derivatives of 6-hydroxy-S, -B and -A

	Eluent[a]	Retention time *t* (min)
6βOH-5αDHS[b]	2	6.3
6βOH-5βDHS	2	8.1
6αOH5αDHS[c]	2	11.8
6αOH-5βDHS[c]	2	12.7
6βOH-5αDHB[d]	3	5.9
6βOH-5βDHB[d]	3	6.4
6αOH-5αDHB[d]	4	8.1
6αOH-5βDHB[d]	4	8.7
6βOH-5α(+5β)DHA[d]	1	~6.2
6αOH-5α(+5β)DHA[d]	4	~6.2

[a]Eluent composition of dichloromethane–methanol–water: 1 (980.8:17.5:1.8, by vol), 2 (978:20:2.0), 3 (975.2:22.5:2.3), and 4 (972.5:25:2.5); [b]the systematic names of the steroids are given in *Abbreviations*; [c]these 2 epimers were not completely separated on the used 15 cm column, but on a 25 cm column; [d]all other epimer pairs were not (completely) separable with the used eluentia.

Tetrahydrosteroids. In Table 2 the capacity factors (*k'*) of the 12 6-hydroxylated tetrahydrocompounds S, B and A show that the 5α-pregnanes elute earlier than

Table 2. Chromatographic (HPLC and GC) data of the A-ring reduced 6-hydroxylated derivatives of S, B and A

		GC		
Abbreviation	HPLC *k'*[a]	MU	ΔMU	Peak area ratio (1st/2nd)
6βOH-5αTHS[b]	2.90[c]	30.07[d]		
6βOH-THS	3.83	29.70		
6αOH-5αTHS	4.83	30.20		
6αOH-THS	5.47	29.63		
6βOH-5αTHB	2.67[c]	30.98/31.40	0.42	5.9
6βOH-THB	3.47	30.84/31.36	0.52	6.3
6αOH-5αTHB	3.20	31.55/32.17	0.62	4.6
6αOH-THB	3.53	31.12/31.78	0.66	9.0
6βOH-5αTHA	1.67[c]	31.37/31.83	0.46	6.0
6βOH-THA	2.90	—/—	—	—
6αOH-5αTHA	3.80	31.58/32.25	0.67	4.8
6αOH-THA	4.07	30.89/31.63	0.74	4.8

[a]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]the systematic names of the steroids are given in *Abbreviations*; [c]the order of elution (using *k'*) is $6\beta5\alpha < 6\beta5\beta < 6\alpha5\alpha < 6\alpha5\beta$, in each of the 3 sets of 4 isomers (except for 6βOH-THB and -5αTHB). Due to slight differences in the eluent composition dichloromethane–methanol–water (943:~55:2.5, by vol) the *k'* factor cannot be used for the order of elution of all 12 steroids together, but all 5β pregnanes elute after the corresponding 5α epimers; [d]the order of elution is $6\beta5\beta < 6\beta5\alpha < 6\alpha5\beta < 6\alpha5\alpha$ in each of the 3 sets of 4 isomers (except for 6βOH- and 6αOH-THS), but (the main peaks of) all six 5α pregnanes are detected after (those of) the corresponding 5β epimers (no data for 6βOH-THA).

the 5β epimers and the 6β-hydroxylated tetrahydrocompounds elute earlier than the 6α-hydroxylated epimers. The *k'* value of 6αOH-THS was the same as that of the product obtained by enzymatic oxidation of 6αOH-20βHHS (not shown).

As each of the 4 6-hydroxy-tetrahydrocompound S isomers was separately obtained from the corresponding 5-dihydropregnanes, the latter 4 steroids could be retrospectively defined to be 6βOH-5αDHS, 6βOH-5βDHS, 6αOH-5αDHS and 6αOH-DHS, and eluted from the normal-phase column in the same sequence as the corresponding tetrahydrocompounds, as shown in the Tables 1 and 2.

GC

Table 2 also shows the MU values of the methoxime-trimethylsilyl ethers of the 6-hydroxy-tetrahydrocompounds S, B, and A. The MU values of 5α-pregnanes are larger than those of the corresponding 5β epimers. The steroids derived from compound B and those from compound A, all showed two E/Z isomers, of which the first peak in the chromatogram was at least 4.6 times larger than the second one. The differences of the MU values of the E/Z isomers of 6αOH-THB (0.66) and 6αOH-5αTHB (0.62) are larger than those of the corresponding 6β epimers, 6βOH-THB (0.52) and 6βOH-5αTHB (0.42).

^{1}H-NMR

The chemical shifts (δ) in the 300 MHz ^{1}H-NMR spectra of the dihydro and the tetrahydro derivatives of 6βOH-S were used to identify the 5α and the 5β epimers. From the chemical shifts of the C-19-H_3 signal in 6βOH-5αTHS and 6βOH-THS in Table 3 it follows that $\delta = 0.87$ can be assigned to the C-19 methyl group in 6βOH-THS (5β) and $\delta = 0.77$ with that in 6βOH-5αTHS. For the corresponding 5-dihydro derivatives of 6βOH-S, 6βOH-5αDHS and -5βDHS, no apparent differences of the chemical shifts at C-19 and C-6 were observed. Therefore, the spectra of 6βOH-5αDHS and -5βDHS were reanalyzed by using the COSY and NOESY techniques. COSY spectra show shift correlation by homonuclear coupling, while NOESY spectra show incoherence transfer (correlation through space). The chemical shifts of the relevant protons are given in Table 4. Starting with $\delta = 3.72$ proton 6α in the presumed 6βOH-5αDHS (see Table 3), and using the 'cross peaks' in the COSY spectrum of spin-coupling of 6α-H only to the 5α, 7α and 7β protons, and, similarly, the spin-coupling of proton 5α only to the 4α, 4β and 6α protons, the chemical shifts were assigned to these five protons. In the corresponding NOESY spectrum of the 5α steroid the absence of an correlation between proton 5 and the protons of the C-19 methyl group (d = 0.37 nm) identified the compound to be the 5α (A-B *trans*) isomer. Further, the existence of correlation through space of the 4β proton to the protons of the C-19 methyl group (d = 0.22 nm) and that of the 2β proton to the C-19 methyl group (d ≃ 0.2 nm) was used to find the chemical shifts of the 4β and the 2β protons (NOE interaction). Coupling of the 4β proton with that of 4α, and that of the 2β proton with the 2α proton resulted in the assignment of the two α protons. A so-called W-coupling between the 4α and 2α protons in the COSY spectrum of the 6βOH-5αDHS was not observed.

By following similar routes the chemical shifts of the same protons in 6βOH-5βDHS were determined. Here the NOESY spectrum showed a coupling through space of the 5β proton to the protons of the C-19 methyl group (d = 0.24 nm) and defined the compound to be the 5β isomer. Overlap of peaks in the COSY spectrum prevented the assignments of the chemical shifts of the 2α and 2β protons, and the discrimination between the 4α and 4β, and between the 7α and 7β protons (see Table 4).

Table 4 shows that the chemical shifts of the protons at C-6, C-21, C-19 and C-18 in 6βOH-5αDHS are nearly the same as those in 6βOH-5βDHS, as also shown in Table 3. The δ values of the protons at these four C atoms in the two steroids 6βOH-5αDHS and

Table 3. Chemical shifts δ in the ^{1}H-NMR spectra of the 6β-hydroxylated derivatives of compound S, 5αDHS, 5βDHS, 5αTHS and THS

	C-4 H	C-6 αH	C-21 H_2	C-19 H_3	C-18 H_3
6βOH-S[a]	5.75[b]	4.25 b	4.62; 4.32 ABq (J = 18 Hz)	1.34	0.65
6βOH-5αDHS	—	3.79 bs	4.67; 4.32 ABq (J = 18 Hz)	1.21	0.72
6βOH-5βDHS	—	3.73	4.67; 4.32 ABq (J = 18 Hz)	1.22	0.71
6βOH-5αTHS	—	3.87 bs	4.43; 4.05 ABq (J = 18 Hz)	0.77	0.42
6βOH-THS	—	3.49 bs	4.43; 4.06 ABq (J = 18 Hz)	0.87	0.40

[a]The systematic names of the steroids are given in *Abbreviations*. The data of 6βOH-S are taken from [7]; [b]the chemical shifts recorded in 30% CD_3OD in $CDCl_3$, or in $CDCl_3$ (6βOH-S) are given in δ units (ppm) relative to Me_4Si (δ = 0). The chemical shifts were not corrected for differences in the concentrations of the compounds. The abbreviations of the NMR date are: ABq, AB quartet; bs, broad singlet; —, unknown. The δ values noted without an abbreviation refer to singlet resonance peaks.

Table 4. Chemical shifts δ[a] in the ^{1}H-NMR spectra of 6βOH-5αDHS and -5βDHS

Proton	6βOH-5αDHS	6βOH-5βDHS
21	4.67; 4.29 ABq[1] (J = 20 Hz)	4.67; 4.29 ABq (J = 20 Hz)
6α	3.72 ND[2]	3.67 ND
4β	2.84	2.37[3,4] (ξ)
4α[1]	2.05	2.13[4] (ξ)
2β	2.45	—[3,5]
2α[1]	1.97	—[5]
7β	1.87	1.73[4] (ξ)
7α	1.25	1.40[4] (ξ)
5	1.55 (α)	1.95 (β)
19	1.21	1.21
18	0.68	0.67

[a]The chemical shifts δ are given in ppm. The COSY and NOESY spectra were calibrated by using δ = 3.35 for the residual CD_2HOD as reference value. The 2 steroids were dissolved in 0.2 ml CD_3OD + 0.5 ml $CDCl_3$ (D = ^{2}H). [1]ABq, AB quartet; [2]ND, narrow doublet; [3]no apparent W-coupling was observed in the COSY spectrum; [4]due to overlapping peaks no information was obtained from the COSY spectrum to discriminate between the protons 4α amd 4β, and between 7α and 7β. However, β protons are normally found at lower field (higher δ value) than the α protons, as reported too for 6β-hydroxy-progesterone and -aldosterone [20]. [5]Not determinable.

-5βDHS in Table 3 are nearly the same as those in Table 4, despite the slightly different experimental conditions.

MM2

The calculated proton–proton distances for the 4 6βOH-DHS and -THS compounds, being relevant for the analysis of the above discussed ^{1}H-NMR spectra, are given in Table 5. The difference in interatomic distances is most striking between H-5α and H-19, and H-4β and H-19 in the 5α steroids compared with the 5β stereoisomers. The MM2 calculated global minimal energy conformations of the investigated compounds showed that the *all trans* 6βOH-5αDHS configuration appeared to be 0.8 kcal/mol more stable than the *A-B cis* 6βOH-5βDHS configuration, and also that 6βOH-5αTHS appeared to be 0.7 kcal/mol more stable than 6βOH-THS (5β). The MM2 program was also used to find the difference between the relative stability of the 6β- and the 6α-hydroxy derivatives of 5αDHS and of 5αTHS. In both cases the calculations showed the 6α-hydroxylated (equatorial) compounds to be about 1 kcal/mol more stable than the corresponding 6β-hydroxylated (axial) epimers.

GC/MS

Figures 1–3 show the mass spectra of 6αOH-5αTHS [Fig. 1(a)] and 6αOH-THS [Fig. 1(b)], 6αOH-5αTHB [Fig. 2(a)] and 6αOH-THB [Fig. 2(b)] and 6αOH-5αTHA [Fig. 3(a)] and 6αOH-THA [Fig. 3(b)]. Table 6 gives the relative intensities of the fragment ions $m/z > 100$ of 11 of the 12 synthesized title compounds.

6OH-THS

The typical fragment ions in the mass spectra ($M^+ = 683$) of the 4 6-hydroxy-tetrahydrocompounds S are recorded in Table 6. It is clear that the spectra are more or less the same, and similar to the spec-

Table 5. Interatomic distances (nm) of the relevant protons in 6βOH-5α/5βDHS and -5α/5βTHS, calculated by use of the MM2 program [12, 13]

	1α	1β	4α	4β	5(α)	6(α)	8(β)
(1) 6βOH-5αDHS (1st) and 6βOH-5αTHS (2nd)[a]							
5α							
1α	—						
1β	0.18	—					
4α	0.41	0.50	—				
4β	0.41	0.43	0.18	—			
5(α)	0.24	0.37	0.24	0.31	—		
6(α)	0.48	0.56	0.25	0.31	0.25	—	
8(β)	0.48	0.46	0.53	0.47	0.40	0.38	—
19	0.36; 0.39[a]	0.24; 0.25	0.38	0.22	0.38	0.38; 0.41	0.23; 0.22
	1α	1β	4α	4β	5(β)	6(α)	8(β)
(2) 6βOH-5βDHS and 6βOH-THS							
5β							
1α	—						
1β	0.18	—					
4α	0.43	0.40	—				
4β	0.50	0.41	0.18	—			
5(β)	0.38	0.25	0.31	0.24	—		
6(α)	0.57	0.49	0.29	0.24	0.25	—	
8(β)	0.46	0.50	0.43	0.52	0.41	0.38	—
19	0.32	0.24	0.48	0.47	0.24	0.40	0.22

[a]The values for the 2 steroids are separately shown, if the difference between those values for the 5α-dihydro- and the 5α-tetrahydrocompounds are >2%. The orientation of the single protons at C-5, C-6 and C-8 are given within parentheses.

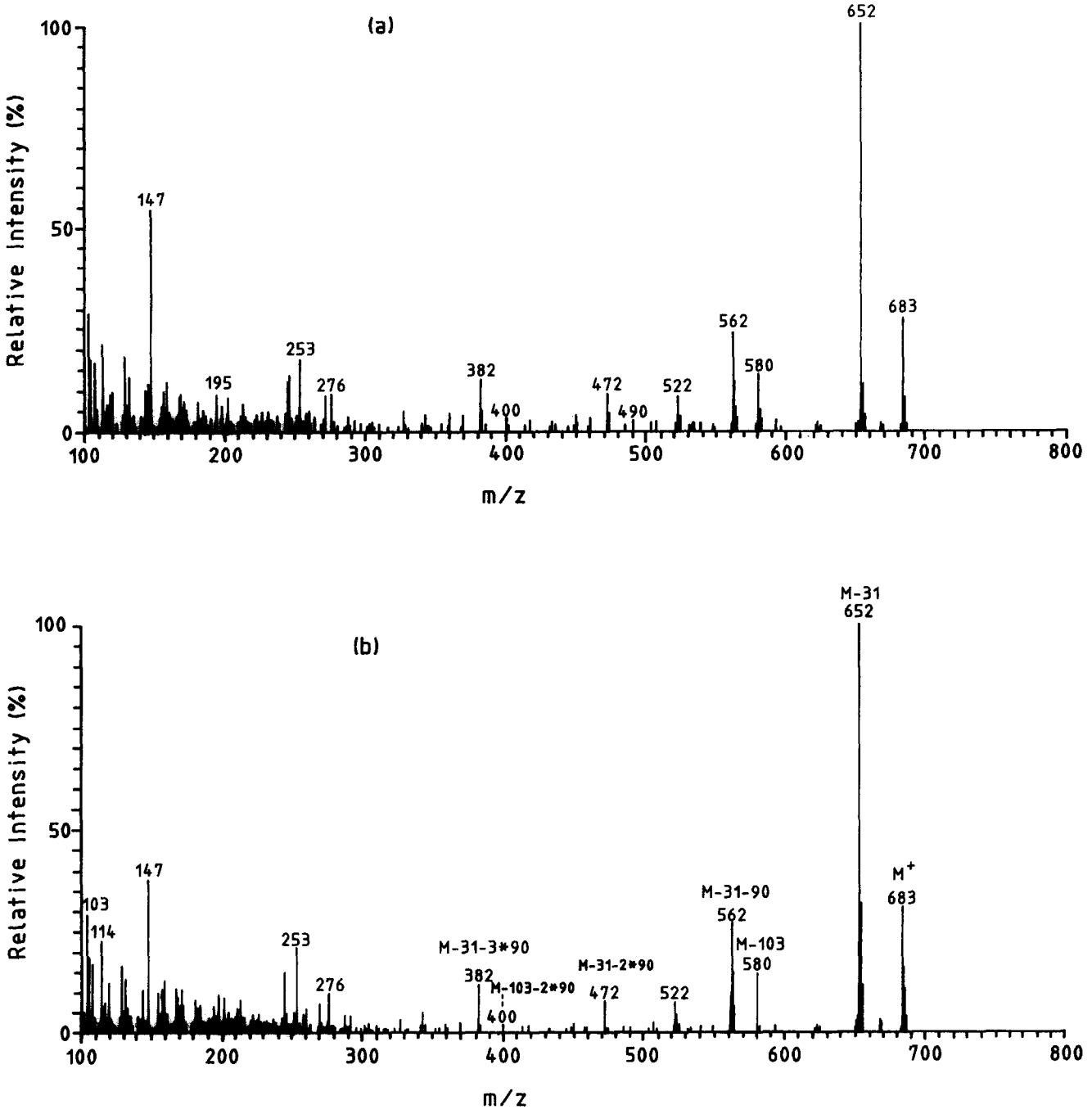

Fig. 1. Electron impact mass spectra at 70 eV of the MO-TMS derivatives of (a) 6αOH-5αTHS (3α,6α,17,21-tetrahydroxy-5α-pregnan-20-one) and (b) 6αOH-THS (3α,6α,17,21-tetrahydroxy-5β-pregnan-20-one).

trum of 3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one (THF). The leading fragment ion m/z 652 $(M-31)^+$ and the ion m/z 562 $(M-31-90)^+ > m/z$ 472 $(M-31-2*90)^+$ as well as the ions m/z 580 $(M-103)^+$, the ions m/z 276, 246 and 244, and the ion m/z 253 characterize the steroidal structure. The ion list of 6αOH-THS obtained by oxidation of the reference steroid 6αOH-20βHHS with NAD^+ and 20β-hydroxysteroid dehydrogenase was identical to that of 6αOH-THS, obtained by A-ring reduction of 6αOH-S and therefore not shown.

6OH-THB

The mass spectra of 6αOH-5αTHB and -THB ($M^+ = 683$) are shown in Fig. 2(a and b), respectively, while the spectral data of all 8 peaks of the 4 6OH-THB isomers are given in Table 6. The 8 spectra are characterized by the dominating fragment ion m/z 188, the fragment m/z 175 and the intensity of the ion m/z 652 $(M-31)^+ <$ ion m/z 562 $(M-31-90)^+ \approx m/z$ 472 $(M-31-2*90)^+$. Furthermore, Table 6 shows that the fragment ion m/z 668 $(M-15)^+$ in each spectrum of the

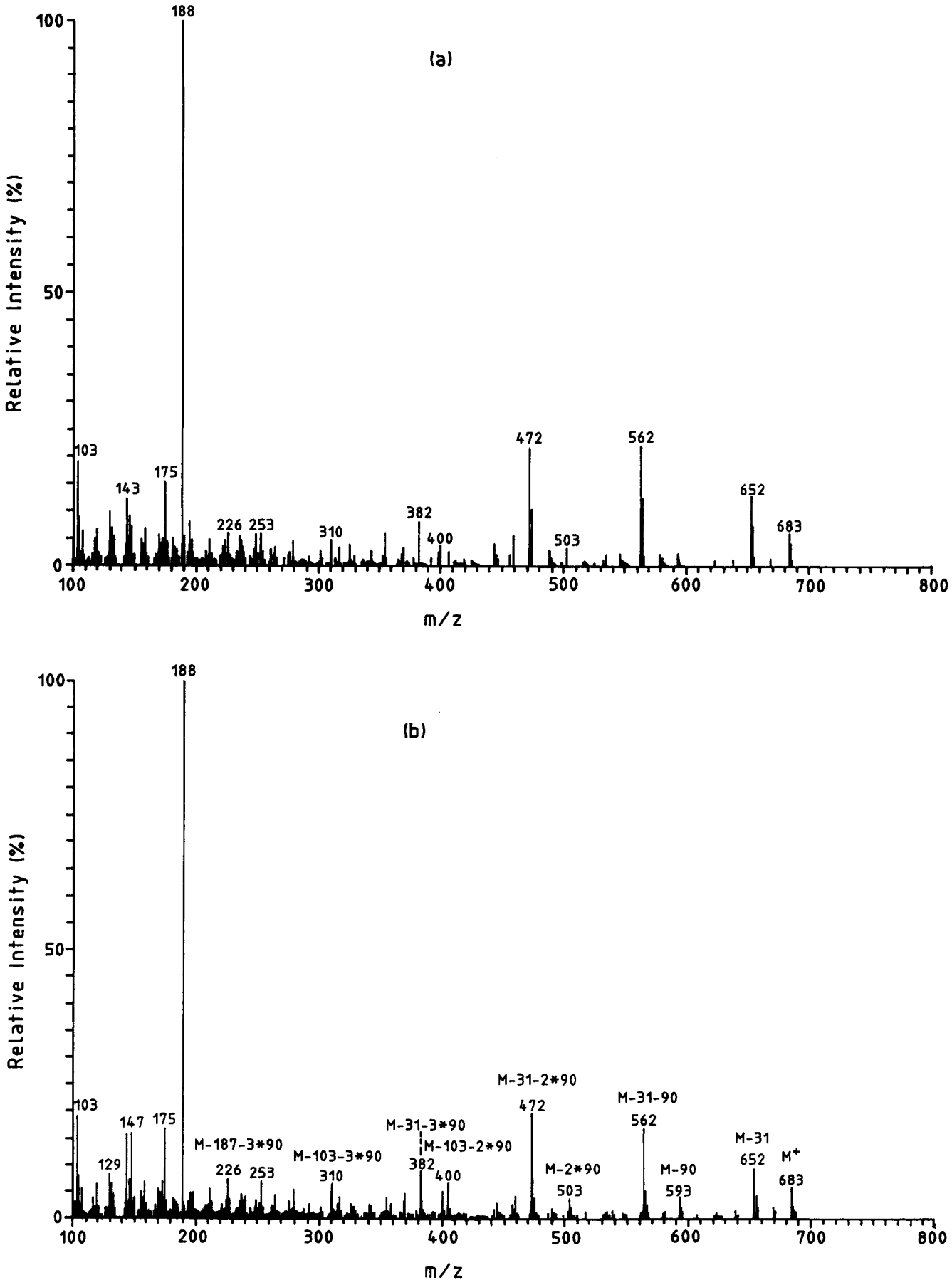

Fig. 2. Electron impact mass spectra at 70 eV of the MO-TMS derivatives of (a) 6αOH-5αTHB (3α,6α,11β,21-tetrahydroxy-5α-pregnan-20-one) and (b) 6αOH-THB (3α,6α,11β,21-tetrahydroxy-5β-pregnan-20-one).

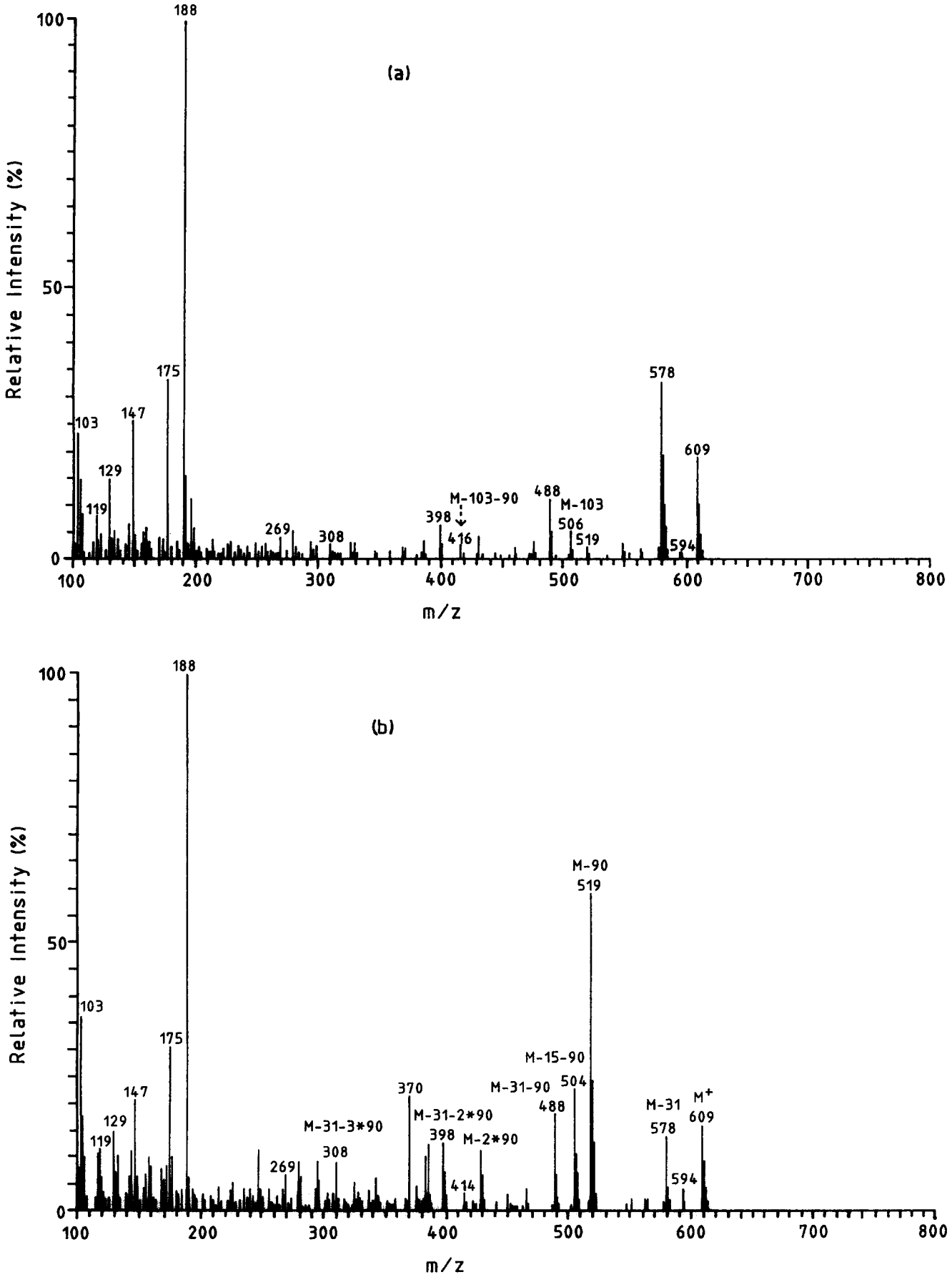

Fig. 3. Electron impact mass spectra at 70 eV of the MO-TMS derivatives of (a) 6αOH-5αTHA (3α,6α,21-trihydroxy-5α-pregnane-11,20-dione) and (b) 6αOH-THA (3α,6α,21-trihydroxy-5β-pregnane-11,20-dione).

Table 6. A-ring reduced 6-hydroxylated derivatives of S, B and A

Steroid (abbreviation)		M^+																						
Fragments *m/z*		683	668	652	580	562	522	507	472	417	382	327	276	270	253	246	244	213	169	159	147	129	114	103
6βOH-5αTHS		20	4	100	7	26	8	3	14	4	10	6	8	5	17	13	11	5	10	7	17	16	20	22
6βOH-THS		19	4	100	10	26	7	3	11	3	13	6	8	3	18	13	12	8	8	11	34	14	18	24
6αOH-5αTHS		29	2	100	14	25	9	2	10	3	13	5	8	5	18	13	13	7	9	12	55	19	22	30
6αOH-THS		31	3	100	15	27	8	2	8	2	12	4	10	7	21	12	15	8	11	13	38	17	23	29
	E/Z isomers																							
Fragments *m/z*		683	668	652	593	580	578	562	503	490	488	472	400	382	310	253	188	175	158	147	143	129	119	103
6βOH-5αTHB	peak 1	15	3	28	7	3	6	53	8	4	7	52	8	18	11	11	100	40	18	20	28	20	13	42
	peak 2	11	22	29	10	6	13	65	11	5	11	57	6	22	11	7	100	37	19	19	36	24	14	66
6βOH-THB	peak 1	14	6	25	7	3	3	47	8	5	5	53	12	25	20	18	100	44	22	40	43	21	14	56
	peak 2	5	15	12	7	2	2	31	5	3	4	29	8	12	8	6	100	17	10	22	28	12	7	38
6αOH-5αTHB	peak 1	6	1	13	2	1	2	23	3	1	3	23	4	8	5	7	100	16	7	8	13	10	7	19
	peak 2	5	13	16	3	2	8	32	9	2	4	31	3	10	7	5	100	17	10	13	20	13	8	33
6αOH-THB	peak 1	7	3	10	4	1	1	17	4	2	2	20	5	9	7	8	100	17	7	16	16	8	7	19
	peak 2	6	13	10	7	2	3	32	6	2	5	34	8	15	9	8	100	18	10	27	29	16	8	38
Fragments *m/z*		609	594	578	519	506	504	488	429	416	414	398	345	326	308	269	188	175	158	147		129	119	103
6βOH-5αTHA	peak 1	16	3	15	59	23	2	19	11	6	5	13	8	6	9	8	100	32	9	21		16	12	36
	peak 2	27	27	8	100	39	0	19	13	8	9	15	8	5	13	4	63	12	14	24		18	7	60
6αOH-5αTHA	peak 1	19	1	33	2	5	1	11	1	2	1	7	<1	3	3	4	100	34	6	27		15	13	24
	peak 2	34	23	49	5	6	1	15	2	3	2	10	1	4	4	2	100	24	11	34		26	7	44
6αOH-THA	peak 1	16	3	14	59	6	23	18	12	5	5	13	2	5	3	7	100	32	9	21		17	12	36
	peak 2	26	27	7	100	6	38	18	13	6	8	14	7	5	5	2	63	12	8	24		18	10	61

The intensities of the molecular ion (M+) and the typical fragment ions $m/z > 100$ are given as the percentage of the base peak.

second isomeric peak of the 4 6OH-THB compounds (peak 2), is larger than that in the corresponding spectrum of the main peak (peak 1). The fragment ion m/z 226 $(M\text{-}3*90\text{-}187)^+$, present in the 4 6OH-THB compounds can be seen in Fig. 2.

6OH-THA

The mass spectra of 6αOH-5αTHA and -THA ($M^+ = 609$) are shown in Fig. 3(a and b), respectively, while the spectral data of these 2 steroids and 6βOH-5αTHA are given in Table 6. The table shows that m/z 188 is the dominant fragment in the spectra of the main peak of the three steroids except for the second peak of 6βOH-5αTHA and 6αOH-THA, where m/z 519 $(M\text{-}90)^+$ is the leading fragment. The spectral data of the three 6OH-THA compounds show that the fragment m/z 594 $(M\text{-}15)^+$ in the second peak is larger than that in the first one.

DISCUSSION

In this study 12 6α- and 6β-hydroxylated A-ring reduced steroids were synthesized from the corresponding 6 precursor compounds: 6α and 6β-hydroxy derivatives of S, B and A which have been described previously [7]. Eleven of the 12 steroids were fully identified by making use of the following experimental observations:

(a) *5α-pregnanes are less polar than the corresponding 5β epimers.* This means that the latter compounds are more retained on the normal-phase HPLC column. This has been proved for 3α,6α,17,21-tetrahydroxy-5β-pregnane-11,20-dione (6αOH-THE) and 3α,6α,17,21-tetrahydroxy-5α-pregnane-11,20-dione (6αOH-5α-THE), 3α,6α,11β,17,21-pentahydroxy-5β-pregnan-20-one (6αOH-THF) and 3α,6α,11β,17,21-pentahydroxy-5α-pregnan-20-one (6αOH-5α-THF), as well as for the corresponding 6β-hydroxylated epimer pairs [15]. The above conclusion is valid too for the epimer pairs 17,21-dihydroxy-5α-pregnane-3,11,20-trione (5αDHE) and 17,21-trihydroxy-5β-pregnane-3,11,20-trione (5βDHE), 11β,17,21-trihydroxy-5α-pregnane-3,20-dione (5αDHF) and 11β,17,21-trihydroxy-5β-pregnane-3,20-dione (5βDHF) [unpublished observations], to 3α,17,21-trihydroxy-5α-pregnane-11,20-dione (5αTHE) and 3α,17,21-trihydroxy-5β-pregnane-11,20-dione (THE), 3α,11β,17,21-tetrahydroxy-5α-pregnan-20-one (5αTHF) and 3α,11β,17,21-tetrahydroxy-5β-pregnane-20-one (THF) [9], and to 15β,17-dihydroxy-5α-pregnane-3,20-dione and 15β,17-dihydroxy-5β-pregnane-3,20-dione, and 3α,15β,17-trihydroxy-5α-pregnan-20-one and 3α,15β,17-trihydroxy-5β-pregnan-20-one [8].

(b) *The methoxime-trimethylsilyl ethers of 5α-pregnanes are more retained on the apolar GC column than the corresponding 5β epimers*, as reported previously [8–10, 15–18]. This means that the MU values of the latter compounds are smaller than those of the corresponding 5α epimers, 3α,20α-dihydroxy-5β-pregnane and 3α,20α-dihydroxy-5α-pregnane excepted. This conclusion holds for all of the above (see paragraph a) described epimer pairs. Table 2 shows that the GC data of 6βOH-THA are missing. The fact that the differences of the MU values of the E/Z isomers of 6αOH-THB (0.66) and 6αOH-5αTHB (0.62) are larger than those of the corresponding 6β epimers, 6βOH-THB (0.52) and 6βOH-5αTHB (0.42), is in accordance to analog results with 3α,6α,21-trihydroxy-5β-pregnan-20-one and 3α,6β,21-trihydroxy-5β-pregnan-20-one and the two corresponding 5α isomers, as reported previously [18].

(c) *Direct proof of structure at C-5 in 6βOH-5αDHS and -5βDHS by ^{1}H-NMR using COSY and NOESY.* As 6βOH-DHS and -5αDHS could be separated by HPLC (see Table 1), about 3 μmol of these 2 epimers were purified on the analytical column, sufficiently to identify these 2 steroids by ^{1}H-NMR. Table 3 shows that the chemical shift of the C-19-H_3 protons was apparently the same for both epimers 6βOH-DHS and -5αDHS, but different for the tetrahydro compounds 6βOH-5αTHS and -THS. The extra shift $\Delta\delta = 0.10$ in these latter 2 compounds was assigned to the extra coupling between the βH at C-5 and C-19-H_3 (*A-B cis*) in 6βOH-THS, in which the distance between these protons (0.24 nm) is shorter than that (0.38 nm) between the αH at C-5 and C-19-H_3 in (*A-B trans*) 6βOH-5αTHS (see the Tables 4 and 5). However, these results were not sufficiently solid to discriminate between the synthesized 5α- and the corresponding 5β-steroids in this study, although in accordance to similar results obtained with 6β-hydroxy (and 6α-hydroxy) derivatives of tetrahydrocortisone (THE) and 5αTHE, and tetrahydrocortisol (THF) and 5αTHF, as reported previously [15]. Therefore, 6βOH-5αDHS and -5βDHS were identified by using the two dimensional NMR techniques, COSY and NOESY [10, 11]. The 2D spectra proved that the presumed 5α compound was 6βOH-5αDHS, and the other steroid was 6βOH-5βDHS indeed. As these 2 steroids were the direct precursors of the corresponding tetrahydro compounds, 6βOH-5αTHS and -THS, respectively, these latter compounds too were correctly identified.

(d) 6αOH-THS was identified by comparing its chromatographic parameters and mass spectrum

with the features of the enzymatically oxidized 5β reference compound 6αOH-20βHHS. The so obtained 6αOH-THS was identical to that synthesized from 6αOH-S.

Therefore, it can be concluded that by using the capacity factors k' (HPLC) and or the MU (GC) values of the 6 epimer pairs in Table 2, all the 12 synthesized A-ring reduced compounds, 6βOH-THA excepted, were correctly defined.

Mass spectra

With respect to the previously published mass spectrum of the urinary steroid 6αOH-THS [6] it can be stated that the fragmental pattern is similar to that of the here synthesized 6αOH-THS [Fig. 2(b), Table 6] except for some minor differences, possibly due to the different experimental conditions. The presence of 6αOH-THS and 5αTHS was confirmed in the urine of an 8 year-old 11OHD boy, as published previously [21]. Similarly, 6αOH-THA and -THB and not the 5α isomers were identified in the reanalyzed urine samples of the two female siblings with CAH due to 17OHD [2], despite more 5αTHB than THB being present in the two urines (unpublished observations). The spectrum of the tentatively identified steroid 6ξ-hydroxytetrahydrocompound A (3α,6ξ,21-trihydroxy-5β-pregnane-11,20-dione) [5] is similar to that of 6αOH-THA shown in Fig. 3(b). Comparing the MU values of 6αOH-THA in Table 2, 30.89/31.63, with the published MU values 30.86/31.52 [16] of the urinary steroid in [5], logically defined as 6α-hydroxytetrahydrocompound A in [16], it can be concluded that the compound in [5] must indeed have been 6αOH-THA (3α,6α,21-trihydroxy-5β-pregnane-11,20-dione).

Stability

From the calculations using the MM2 program it can be concluded that the 5α oriented steroid (*A-B trans*) is slightly more stable than the corresponding 5β configured isomer (*A-B cis*). Secondly, that 6α-hydroxylated (equatorial) compounds are about 1 kcal/mol more stable than the corresponding 6β-hydroxylated (axial) epimers. This is in good agreement with the knowledge that the most stable orientation of hydroxy substituents on cyclohexane rings is usually equatorial [22].

Finally it can be concluded that by the results of this study, most of the tetrahydro (A-ring) derivatives of 6α-hydroxylated adrenocorticosteroids in the urine are identified and characterized: 3α,6α,21-trihydroxy-5α(and β)-pregnan-20-one [18]; 3α,6α,17,21-tetrahydroxy-5α(and β)-pregnan-20-one, 3α,6α,11β,21-tetrahydroxy-5α(and β)-pregnan-20-one and 3α,6α,21-trihydroxy-5α(and β)-pregnane-11,20-dione (this study); and 3α,6α,17,21-tetrahydroxy-5α(and β)-pregnane-11,20-dione and 3α,6α,11β,17,21-pentahydroxy-5α-(and β)-pregnan-20-one [15]. The data of these studies can be used for searching for 6α-hydroxylated steroids in the urine of diseased children and adults.

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