Available online at www.sciencedirect.com
SCIENCE DIRECT®
Steroids 68 (2003) 31–42
Steroids
www.elsevier.com/locate/steroids

Chemical synthesis of 7- and 8-dehydro derivatives of pregnane-3,17α,20-triols, potential steroid metabolites in Smith–Lemli–Opitz syndrome

Li-Wei Guo [a], William K. Wilson [a,*], Jihai Pang [a], Cedric H.L. Shackleton [b]

[a] *Department of Biochemistry and Cell Biology, Rice University, 6100 Main Street, Houston, TX 77005-1892, USA*
[b] *Childrens Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609, USA*

Received 2 May 2002; accepted 27 June 2002

Abstract

Pregnane-3,17α,20-triols bearing unsaturation at Δ^7, Δ^8, $\Delta^{5,7}$, or $\Delta^{5,8}$ have been tentatively identified as steroid metabolites in Smith–Lemli–Opitz syndrome (SLOS). Starting with 17α-hydroxypregnenolone diacetate, we have synthesized 13 unsaturated C_{21} triols by four different routes in one to four steps. These multifunctional steroids were prepared by a series of regio- and stereoselective transformations chosen to minimize facile olefin isomerization and 17-deoxygenation. The results include a study of stereoselectivity in the reduction of 17α-hydroxy-20-ketosteroids, an alternative method for reducing diethyl azodicarboxylate adducts of $\Delta^{5,7}$ steroids, and an efficient oxidation–isomerization of a $\Delta^{5,7}$ steroid using cholesterol oxidase. The 13 triols and their synthetic precursors were fully characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The NMR data, together with molecular modeling, indicated unanticipated conformational heterogeneity for two synthetic intermediates, 17α-hydroxypregna-4,7-diene-3,20-dione and 17α-hydroxy-5β-pregn-7-ene-3,20-dione. The unsaturated C_{21} triols are useful as reference standards to study adrenal steroid production in SLOS and to develop methods for pre- and postnatal diagnosis of this congenital disorder.

Keywords: Steroid metabolites; Smith–Lemli–Opitz syndrome; Conformational analysis; NMR; Mass spectrometry

1. Introduction

Congenital adrenal hyperplasia and other classical enzyme disorders of adrenal steroid synthesis involve defects in the pathway between cholesterol and either cortisol or aldosterone [1,2]. An additional cause of attenuated steroid production was suggested by the identification of defective 7-dehydrocholesterol-Δ^7-reductase (DHCR7) as the cause of Smith–Lemli–Opitz syndrome (SLOS) [3]. This genetic disorder is characterized by mental retardation, physical anomalies, and physiological aberrations [4] leading to cholesterol deficiency and occasional adrenal insufficiency [4–6]. The hallmark of SLOS is an overproduction of 7-dehydrocholesterol (7DHC) and 8-dehydrocholesterol (8DHC) [3,4,7], the two isomers being interconverted by the Δ^8–Δ^7 sterol isomerase [8,9].

In contrast to previously studied enzymatic defects of adrenal steroid synthesis, in which only known and normal intermediates accumulate, SLOS has the potential of producing a new class of steroids if 7- or 8DHC can be utilized as steroid precursor since the resulting metabolites would likely have Δ^7 or Δ^8 unsaturation. This type of steroid was unknown in humans but had long ago been found in the equine [10]. In studies of steroids excreted by child and adult SLOS patients, we found evidence for the presence of ring B unsaturated compounds, particularly 17-hydroxylated pregnene derivatives [11–13]. Additionally, in neonates and during pregnancy we found evidence of 16α-hydroxylated dehydrosteroids, including a dehydroestriol [14]. A major compound in SLOS urine was tentatively identified as a 7- or 8-dehydro-3,17α,20-triol, and this C_{21} steroid was potentially a good analyte for diagnosing SLOS by urine analysis [11–13]. The amount of isolated analytes was insufficient for de novo structure determination, and no reference compounds were available as aids for identification. To address this deficiency, we have undertaken the preparation of authentic standards. We describe here the chemical synthesis of 7- and 8-dehydro derivatives of pregnane-3,17α,20-triols.

* Corresponding author. Tel.: +1-713-348-4914; fax: +1-713-348-5154.
E-mail address: billw@rice.edu (W.K. Wilson).

0039-128X/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0039-128X(02)00113-7

2. Experimental procedures and results

2.1. Materials and methods

1,3-Dibromo-5,5-dimethylhydantoin (dibromantin), diisobutylaluminum hydride (DIBALH), L-Selectride, diethyl azodicarboxylate (DEAD), 4-methoxypyridine-*N*-oxide, tetrabutylammonium bromide, tetrabutylammonium fluoride, palladium on carbon, and aluminum–nickel alloy were purchased from Aldrich (Milwaukee, WI). TES buffer (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, sodium salt), cholesterol oxidase from *Streptomyces* sp., catalase, and 17α-hydroxypregnenolone diacetate (**1**) and 3-acetate (**17**) were obtained from Sigma (St. Louis, MO). Pyrophoric Raney nickel was prepared by slowly adding sodium hydroxide pellets (27 g) to a suspension of aluminum–nickel alloy 50/50 w/w (14 g) in water (200 ml), heating at 90 °C for 1.5 h, and then washing with water (10 × 200 ml) and ethanol (4 × 200 ml). 4-Methoxypyridine was prepared by hydrogenation of 4-methoxypyridine-*N*-oxide over Raney nickel. Solvents were Omnisolve grade from EM Science (Gibbstown, NJ).

Low- and high-resolution mass spectra (HR-MS) were acquired on a ZAB-HF or MAT-95 mass spectrometer in the electron-impact mode. Mass spectral data are presented as *m*/*z* (% relative abundance); fragment ions representing loss of H_2O, CH_3, CH_3COOH, CH_3CHOH (side chain of 20-hydroxysteroids), CH_3CO (side chain of 20-ketosteroids), and combinations thereof are marked by an asterisk (∗). Medium pressure liquid chromatography (MPLC) was done on glass columns dry-packed with silica gel (230–400 mesh). High performance liquid chromatography (HPLC) was carried out on a preparative 5 μm Prodigy ODS(3) column (250 mm × 21.2 mm i.d.; Phenomenex, Torrance, CA) with UV detection at 210 nm. Nuclear magnetic resonance (NMR) spectra of steroids (2–15 mM in $CDCl_3$) were acquired at 25 °C as described [15] on an AMX500, Avance 500, or Inova 500 spectrometer and referenced to internal tetramethylsilane (^1H) or $CDCl_3$ at 77.0 ppm (^{13}C). NMR signal assignments were made from 1D and 2D spectra as described previously [15], and chemical shifts were corrected for effects of strong coupling. The purity of steroid samples was estimated by ^1H NMR (500 MHz spectrum; methyl region and δ 2.5–7.0 region). Steroid structures were modeled by molecular mechanics with PCMODEL 7.0 (Serena Software, Bloomington, IN).

2.2. Chemical syntheses

The chemical synthesis of compounds **2**–**15** are summarized in Figs. 1 and 2. Because of the reported lability of 17-hydroxy-20-ketosteroids in acidic media [16], reactions were conducted under neutral or basic conditions (except for attempted diene isomerizations in HCl).

2.2.1. 3β,17α-Dihydroxypregna-5,7-dien-20-one diacetate (2)

To a solution of 17α-hydroxypregnenolone diacetate (**1**; 2.03 g, 4.8 mmol) in benzene–hexane 1:1 (120 ml) was added dibromantin (0.84 g, 2.92 mmol, 1.2 equivalents) and 2,2′-azobisisobutyronitrile (32 mg). The mixture was refluxed under nitrogen for 10 min in a preheated 100 °C oil bath and then placed in an ice bath to cool. Insoluble material was removed by suction filtration, followed by evaporation of the filtrate to a yellow solid. To a solution of this yellow solid in anhydrous tetrahydrofuran (40 ml) was added tetrabutylammonium bromide (0.4 g). The resulting solution was stirred for 75 min under nitrogen at room temperature. To this reaction mixture was added tetrabutylammonium fluoride (10 ml, 1 M solution in tetrahydrofuran, 10 mmol, 2.1 equivalents). The resulting dark brown solution was stirred for an additional 50 min, followed by rotary evaporation at 40–45 °C to a brown solid. A solution of this solid in ethyl acetate (200 ml) was washed with water (3 × 50 ml) and dried over anhydrous Na_2SO_4. Evaporation of solvent gave crude **2** (2.03 g), which was subjected to MPLC (370 mm × 25 mm i.d. column; elution with hexane–ethyl acetate 95:5, 4000 ml and hexane–ethyl acetate 92:8, 2000 ml). Fraction volumes were 20 ml. After elution of **1** in fractions 161–198, the $\Delta^{5,7}$ product was eluted in fractions 199–257, which were evaporated to give **2** (0.97 g, 49% yield) of ∼96% purity (containing 0.5–1.3% each of several minor olefins). ^1H and ^{13}C NMR (Tables 1 and 2); HR-MS, calculated for $C_{23}H_{30}O_3$ (M–AcOH) 354.2195, found 354.2190; MS *m*/*z* 354 (5), 294* (7), 279* (25), 251* (100).

2.2.2. 3β,17α-Dihydroxypregna-5,7-dien-20-one (3)

To a solution of diacetate **2** (100 mg) in a 1:2 mixture of tetrahydrofuran and methanol (24 ml) was added potassium carbonate (140 mg). The resulting mixture was sparged with nitrogen and then stirred at room temperature under nitrogen for 46 h. After completion of the reaction as judged by TLC, water (60 ml) was added. The resulting mixture was extracted with ethyl acetate (2 × 60 ml). The combined organic phase was washed with water (2 × 30 ml) and dried over anhydrous Na_2SO_4. Evaporation to a white solid (64 mg) and recrystallization from methanol–water gave **3** as a white solid (56 mg, 71% yield) of ∼90% purity (containing 1–3% each of several minor olefins). ^1H and ^{13}C NMR (Tables 1 and 2); HR-MS, calculated for $C_{21}H_{30}O_3$ 330.2195, found 330.2200; MS *m*/*z* 330 (53, M^+), 312* (60), 297* (46), 287* (45), 284 (45), 279* (63), 269* (100), 251* (99), 244 (76), 211 (99), 143 (73).

2.2.3. 17α-Hydroxypregna-4,7-diene-3,20-dione (4)

A solution of **3** (25 mg, 0.075 mmol) in butyl acetate (7 ml) was added to a TES buffer solution (6 ml, 50 mM, pH 7.5) containing cholesterol oxidase from *Streptomyces* sp. (50 units, 2.6 mg, 19 units/mg solid) and catalase (40,000 units). The two-phase mixture was stirred in a vial

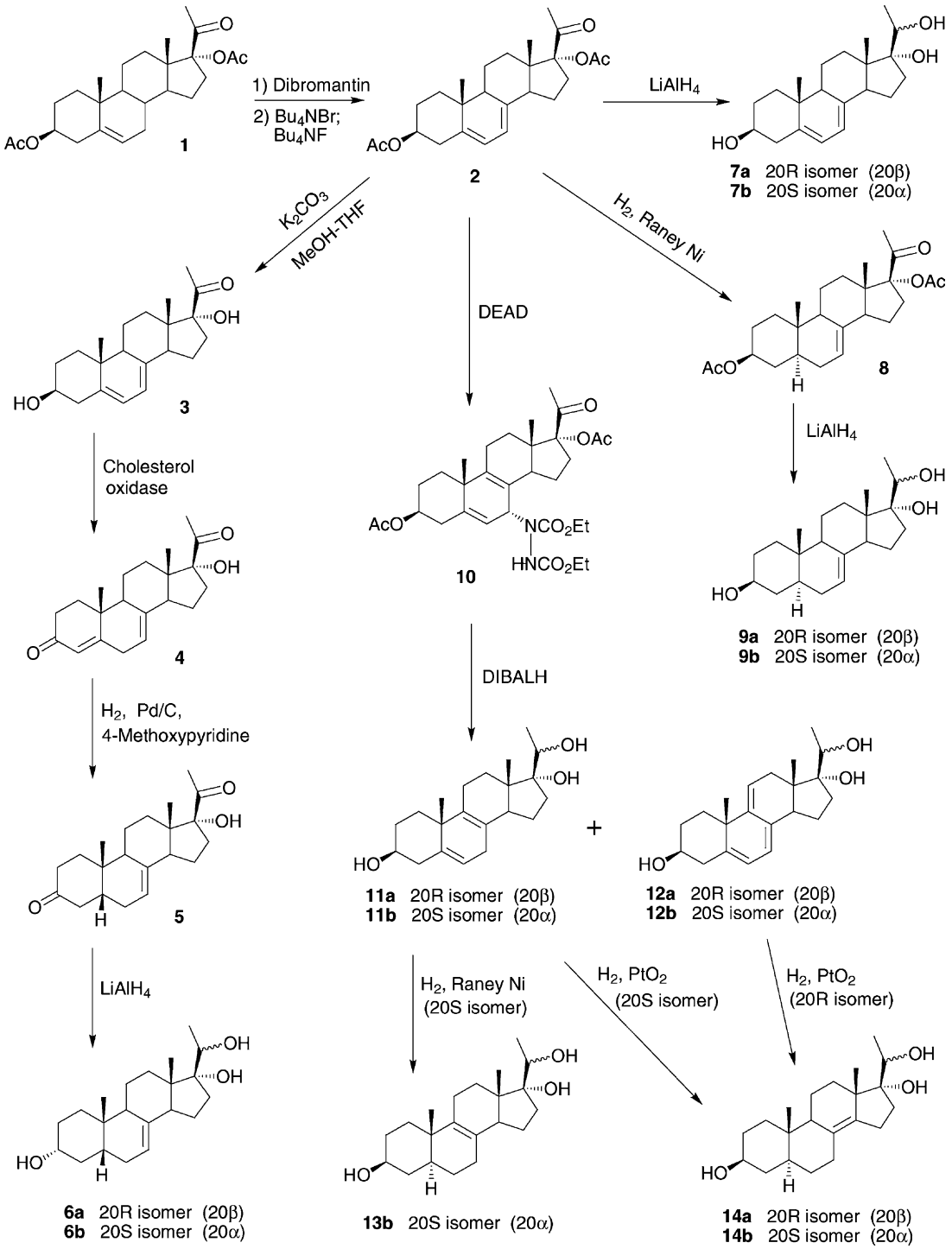

Fig. 1. Chemical synthesis of pregnane-3,17α,20-triols with unsaturation in rings B and C.

at room temperature with a magnetic stirrer for 17 h. The mixture was extracted with ethyl acetate (20 ml), and the separated organic phase was washed with water and brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave crude **4** (24 mg) as a nearly white solid in ~80% purity (containing 6% of the $\Delta^{4,6}$ isomer and 1–11% each of several minor olefins). ^1H and ^{13}C NMR (Tables 1 and 2); HR-MS, calculated for $C_{21}H_{28}O_3$ 328.2038, found 328.2029; MS *m/z* 328 (38, M^+), 310* (37), 285* (100), 267* (50), 242 (53), 227 (58), 215 (43), 105 (38).

2.2.4. 17α-Hydroxy-5β-pregn-7-ene-3,20-dione (5)

To a solution of **4** (20 mg) in 4-methoxypyridine (1.5 ml) was added palladium on carbon (20 mg; 10% Pd). This

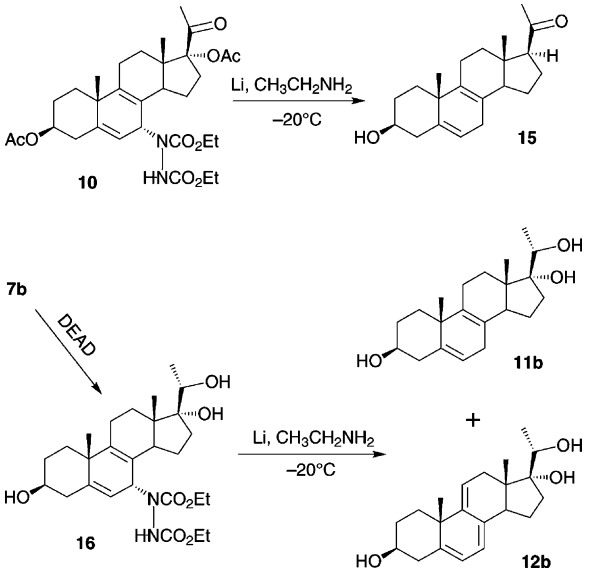

Fig. 2. Lithium-ethylamine reductions of $\Delta^{5,8}$ DEAD adducts **10** and **15**.

mixture was stirred at room temperature under a hydrogen-filled balloon for 16 h. The catalyst was removed by filtration through Celite. Removal of solvent by bulb-to-bulb distillation gave crude **5** (20 mg) as a nearly white solid in ~75% purity (containing 1–10% each of several minor olefins). ^{1}H and ^{13}C NMR (Tables 1 and 2); HR-MS, calculated for $C_{21}H_{30}O_3$ 330.2195, found 330.2190; MS *m/z* 330 (68, M^+), 312* (12), 297* (13), 287* (85), 269* (66), 244 (72), 229 (63), 211 (71), 105 (100). Several of the ^{13}C NMR signals were severely broadened, as shown in Fig. 3A.

*2.2.5. 5β-Pregn-7-ene-3α,17α,20R-triol (**6a**) and its 20S epimer **6b***

To a solution of crude **5** (20 mg) in ether (5 ml) was added $LiAlH_4$ (50 mg). The resulting mixture was refluxed for 2 h, followed by addition of cold 5% HCl (5 ml) to quench the reaction. The organic phase was separated, washed with water, and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude product (21 mg) comprising an 8:9 mixture of

Table 1
^{1}H NMR chemical shifts of unsaturated pregnanetriols **6a**–**7b** and their synthetic precursors **1**–**5**[a,b]

	Δ^5	$\Delta^{5,7}$	$\Delta^{5,7}$	$\Delta^{4,7}$	5β-Δ^7	5β-Δ^7		$\Delta^{5,7}$	
	1[c]	**2**[c]	**3**	**4**	**5**	3α,20β (**6a**)	3α,20α (**6b**)	3β,20β (**7a**)	3β,20α (**7b**)
H-1α	1.166	1.378	1.317	1.982†	2.053	1.760†	1.753†	1.315	1.317
H-1β	1.878†	1.908	1.892†	1.915†	1.458	1.058	1.063	1.888†	1.892†
H-2α	1.879†	1.933†	1.893†	2.371†	2.466	1.451	1.437	1.899†	1.892†
H-2β	1.600	1.582	1.498	2.350†	2.284	1.776†	1.777†	1.499	1.500
H-3	4.611	4.715	3.651			3.612	3.613	3.650	3.651
H-4α	2.344†	2.514	2.481	5.805	2.349	1.364†	1.361†	2.475	2.478
H-4β	2.317†	2.367	2.285†		2.11	1.515	1.518	2.286	2.286
H-5					1.817	1.361†	1.361†		
H-6(α)	5.386	5.577	5.582	2.694	1.655	1.622	1.633	5.573	5.580
H-6β				3.159	2.415†	2.415	2.415		
H-7(α)	1.638	5.451	5.455	5.265	5.211	5.128	5.166	5.414	5.446
H-9α	1.053	2.060	2.008	2.241†	–[d]	2.124	2.120†	1.992†	1.992†
H-11α	1.655	1.72	1.70	1.755	1.734	1.587	1.589†	1.67	1.66
H-11β	1.468	1.71	1.68	1.606	1.511	1.458	1.432	1.73	1.72
H-12α	1.981	2.047	1.776†	1.856	1.864	1.713†	1.755	1.70	1.74*
H-12β	1.558	1.650	1.473	1.479	1.464	1.760†	1.598†	1.82	1.66*
H-14α	1.700†	2.616	2.608	2.562†	2.609	2.525	2.560	2.599	2.634
H-15α	1.704†	1.853†	1.961	1.823†	1.797†	1.673†	1.693	1.860†	1.881
H-15β	1.277	1.579†	1.669†	1.688†	1.708†	1.482†	1.527	1.465	1.507
H-16α	1.753	1.840†	1.705†	1.684†	1.694†	1.536†	1.769	1.564	1.806
H-16β	2.936	2.990	2.699	2.695	2.687	1.675†	2.077	1.711	2.109
H-18	0.643	0.587	0.698	0.662	0.647	0.680	0.613	0.756	0.691
H-19	1.025	0.949	0.948	1.182	1.000	0.870	0.862	0.959	0.949
H-20						4.021	3.835	4.050	3.866
H-21	2.118	2.106	2.301	2.298	2.298	1.185	1.206	1.194	1.226

Chemical shifts given to two (three) decimal places are accurate to ±0.01 (±0.001) ppm except that values marked by dagger (†) are accurate to ca. ±0.003 ppm. Assignments of diastereotopic pairs marked with an asterisk (*) may be interchanged.

[a] Data obtained at 500 MHz in 5–15 mM $CDCl_3$ solution at 25 °C and referenced to $Si(CH_3)_4$.

[b] Coupling constants were generally similar to those of C_{27} sterols with the same double bond configuration (described in [15]) except for H-20 (q, 6.3 Hz) and ring-A couplings of 5β steroids.

[c] Acetate signals: **1**, δ 2.039 (s), 2.043 (s); **2**, δ 2.050 (s), 2.075 (s); other signals for **1**: δ 1.553 (tdd, 12.0, 10.4, 5.1 Hz, H-8β), 2.023 (m, H-7β).

[d] Not measured.

Table 2
^{13}C NMR chemical shifts of unsaturated pregnanetriols **6a**–**7b** and their synthetic precursors **1**–**5**[a,b]

	Δ^5	$\Delta^{5,7}$	$\Delta^{5,7}$	$\Delta^{4,7}$	5β-Δ^7	5β-Δ^7		$\Delta^{5,7}$	
	1	**2**	**3**	**4**	**5**[c]	3α,20β (**6a**)	3α,20α (**6b**)	3β,20β (**7a**)	3β,20α (**7b**)
C-1	36.97	37.88	38.28	33.16	35.5†	34.71	34.67	38.32	38.32
C-2	27.72	28.03	31.94	34.08	37.70	31.49	31.45	31.97	31.96
C-3	73.79	72.59	70.32	199.21	212.46	71.31	71.26	70.39	70.37
C-4	38.04	36.59	40.73	122.88	43.47	37.65	37.61	40.75	40.75
C-5	139.63	139.13	140.11*	168.28	41.9†	40.96	40.90	139.73	139.82
C-6	122.20	120.02	119.45	32.80	28.34	28.66	28.66	119.51	119.55
C-7	31.79	117.76	117.37	116.72	115.99	115.65	116.05	116.58	116.93
C-8	31.92	139.52	140.17*	138.71	137.25	137.53	–[d]	141.48	140.94
C-9	49.39	45.65	45.93	45.51	~36 ± 2	36.69	36.62	46.11	46.01
C-10	36.56	37.10	37.07	37.97	33.82	33.54	33.51	37.02	37.00
C-11	20.64	20.55	20.57	21.38	21.7†	21.34	21.12	20.81	20.62
C-12	31.06	30.67	29.57	29.49	30.11	32.33	31.22	31.67	30.65
C-13	46.76	46.90	48.66	49.01	49.5†	48.26	46.6†	47.54	45.97
C-14	51.83	49.94	49.07	49.54	49.87	49.42	50.28	48.88	49.80
C-15	24.03	22.83	22.98	22.68	22.49	22.31	21.99	22.63	22.29
C-16	30.43	30.50	33.67	33.54	33.61	33.81	37.61	33.82	37.73
C-17	97.04	97.06	90.15	89.92	90.05	85.51	85.9†	85.55	86.03
C-18	14.25	14.31	15.45	15.45	15.49	15.27	14.19	15.45	14.22
C-19	19.28	16.22	16.34	21.29	23.47	24.50	24.47	16.34	16.31
C-20	204.19	203.86	211.40	211.23	211.42	70.57	72.35	70.39	72.27
C-21	26.38	26.39	27.87	27.81	27.83	18.67	18.75	18.71	18.74

Assignments marked by an asterisk (*) may be interchanged. Values marked by dagger (†) were estimated to ca. ±0.1 ppm from HSQC, HMBC, or 1D spectra.

[a] Data obtained at 125 MHz at 25 °C in 2–30 mM $CDCl_3$ solution and referenced to the $CDCl_3$ signal at 77.0 ppm.

[b] Acetate signals: **1**, δ 21.27, 21.42, 170.56, 170.79; **2**, δ 21.21, 21.41, 170.60, 170.71.

[c] Several signal of **5** were severely broadened at 25 °C (see Fig. 3A).

[d] Not measured.

C-20 epimers **6a** and **6b**. A portion (10 mg) of this mixture was subjected to preparative reverse-phase HPLC (elution with methanol–water 8:2 at 9 ml/min) to give homogenous samples of the 20*R* isomer **6a** (t_R 21.1 min, 3.4 mg, ~97% purity) and the 20*S* isomer **6b** (t_R 23.1 min, 3.1 mg, ~98% purity). ^{1}H and ^{13}C NMR for **6a** and **6b** (Tables 1 and 2); HR-MS, calculated for $C_{21}H_{34}O_3$ 334.2508, found 334.2520 for **6a**, found 334.2510 for **6b**; MS of **6a** and **6b** (Table 3).

2.2.6. *Pregna-5,7-diene-3β,17α,20R-triol (**7a**) and its 20S epimer **7b***

To an ice-cold solution of $\Delta^{5,7}$ diacetate **2** (51 mg, 0.124 mmol) in anhydrous tetrahydrofuran (10 ml) was added an excess of $LiAlH_4$ (73 mg, 1.92 mmol). The resulting mixture was stirred under nitrogen in an ice bath for 1.5 h, followed by addition of cold 5% HCl (15 ml) and extraction with ethyl acetate (3 × 15 ml). The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated to a white crystalline solid (41 mg) consisting of a 3:4 mixture of **7a** and **7b**. This mixture was separated by MPLC (elution with toluene–ethyl acetate 10:3; 6-ml fraction volumes). Evaporation of fractions 42–57 gave **7a** (15 mg, 97% purity), and evaporation of fractions 73–100 gave **7b** (16 mg, 98% purity). ^{1}H and ^{13}C NMR of **7a** and **7b** (Tables 1 and 2); HR-MS, calculated for $C_{21}H_{32}O_3$

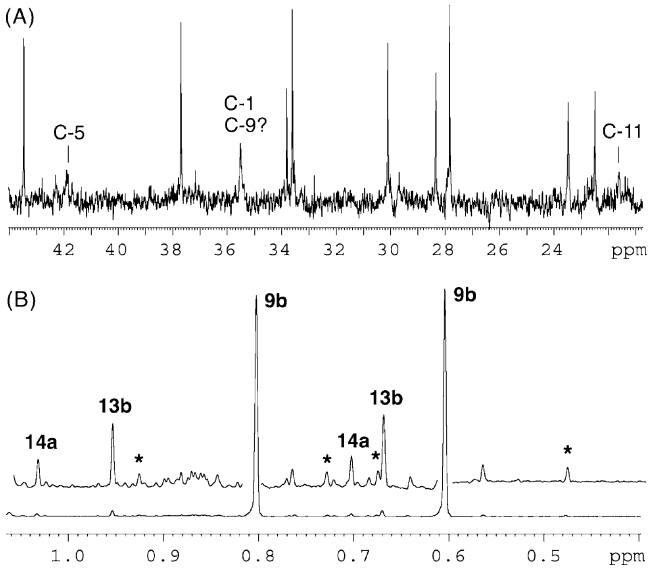

Fig. 3. (A) ^{13}C NMR spectrum (125 MHz; upfield portion) of 5β-Δ^7-3,20-diketone **5**, showing the broadening of certain signals in rings B and C. The position of C-9 is uncertain. (B) ^{1}H NMR spectrum (500 MHz; upfield methyl region) of **9b**, giving a representative illustration of the purity of the unsaturated pregnane-3,17α,20-triols. Signals of the major contaminants (3% **13b** and 1.5% **14a**) are labeled, and ^{13}C satellites are marked by an asterisk (*).

Table 3
Comparison of ion abundances in the electron-impact mass spectra of unsaturated pregnanetriols[a,b,c]

Ion assignment	m/z	Relative abundance (%)							m/z	Relative abundance (%)			
		Δ^7				Δ^8	$\Delta^{8(14)}$			$\Delta^{5,7}$		$\Delta^{5,8}$	
		6a	**6b**	**9a**	**9b**	**13b**	**14a**	**14b**		**7a**	**7b**	**11a**	**11b**
M^+	334	5	1	0	0	0.4	36	28	332	4	0	2	2
$M–H_2O$	316	3	2	4	10	3	13	17	314	7	9	10	8
$M–H_2O–CH_3$	301	12	7	21	18	12	18	16	299	3	1	16	14
$M–2H_2O$	298	10	3	4	12	1	10	9	296	3	22	6	4
M–SC	289	1	1	1	3	1	33	29	287	1	1	2	1
$M–2H_2O–CH_3$	283	17	7	5	25	4	12	11	281	8	46	28	23
$M–H_2O–SC$	271	100	100	100	100	100	31	73	269	100	78	100	100
$M–2H_2O–SC+2H$	255	7	4	5	36	2	7	11	253	5	47	19	6
$M–2H_2O–SC$	253	39	23	9	16	3	8	11	251	21	33	15	15
$M–SC–C_2H_3O$	246	8	4	2	6	3	100	100	244	1	3	6	2
$M–SC–C_3H_6O$	231	4	2	5	9	5	29	28	229	2	8	9	4
$M–H_2O–SC–C_2H_3O$	228	9	4	1	5	1	21	18	226	2	12	8	6
$M–H_2O–SC–C_3H_6O$	213	18	10	9	26	6	39	35	211	9	36	18	18

SC, side chain (C_2H_4O).
[a] Spectra of underivatized triols were obtained by direct probe using electron-impact ionization (70 eV).
[b] Molecular formulas of representative fragment ions were confirmed by HR-MS. Losses of C_2H_3O and C_3H_6O evidently arose from cleavage in ring D.
[c] Additional ions: **6a**, 105 (29); **6b**, 105 (18); **7a**, 143 (14); **7b**, 263 (41), 143 (100); **9a**, 105 (21); **9b**, 105 (62); **11a**, 143 (28); **11b**, 143 (24); **13b**, 105 (14); **14a**, 147 (54); **14b**, 147 (57).

332.2351, found 332.2355 for **7a**, found 332.2351 for **7b**; MS of **7a** and **7b** (Table 3).

*2.2.7. 3β,17α-Dihydroxy-5α-pregn-7-en-20-one diacetate (**8**)*

Freshly prepared Raney nickel was added to a solution of $\Delta^{5,7}$ diacetate **2** (50 mg) in freshly distilled ethyl acetate (15 ml). The system was repeatedly evacuated and filled with hydrogen. The mixture was then stirred at room temperature under a hydrogen-filled balloon for 1 h. After filtration of the catalyst, the solvent was evaporated to give **8** as a white solid (55 mg, ~94% purity, containing 3% of the Δ^8 isomer). ^1H and ^{13}C NMR (Tables 4 and 5); HR-MS, calculated for $C_{25}H_{36}O_5$ 416.2563, found 416.2566; MS *m/z* 356 (27, M–AcOH), 341* (27), 313* (100), 281* (12), 253* (14); NMR data for the Δ^8 isomer: δ_H 0.565 (s, H-18), 0.966 (s, H-19); δ_C 13.7 (C-18), 17.8 (C-19). Similar hydrogenation of diol **3** resulted in concomitant hydrogenation of the 20-keto group, producing a mixture of Δ^7 and $\Delta^{8(14)}$ triols epimeric at C-20 (Table 6).

*2.2.8. 5α-Pregn-7-ene-3β,17α,20R-triol (**9a**) and its 20S epimer **9b***

To an ice-cold solution of diacetate **8** (50 mg, 0.120 mmol) in anhydrous tetrahydrofuran (10 ml) was added an excess of $LiAlH_4$ (72 mg, 1.91 mmol). The resulting mixture was stirred under nitrogen in an ice bath for 1.5 h, followed by addition of cold 5% HCl (15 ml) and extraction with ethyl acetate (3 × 15 ml). The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated to a white crystalline solid (39 mg) consisting of a 2:3 mixture of **9a** and **9b**. This mixture was separated by MPLC (elution with toluene–ethyl acetate 10:3; 6-ml fraction volumes). Evaporation of fractions 49–70 gave **9a** (13 mg, 95% purity, containing 3% of the 20*R* epimer of **13b**), and evaporation of fractions 77–110 gave **9b** (16 mg, 95% purity, containing 3% **13b** and 1.5% **14a**, as shown in Fig. 3B). ^1H and ^{13}C NMR of **9a** and **9b** (Tables 4 and 5); HR-MS of **9a**, calculated for $C_{21}H_{34}O_3$ 334.2508, found 334.2512; HR-MS of **9b**, calculated for $C_{21}H_{32}O_2$ ($M–H_2O$) 316.2402, found 316.2408; MS of **9a** and **9b** (Table 3). NMR data for the 20*R* epimer of **13b**: δ_H 0.747 (s, H-18), 0.961 (s, H-19), 2.726 (m, H-14α); δ_C 14.3 (C-18), 17.9 (C-19).

*2.2.9. 3β,17α-Dihydroxy-7α-[N,N′-bis(ethoxycarbonyl) hydrazino]pregna-5,8-dien-20-one 3,17-diacetate (**10**)*

A solution of **2** (0.30 g, 0.72 mmol) and DEAD (0.45 g, 2.58 mmol, 3.6 equivalents) in benzene (5 ml) was refluxed under nitrogen at 100 °C for 4.5 h. After cooling, this solution was subjected directly to MPLC (elution with hexane–ethyl acetate 7:3; 15-ml fraction volumes). Fractions 11–13 contained the Diels–Alder adduct (41 mg; NMR δ_H 0.748 (s), 0.954 (s), 2.031 (s), 2.059 (s), 2.178 (s), 6.092 (d, 8.3 Hz), 6.559 (d, 8.3 Hz)), and fractions 17–18 contained the $\Delta^{5,8(14)}$ adduct (7 mg). Evaporation of fractions 20–41 gave the $\Delta^{5,8}$ adduct **10** as the major product (0.32 g, ~98% purity). ^1H NMR, δ 0.613 (s, H-18), 1.195 (s, H-19), 2.079 (s, H-21), 2.03 and 2.14 (br s, acetates), 3.04 (m), 4.0–6.2 (many broad signals); HR-MS, calculated for $C_{31}H_{44}N_2O_9$ 588.3047, found 588.3036; MS *m/z* 412 (7, $M–C_6H_{12}N_2O_4$), 397* (3), 353* (69), 293* (43), 277* (44), 250* (100), 235 (30), 157 (30).

Table 4
^{1}H NMR data for unsaturated pregnanetriols **9a**–**14b** and 20-ketosteroids **8** and **15**[a]

	5α-Δ^7	5α-Δ^7		Δ^{5,8}		Δ$^{5,7,9(11)}$		5α-Δ^8	5α-Δ$^{8(14)}$		Δ^{5,8}
		3β,20β	3β,20α	3β,20β	3β,20α	3β,20β	3β,20α	3β,20α	3β,20β	3β,20α	
	8[b]	**9a**	**9b**	**11a**	**11b**	**12a**	**12b**	**13b**	**14a**	**14b**	**15**[b]
H-1α	1.160	1.093	1.095	1.370	1.368	1.529	1.526†	1.228	1.114	1.115	1.378
H-1β	1.858†	1.836	1.829	1.889†	1.875†	1.712†	1.701†	1.761	1.715	1.703	1.886
H-2α	1.832†	1.800	1.802	1.892†	1.892†	1.934	1.933†	1.851	1.826	1.827	1.900
H-2β	1.486	1.391	1.391	1.561	1.563	1.710†	1.706†	1.444	1.357	1.354	1.568
H-3α	4.703	3.604	3.606	3.554	3.556	3.614	3.613†	3.625	3.627	3.627	3.551
H-4α	1.759	1.723	1.728	2.351†	2.355†	2.382	2.384	1.655	1.639	1.641	2.357†
H-4β	1.364	1.276	1.277	2.314†	2.313†	2.473	2.474	1.327	1.265†	1.265†	2.313†
H-5α	1.464	1.410	1.410					1.414	1.260†	1.263†	
H-6(α)	1.78	1.763†	1.77*	5.434	5.440	5.682	5.689	1.392	1.339	1.344	5.433
H-6β	1.74	1.744†	1.75*					1.519	1.225†	1.214†	
H-7(α)	5.232	5.184	5.221	2.544†	2.555†	5.432	5.453	1.99*	1.834	1.837	2.530†
H-7β				2.582†	2.594†			2.01*	2.400	2.410	2.580†
H-9α	1.734†	1.671†	1.658†						1.705†	1.698†	
H-11(α)	1.708†	1.651†	1.652†	2.18*	2.18	5.579	5.542	2.202†	1.676	1.675†	2.22
H-11β	1.472†	1.484†	1.458	2.19*	2.18			2.064†	1.508	1.491†	2.24
H-12α	1.573	1.69	1.713	1.838	1.874	2.578	2.692	1.810	1.593	1.670†	1.704
H-12β	2.038†	1.74	1.581	1.746	1.556	2.081	1.919	1.538	1.693	1.490†	2.068
H-14α	2.526	2.503	2.533	2.799	2.826	2.827	2.855	2.751			2.273†
H-15α	1.690	1.673†	1.704†	1.769	1.792†	1.915	1.912†	1.758†	2.402†	2.384†	1.731†
H-15β	1.569	1.463	1.510	1.352	1.408	1.502	1.539†	1.383	2.326†	2.333†	1.421
H-16α	1.782	1.528†	1.761†	1.561	1.793†	1.613	1.857†	1.760†	1.528	1.867	1.727†
H-16β	2.948	1.663†	2.068	1.741	2.149	1.760	2.119	2.135†	1.747	2.006	2.276
H-18	0.501	0.670	0.604	0.789	0.709	0.697	0.614	0.670	1.033	0.948	0.606
H-19	0.813	0.810	0.802	1.199	1.191	1.255	1.251	0.953	0.702	0.696	1.186
H-20		4.022	3.831	4.058	3.855	4.045	3.880	3.835	4.022	3.869	
H-21	2.111	1.180	1.201	1.191	1.216	1.199	1.209	1.205	1.198	1.226	2.144

Chemical shifts given to two (three) decimal places are accurate to ±0.01 (±0.001) ppm except that values marked by dagger (†) are accurate to ca. ±0.003 ppm. Assignments of diastereotopic pairs marked with an asterisk (*) may be interchanged.

[a] Data obtained at 500 MHz in 5–15 mM $CDCl_3$ solution at 25 °C and referenced to $Si(CH_3)_4$. Coupling constants were generally similar to those of C_{27} sterols with the same double bond configuration (described in [15]) except for H-20 (q, 6.3 Hz) and ring-A couplings of 5β steroids.

[b] Additional signals: acetate signals for **8**: δ 2.033 (s), 2.053 (s); H-17α of **15**, δ 2.601 (br t, 8.8 Hz).

*2.2.10. Pregna-5,8-diene-3β,17α,20R-triol (**11a**), its 20S epimer **11b**, pregna-5,7,9(11)-triene-3β,17α,20R-triol (**12a**), and its 20S epimer **12b***

To a solution of DEAD derivative **10** (170 mg, 0.29 mmol) in anhydrous tetrahydrofuran (25 ml) was added dropwise DIBALH (1.0 M solution in tetrahydrofuran, 7.2 ml, 25 equivalents). The resulting solution was stirred at room temperature for 50 min, followed by addition of cold 5% HCl (20 ml). After extraction with ethyl acetate (3 × 20 ml), the combined organic phase was dried over anhydrous Na_2SO_4. Evaporation of solvent gave a 3:3:3:4 mixture of **11a**, **11b**, **12a**, and **12b** as a white solid (138 mg). Although this mixture was essentially inseparable on silica gel using toluene–ethyl acetate 10:3, a portion of the product (60 mg) was separated on reverse-phase HPLC (elution with methanol–water 65:35 at 8 ml/min) to give **12b** (t_R 28.5 min, 13 mg, 98% purity), **11b** (t_R 32.8 min, 12 mg, ~97% purity), **12a** (t_R 33.9 min, 6 mg, 98% purity), and **11a** (t_R 42.2 min, 10 mg, 97% purity). ^{1}H and ^{13}C NMR of **11a**, **11b**, **12a**, and **12b** (Tables 4 and 5); HR-MS of **11a**, calculated for $C_{21}H_{32}O_3$ 332.2351, found 332.2346; HR-MS of **11b**, calculated for $C_{21}H_{30}O_2$ (M–H_2O) 314.2246, found 314.2229; MS of **11a** and **11b** (Table 3); HR-MS of trienetriols, calculated for $C_{21}H_{30}O_3$ 330.2195, found 330.2189 for **12a**, found 330.2191 for **12b**; MS of **12a** *m/z* 330 (21, M^+), 312* (10), 297* (22), 294* (18), 279* (90), 276 (41), 267* (61), 261 (100), 251 (40), 209 (92); MS of **12b** *m/z* 330 (33, M^+), 312* (5), 297* (19), 279* (73), 276 (47), 269 (100), 267* (63), 261 (70), 251 (25), 209 (54).

*2.2.11. 5α-Pregn-8-ene-3β,17α,20S-triol (**13b**)*

To a solution of **11b** (8 mg) in freshly distilled ethyl acetate (2 ml) was added Raney nickel (~5 mg). The resulting mixture was stirred at room temperature under a hydrogen-filled balloon. Analysis of reaction aliquots by NMR after 10, 20, 30, and 40 min indicated a mixture of Δ^7, Δ^8, and Δ$^{8(14)}$ triols (Table 7). The catalyst was removed by filtration through Celite, and the filtrate was evaporated to a white solid (8 mg). This isomeric mixture was subjected to reverse-phase HPLC (elution with methanol–water

Table 5
^{13}C NMR data for unsaturated pregnanetriols **9a**–**14b** and 20-ketosteroids **8** and **15**[a]

	5α-Δ^7	5α-Δ^7		Δ^{5,8}		Δ$^{5,7,9(11)}$		5α-Δ^8	5α-Δ$^{8(14)}$		Δ^{5,8}
	3β,20β	3β,20α		3β,20β	3β,20α	3β,20β	3β,20α	3β,20α	3β,20β	3β,20α	
	8[b]	**9a**	**9b**	**11a**	**11b**	**12a**	**12b**	**13b**	**14a**	**14b**	**15**
C-1	36.81	37.12	37.12	35.76	35.69	38.45	38.43	35.20	36.46	36.45	35.62
C-2	27.42	31.45	31.43	31.96	31.90	32.24	32.22	31.66	31.52	31.50	31.88
C-3	73.29	71.00	70.98	71.45	71.43	72.24	72.25	71.15	71.15	71.13	71.34
C-4	33.69	37.95	37.92	42.22	42.18	41.57	41.55	38.31	38.25	38.24	42.16
C-5	40.05	40.25	40.21	138.88	138.82	141.51	141.54	40.77	44.12	44.09	138.82
C-6	29.46	29.64	29.65	119.35	119.34	118.14	118.16	25.45	28.66	28.64	119.26
C-7	119.20	117.76	118.18	29.11	29.08	115.99	116.15	27.26	29.50	29.55	29.01
C-8	138.02	139.61	139.09	126.68	126.40	135.47	135.10	128.29	129.50	129.35	125.97
C-9	48.81	49.28	49.17	131.62	131.80	143.58	143.70	134.75	49.01	48.79	132.22
C-10	34.21	34.22	34.20	37.43	37.41	39.40	39.41	35.77	36.81	36.75	37.42
C-11	20.96	21.19	20.97	22.04	22.04	122.70	122.05	22.49	19.57	19.26	22.21
C-12	30.90	31.95	30.85	29.80	28.75	35.27	34.26	28.68	29.33	28.15	35.87
C-13	47.26	47.95	46.38	46.68	45.39	47.07	45.30	45.36	47.74	46.23	43.56
C-14	50.53	49.34	50.22	46.17	46.99	45.69	46.44	47.09	139.10	139.68	51.90
C-15	22.72	22.41	22.11	22.65	22.20	22.30	21.73	23.06	24.45	24.07	23.29
C-16	30.38	33.65	37.51	34.68	38.54	34.24	38.05	38.51	32.25	34.71	23.18
C-17	97.15	85.52	86.05	84.50	84.99	85.15	85.25	85.07	85.75	86.03	62.26
C-18	14.31	15.24	14.17	14.41	13.69	14.87	13.97	13.47	22.74	21.67	12.62
C-19	12.94	13.06	13.06	22.87	22.80	30.41	30.38	17.87	12.85	12.86	22.84
C-20	203.95	70.54	72.34	70.80	72.49	70.28	72.21	72.49	69.43	71.45	209.43
C-21	26.34	18.65	18.74	18.59	18.67	18.61	18.32	18.71	19.06	18.62	31.39

[a] Data obtained at 125 MHz at 25 °C in 2–30 mM $CDCl_3$ solution and referenced to the $CDCl_3$ signal at 77.0 ppm.
[b] Acetate signals for **8**: δ 21.44, 170.72, 21.22, 170.72.

55:45) to give **13b** (4 mg, containing 13% **9b**). ^{1}H and ^{13}C NMR (Tables 4 and 5); HR-MS, calculated for $C_{21}H_{32}O_2$ (M–H_2O) 316.2402, found 316.2398; MS (Table 3).

2.2.12. 5α-Pregn-8(14)-ene-3β,17α,20R-triol (14a)

The Δ$^{5,7,9(11)}$ triol **12a** (10 mg) was hydrogenated for 3.5 h over PtO_2 (7 mg) as described for the conversion of **11b** to **14b**. MPLC (elution with hexane–ethyl acetate 7:3) of this crude product (10 mg) gave **14a** (6.5 mg, 98% purity). ^{1}H and ^{13}C NMR (Tables 4 and 5); HR-MS, calculated for $C_{21}H_{34}O_3$ 334.2508, found 334.2530; MS (Table 3).

Table 7
Products formed in the catalytic hydrogenation of Δ^{5,8} triol **11b** over Raney nickel after reaction times of 10–40 min[a]

Reaction time (min)	Percentage composition			
	Δ^{5,8} (**11b**)	Δ^7 (**9b**)	Δ^8 (**13b**)	Δ$^{8(14)}$ (**14b**)
10	23	37	30	10
20	5	44	37	13
30	0	44	45	10
40	0	48	30	22

[a] Reaction aliquots were taken after the indicated reaction time and analyzed by ^{1}H NMR.

Table 6
Ratio of 20*R* and 20*S* isomers produced by reduction of 3β,17α-dihydroxy-20-ketosteroid derivatives[a,b]

Steroid	Reagent	20*R* (%)	20*S* (%)
1 (Δ^5-diacetate)	$LiAlH_4$, 60 equivalents, 0.5 h	38[c]	62[c]
17 (Δ^5-3-acetate)	$LiAlH_4$, 58 equivalents, 1.5 h	61[c]	39[c]
1 (Δ^5-diacetate)	DIBALH, 80 equivalents, 0.5 h	80[c]	20[c]
17 (Δ^5-3-acetate)	DIBALH, 86 equivalents, 1.5 h	66[c]	34[c]
3 (Δ^{5,7}-diol)	H_2, Raney nickel	21[d]	79[d]

[a] All hydride reductions were conducted in tetrahydrofuran at room temperature, followed by addition of water, extraction with ethyl acetate, evaporation of the organic extracts, and NMR analysis. In the case of catalytic hydrogenation in ethyl acetate, the reaction mixture was filtered to remove the catalyst and then evaporated to dryness for NMR analysis.
[b] A large excess of hydride reducing agent was used to insure complete reduction of acetate and keto groups. Use of 15–20 equivalents of hydride resulted in mixtures containing little 20-hydroxysteroid.
[c] These percentages exclude a small amount (~10%) of an unidentified byproduct: NMR, δ 0.664 (s), 1.020 (s), 1.285 (d, 6.5 Hz), 4.426 (q, 6.5 Hz).
[d] These percentages were derived from the total product composition of 55% **9b**, 15% **9a**, 13% **14b**, 0.3% **14a**, 2% 20-ketosteroid species, and 16% unidentified.

*2.2.13. 5α-Pregn-8(14)-ene,3β,17α,20S-triol (**14b**)*

To a solution of $\Delta^{5,8}$ triol **11b** (10 mg) in freshly distilled ethyl acetate (2 ml) was added PtO_2 (8 mg). The resulting mixture was stirred at room temperature under a hydrogen-filled balloon for 2 h. The catalyst was removed by filtration through Celite, and the filtrate was evaporated to give **14b** as a white solid (8 mg, ∼95% purity). ^{1}H and ^{13}C NMR (Tables 4 and 5); HR-MS, calculated for $C_{21}H_{34}O_3$ 334.2508, found 334.2498; MS (Table 3).

*2.2.14. Lithium-ethylamine reductions of DEAD adducts **10** and **16***

Freshly cut lithium (88 mg, 12.7 mmol) was added to a solution of **10** (44 mg, 0.075 mmol) in ethylamine (2 ml) at −78 °C under nitrogen. After the blue color of the reaction solution appeared, the reaction temperature was elevated to −20 °C. The reaction solution was stirred for an additional 30 min, cooled to −78 °C, and quenched with *t*-butanol (2 ml). After aqueous workup, the crude product was subjected to MPLC (elution with hexane–ethyl acetate 7:3) to give 3β-hydroxypregna-5,8-dien-20-one (**15**; 15 mg). ^{1}H and ^{13}C NMR (Tables 4 and 5); MS *m/z* 314 (48, M^+), 299* (8), 281* (84), 255 (32), 211 (27), 159 (36), 143 (58). Similar reduction of **16** (14 mg, 0.028 mmol) with lithium (14 mg, 2 mmol) in ethylamine (1 ml) and anhydrous tetrahydrofuran (0.5 ml) gave a 2:1 mixture (6 mg) of **11b** and **12b**.

*2.3. Conformational analysis of **4***

^{1}H NMR analysis of the $\Delta^{4,7}$-3-ketosteroid **4** indicated the following ^{1}H NMR coupling constants: $J_{1\alpha-2\alpha}$ 5.8 Hz; $J_{1\alpha-2\beta}$ 7.2 Hz; $J_{1\beta-2\alpha}$ 8.7 Hz; $J_{1\beta-2\beta}$ 6.0 Hz. Molecular modeling using the MMX or MM3 force fields suggested two conformers: (I) the usual 1α,2β-half chair and (II) a 1β,2α-half chair at a slightly higher relative energy (0.5 kcal/mol for MMX; 0.3 kcal/mol for MM3). In conformer I, C-19 is axial in both rings A and B, whereas C-19 is equatorial in ring A of conformer II. Predicted couplings for MM3 geometries of conformer I ($J_{1\alpha-2\alpha}$ 4.49 Hz; $J_{1\alpha-2\beta}$ 13.24 Hz; $J_{1\beta-2\alpha}$ 2.69 Hz; $J_{1\beta-2\beta}$ 3.99 Hz) and conformer II ($J_{1\alpha-2\alpha}$ 3.72 Hz; $J_{1\alpha-2\beta}$ 2.92 Hz; $J_{1\beta-2\alpha}$ 13.32 Hz; $J_{1\beta-2\beta}$ 4.30 Hz) gave the best fit to the observed data when the ratio of I:II was 43:57. This model roughly corresponds to the experimental data but does not exclude the possibility of an additional low-energy conformer.

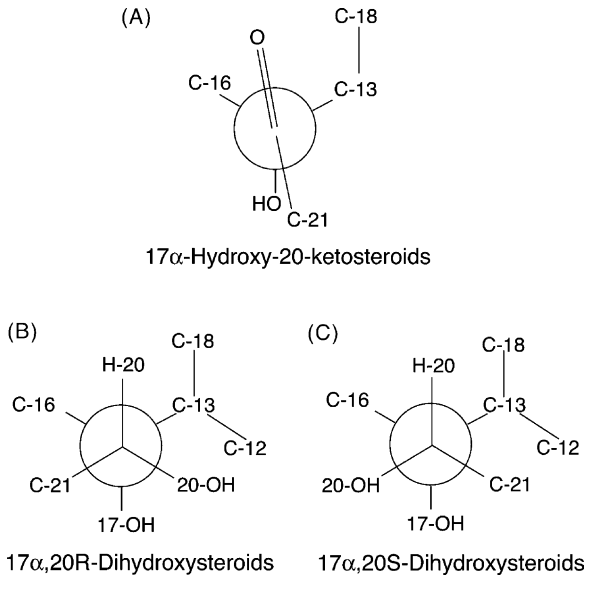

Fig. 4. Newman projections showing the best conformers of 17α-hydroxy-20-ketosteroids (A) and 17α,20-dihydroxysteroids (B, C). The view is from C20 to C17.

2.4. Side-chain conformations of 17α-hydroxysteroids

Molecular mechanics energies were calculated for the rotamers about the C17–C20 bond of 5α-pregnane-3β,17α,20*R*-triol, its 20*S* epimer, and 3β,17α-dihydroxy-5α-pregnan-20-one. The best conformers are shown in Fig. 4. Other rotamers were at least 1 kcal/mol higher in energy and represent minor conformers (≤15% of the total population).

3. Discussion

We describe the chemical synthesis of a series of C_{21} steroids having hydroxyl at C-3, C-17, and C-20 and unsaturation in rings B and/or C. These synthetic targets are candidate metabolites for SLOS. Apart from Δ^5 steroids, very few C_{21} steroids of any kind have been reported with double bonds in rings B and C. Consequently, we designed synthetic routes based on known methods for preparing unsaturated sterols in the cholesterol and ergosterol series [15,17–21]. As shown in Fig. 1, all the unsaturated C_{21} triols were made from a single precursor, 17α-hydroxy-7-dehydropregnenolone diacetate (**2**). This intermediate was prepared on a multi-gram scale in ∼50% yield from the commercially available 17α-hydroxypregnenolone diacetate (**1**). The conversion of **1** to **2** was based on the analogous bromination–dehydrobromination of pregnenolone acetate using tetrabutylammonium fluoride [22].

Key intermediate **2** was converted to various unsaturated pregnanetriols by four different methods (Fig. 1). Reduction of **2** with excess $LiAlH_4$ gave the $\Delta^{5,7}$ triols **7a** and **7b**, which were isolated by MPLC in 37% and 39% yield, respectively. Hydrogenation of **2** over Raney nickel gave the Δ^7-20-keto-diacetate **8**, which was also reduced with excess $LiAlH_4$ to provide, after chromatographic separation, the Δ^7 triols **9a** and **9b** in 33% and 42% isolated yields. Interestingly, when the hydrogenation was carried out on diol **3** instead of diacetate **2**, the carbonyl group at C-20 was also reduced, giving **9a** and **9b** directly as a 1:4 mixture (Table 6). Despite this favorable ratio of C-20 epimers, the product was contaminated by $\Delta^{8(14)}$ steroids and other byproducts, a result that led us to avoid hydrogenation of 17α-hydroxy-20-ketosteroids in favor of $LiAlH_4$ reductions.

The synthesis of **6a** and **6b**, the 3α-hydroxy-5β stereoisomers of **9a** and **9b**, began with solvolysis of diacetate **2** to diol **3**, followed by conversion to the $\Delta^{4,7}$-3-ketosteroid **4** with cholesterol oxidase under conditions we have used previously for the selective oxidation of oxysterols [23]. The enzymatic oxidation–isomerization was mild enough to minimize formation of the unwanted $\Delta^{4,6}$ isomer. Hydrogenation of **4** over palladium on carbon in 4-methoxypyridine [24] produced predominantly the desired 5β-steroid **5**. Reduction of this 3,20-diketone with $LiAlH_4$ gave a 9:10 mixture of 20*R* and 20*S* epimers **6a** and **6b**, which were isolated by preparative reverse-phase HPLC. The 3α-hydroxy-5β-H stereochemistry was confirmed by comparison with NMR data reported for sterols having similar 3-hydroxy-Δ^7 functionality [15,25]. We also attempted to prepare **6a** and **6b** by a remarkable transformation in which a 3β-hydroxy-$\Delta^{5,7}$ sterol is converted to the 3α-hydroxy-5β-Δ^7 sterol by heating with a copper/alumina catalyst in cyclohexanol [26]. In our hands, the reported transformation with ergosterol gave a more complex mixture than originally described [26], although some 5β-ergosta-7,22*E*-dien-3α-ol was observed. However, treatment of either **3** or **7a** under the same reaction conditions gave a complex mixture, devoid of the desired Δ^7 stereoisomers.

We initially attempted to synthesize the $\Delta^{5,8}$ triols **11a** and **11b** by the classical method for converting $\Delta^{5,7}$ to $\Delta^{5,8}$ sterols via an ene adduct with DEAD [27,28]. Although the DEAD adduct **10** was readily prepared in good yield from the $\Delta^{5,7}$ diacetate **2**, reduction of **10** with lithium in ethylamine at −20 °C led, as expected [29], to the formation of the 17-deoxygenated product **15** (Fig. 2). Lithium-ethylamine reduction of the unprotected DEAD adduct **16** did give **11b**, albeit accompanied by considerable $\Delta^{5,7,9(11)}$ triol **12b** (Fig. 2). However, we found that adduct **10** could be reduced directly to triols **11a** and **11b** with DIBALH. This single step achieved reduction of the 20-keto group, the 3β- and 17α-acetates, and the 7α-[*N*,*N*′-bis(ethoxycarbonyl)hydrazino] moiety without any deoxygenation at C-17. Although the $\Delta^{5,8}$ products were accompanied by similar amounts of $\Delta^{5,7,9(11)}$ byproducts, the mixture was separable on reverse-phase HPLC. Interestingly, attempted reduction of **10** with sodium borohydride, lithium aluminum hydride, or L-Selectride gave none of the target $\Delta^{5,8}$ steroids.

Preparation of the Δ^8 triol **13b** was more challenging than the other syntheses. Although Δ^8 sterols are accessible from $\Delta^{5,7}$ sterols by acid isomerization [17–20], followed by careful hydrogenation of the resulting $\Delta^{8,14}$ dienol [21], our efforts to isomerize either **2** or **7a** with aqueous or anhydrous HCl gave only mixtures containing little or none of the $\Delta^{8,14}$ product. This isomerization also failed for C_{19} steroids [30]. Few other methods are available for preparing Δ^8 steroids. Our attempts to follow a questionable [19,31] report [32] describing the catalytic hydrogenation of a $\Delta^{5,8}$ sterol to a Δ^8 sterol using platinum oxide gave almost exclusively the $\Delta^{8(14)}$ triol **14b** from **11b**, with no detectable Δ^8 triol **13b**. Similar hydrogenation of the $\Delta^{5,7,9(11)}$ triol **12a** also produced mainly the $\Delta^{8(14)}$ triol **14a**. Nevertheless, hydrogenation of **11b** over Raney nickel in redistilled ethyl acetate gave **13b**, accompanied by the Δ^7 and $\Delta^{8(14)}$ triols **9b** and **14b**. As indicated in Table 7, a mixture of mainly Δ^7 and Δ^8 isomers formed initially, but extended reaction times resulted in isomerization to the $\Delta^{8(14)}$ byproduct.

Our syntheses of unsaturated triols produced both C-20 epimers. We initially hoped to reduce the 20-ketosteroid intermediates stereoselectively to the natural 20*S* isomer without resorting to microbiological methods [33]. Compared with hydride reductions of 17-deoxysteroids, in which the 20*R* isomer is strongly favored [34], 17α-hydroxy-20-ketosteroids give increased amounts of 20*S* epimer [16]. However, bulky hydrides lead predominantly or exclusively [16] to the 20*R* epimer, as confirmed by our results with large (DIBALH) and small ($LiAlH_4$) reagents and with a 17α-hydroxy or 17α-acetoxy group (Table 6). Acetylation of the 17-hydroxyl appeared to favor formation of the 20*S* epimer in $LiAlH_4$ reductions (Table 6), and we observed somewhat lower 20*R*:20*S* ratios in reductions of 17α-acetoxysteroids (**7a**:**7b**, 3:4; **9a**:**9b**, 2:3) than in that of a 17α-hydroxysteroid (**6a**:**6b**, 8:9). Although the model DIBALH reductions described in Table 6 favored the 20*R* epimer, DIBALH reduction of adduct **10** gave a 1:1 ratio of $\Delta^{5,8}$ triols and a 3:4 ratio of $\Delta^{5,7,9(11)}$ steroids epimeric at C-20 (20*R*:20*S*). In one example, catalytic hydrogenation gave the largest percentage of 20*S* product, but this material was accompanied by isomeric byproducts that were not easily removed chromatographically.

All pairs of C-20 epimers could be resolved chromatographically and spectroscopically. Some epimeric pairs ($\Delta^{5,7}$ and 5α-Δ^7 triols) were resolved on MPLC ($t_R(20S) > t_R(20R)$) but others required separation on reverse-phase HPLC. The 20*R* and 20*S* isomers were easily distinguished by ^1H and ^{13}C NMR, as described previously [16,35,36]. NMR signals for H-12β and H-18 were deshielded in the 20*R* epimer due to their proximity to the 20-hydroxyl in the major conformer (Fig. 4), and H-16β was similarly deshielded in the 20*S* epimer. These and other effects resulted in notable chemical shift differences between the C-20 epimers for H-16α, H-16β, H-18, H-20, C-18, and C-20 (Tables 1, 2, 4, and 5). The best NMR reporter peaks were the H-20 signals, which resonated at δ 4.0–4.1 in the 20*R* isomers and δ 3.8–3.9 in the 20*S* isomers. These H-20 chemical shift differences are much larger than in the corresponding 17-deoxy analogs (δ 3.73 versus 3.72). The C-20 epimers (and most other pairs of isomers) were not readily distinguished from their mass spectra (Table 3).

In the course of characterizing the steroids by 1D and 2D NMR, we encountered two examples of conformational heterogeneity. In the ^1H NMR spectrum of the $\Delta^{4,7}$-3-ketosteroid **4**, coupling constants for the C-1 and C-2 protons showed major deviations from the values normally observed in ring A of Δ^4-3-ketosteroids [37,38]. Molecular modeling suggested that **4** is populated by a 1β,2α-half chair

as well as the usual 1α,2β-half chair conformer adopted by most Δ^4-3-ketosteroids. This influence of the Δ^7 bond on the conformation of ring A may have been responsible for the reduced stereoselectivity in the hydrogenation of **4** compared to similar reductions of Δ^4-3-ketosteroids lacking a Δ^7 bond [24]. Effects of ring-B double bonds [38] and other functionality ([37,39] and references therein) on the ring-A conformation of 3-ketosteroids have been described previously. The ring-A conformers of **4** interconvert rapidly on the NMR time scale, and only one set of averaged chemical shifts was observed. By contrast, several ^{13}C signals in the NMR spectrum of 5β-Δ^7-3,20-diketone **5** were severely broadened or missing entirely (Fig. 3A). A 'nonsteroidal' conformation for rings A and B is possible for 5β-sterols [25,40]. In the case of a 3α-hydroxy-Δ^7 sterol, only the usual steroidal conformation was observed [25], whereas a nonsteroidal conformation was found in an X-ray structure of a derivative of 5β-cholesta-8,14-dien-3β-ol [40]. The latter report also noted severe broadening of certain ^{13}C NMR signals.

The unsaturated pregnanetriols were isolated by chromatographic methods in 95–98% purity (except for **13b**) as judged by 500 MHz ^1H NMR spectra (see Fig. 3B). Considering that these steroids are labile and differ little in chromatographic mobility, this level of purity is quite high and should be ample for the intended use as reference standards.

Acknowledgments

This work was supported by the National Institutes of Health (Grants HL-49122 and R03HD-39707).

References

[1] White PC. Genetic diseases of steroid metabolism. Vitam Horm 1994;49:131–95.
[2] New MI. Genetic disorders of adrenal hormone synthesis. Horm Res 1992;37(Suppl 3):22–33.
[3] Tint GS, Irons M, Elias MR, Batta AK, Frieden R, Chen TS, Salen G. Defective cholesterol biosynthesis associated with the Smith–Lemli–Opitz syndrome. N Engl J Med 1994;330:107–13.
[4] Kelley RI, Hennekam RC. The Smith–Lemli–Opitz syndrome. J Med Genet 2000;37:321–35.
[5] Andersson HC, Frentz J, Martinez JE, Tuck-Muller CM, Bellizaire J. Adrenal insufficiency in Smith–Lemli–Opitz syndrome. Am J Med Genet 1999;82:382–4.
[6] Nowaczyk MJ, Siu VM, Krakowiak PA, Porter FD. Adrenal insufficiency and hypertension in a newborn infant with Smith–Lemli–Opitz syndrome. Am J Med Genet 2001;103:223–5.
[7] Ruan B, Wilson WK, Pang J, Gerst N, Pinkerton FD, Tsai J, Kelley RI, Whitby FG, Milewicz DM, Garbern J, Schroepfer Jr GJ. Sterols in blood of normal and Smith–Lemli–Opitz subjects. J Lipid Res 2001;42:799–812.
[8] Batta AK, Tint GS, Shefer S, Abuelo D, Salen G. Identification of 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol) in patients with Smith–Lemli–Opitz syndrome. J Lipid Res 1995;36:705–13.
[9] Ruan B, Wilson WK, Tsai J, Schroepfer Jr GJ. Aberrant pathways in the late stages of cholesterol biosynthesis. Origin and metabolic fate of unsaturated sterols relevant to the Smith–Lemli–Opitz syndrome. J Lipid Res 2000;41:1772–82.
[10] Bhavnani BR. The saga of the ring B unsaturated equine estrogens. Endocr Rev 1988;9:396–416.
[11] Shackleton CH, Roitman E, Kratz L, Kelley R. Dehydro-oestriol and dehydropregnanetriol are candidate analytes for prenatal diagnosis of Smith–Lemli–Opitz syndrome. Prenat Diagn 2001;21:207–12.
[12] Shackleton CH, Roitman E, Kratz LE, Kelley RI. Midgestational maternal urine steroid markers of fetal Smith–Lemli–Opitz (SLO) syndrome (7-dehydrocholesterol 7-reductase deficiency). Steroids 1999;64:446–52.
[13] Shackleton CH, Roitman E, Kelley R. Neonatal urinary steroids in Smith–Lemli–Opitz syndrome associated with 7-dehydrocholesterol reductase deficiency. Steroids 1999;64:481–90.
[14] Guo L-W, Shackleton CH, Wilson WK. Synthesis of ring B unsaturated estriols. Confirming the structure of a diagnostic analyte for Smith–Lemli–Opitz syndrome. Org Lett 2001;3:2547–50.
[15] Wilson WK, Sumpter RM, Warren JJ, Rogers PS, Ruan B, Schroepfer Jr GJ. Analysis of unsaturated C_{27} sterols by nuclear magnetic resonance spectroscopy. J Lipid Res 1996;37:1529–55.
[16] Shull BK, Koreeda M. (*E*)-3β-Hydroxy-5,17(20)-pregnadiene 3-pivaloate and (*Z*)-3β,16α-dihydroxy-5,17(20)-pregnadiene 3-pivaloate. J Org Chem 1990;55:99–105.
[17] Wilson WK, Schroepfer Jr GJ. Acid-catalyzed isomerization of 7-dehydrocholesteryl benzoate. A revised mechanism and improved synthetic procedure. J Org Chem 1988;53:1713–9.
[18] Dolle RE, Schmidt SJ, Eggleston D, Kruse LI. Studies on the acid-catalyzed homonuclear steroidal diene isomerization. J Org Chem 1988;53:1563–6.
[19] Fieser LF, Fieser M. Steroids. New York, NY: Reinhold; 1959, p. 111–9 and 161.
[20] Fieser LF, Ourisson G. Cholesterol and companions. Oxidation of Δ^7 sterols with selenium dioxide. J Am Chem Soc 1953;75:4404–14.
[21] Lee WH, Kammereck R, Lutsky BN, McCloskey JA, Schroepfer Jr GJ. Studies on the mechanism of the enzymatic conversion of Δ^8-cholesten-3β-ol to Δ^7-cholesten-3β-ol. J Biol Chem 1969;244:2033–40.
[22] Siddiqui AU, Wilson WK, Swaminathan S, Schroepfer Jr GJ. Efficient preparation of steroidal 5,7-dienes of high purity. Chem Phys Lipids 1992;63:115–29.
[23] Li S, Pang J, Wilson WK, Schroepfer Jr GJ. Sterol synthesis. Preparation and characterization of fluorinated and deuterated analogs of oxygenated derivatives of cholesterol. Chem Phys Lipids 1999;99:33–71.
[24] Tsuji N, Suzuki J, Shiota M. Highly stereoselective hydrogenation of 3-oxo-4-ene and -1,4-diene steroids to 5β compounds with palladium catalyst. J Org Chem 1980;45:2729–31.
[25] Andrews AT de B, Boul AD, Meakins GD, Sledge MJ. Hydroxysteroids. Part XIII. 5β-Ergosta-7,22-dien-3-one and related compounds. J Chem Soc (C) 1970;1052–5.
[26] Ravasio N, Gargano M. Selective hydrogenations promoted by copper catalysts. Hydrogen-transfer reactions leading to stereoselective hydrogenation of Δ^5-3β-sterols to 5β derivatives. J Org Chem 1993;58:1259–61.
[27] Anastasia M, Fiecchi A. Synthesis of lichesterol [ergosta-5,8(9),22-trien-3β-ol]. J Chem Soc, Perkin Trans 1 1981:2125–6.
[28] Anastasia M, Fiecchi A, Galli G. Synthesis of cholesta-5,8-dien-3β-ol. J Org Chem 1981;46:3421–2.
[29] Dryden Jr HL. Reductions of steroids by metal–ammonia solutions: In: Fried J, Edwards JA, editors. Organic reactions in steroid chemistry, vol. 1. New York, NY: Van Nostrand Reinhold; 1972, p. 1–60.
[30] Antonucci R, Bernstein S, Giancola D, Sax KJ. $\Delta^{5,7}$ Steroids. X. Transformation products of $\Delta^{5,7}$-androstadiene-3β,17β-diol. J Org Chem 1951;16:1891–8.
[31] Barton DHR, Corrie JET, Widdowson DA, Bard M, Woods RA. Biosynthesis of terpenes and steroids. Part IX. The sterols of some mutant yeasts and their relationship to the biosynthesis of ergosterol. J Chem Soc, Perkin Trans 1 1974:1326–33.

[32] Tsuda K, Arima K, Hayatsu R. Cholesterol and related compounds. Structure of a new non-conjugated cholestadienol from 7-bromocholesterol. J Am Chem Soc 1954;76:2933–8.
[33] Madyastha KM, Joseph T. Transformation of 16-dehydroprogesterone and 17α-hydroxyprogesterone by *Mucor piriformis*. Appl Microbiol Biotechnol 1994;41:170–7.
[34] Wheeler DMS, Wheeler MM. Reductions of steroidal ketones. In: Fried J, Edwards JA, editors. Organic reactions in steroid chemistry, vol. 1. New York, NY: Van Nostrand Reinhold; 1972, p. 61–110.
[35] Joannou GE, Reeder AY. 15β-Hydroxysteroids (part III). Steroids of the human perinatal period: the synthesis of 3β,15β,17α-trihydroxy-5-pregnen-20-one. Application of *n*-butyl boronic acid protection of a 17,20-glycol. Steroids 1996;61:11–7.
[36] Joannou GE, Reeder AY. 15β-Hydroxysteroids (part V). Steroids of the human perinatal period: the synthesis of 3β,15β,17α-trihydroxy-5-pregnen-20-one from 15β,17α-dihydroxy-4-pregnen-3,20-dione. Steroids 1996;61:18–21.
[37] Marat K, Templeton JF, Kumar VPS. An NMR study of ring-A conformation in some 4-en-3-one steroids. Magn Reson Chem 1987;25:25–30.
[38] Ruan B, Wilson WK, Pang J, Schroepfer Jr GJ. Synthesis of [3α-^{3}H]cholesta-5,8-dien-3β-ol and tritium-labeled forms of other sterols of potential importance in the Smith–Lemli–Opitz syndrome. Steroids 2000;65:29–39.
[39] Marat K, Templeton JF, Gupta RK, Kumar VPS. Ring-A conformation in steroids. NMR study of C-2 monomethyl- and dimethyl-substituted 5α-androstan-3-ones. Magn Reson Chem 1987;25:730–3.
[40] Wilson DK, Wilson WK, Quiocho FA, Schroepfer Jr GJ. Concerning the structure of 3β-benzoyloxy-5β-cholesta-8,14-diene, a major byproduct in the chemical synthesis of 5α-cholest-8(14)-en-3β-ol-15-one. Chem Phys Lipids 1988;47:273–82.