Bile acids. LXXXI. Synthesis and structural assignment of E/Z isomers of substituted methyl hydroxy-5β-cholest-24-en-26-oates

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Syntheses of the E and Z isomers of methyl 3α-, 3α,7α-, 3α,12α-, and 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oates are reported. Mass spectral studies show fragmentation patterns in support of assignment as the E or Z isomers, especially in differences in loss of the side chain. Chromatographic procedures, primarily gas chromatography and high-performance liquid chromatography, support these assignments. The E isomer predominates in either of two methods of synthesis. (*Steroids* **56**:505–512, 1991)

Keywords: steroids; bile acids; C_{27} bile acids; mass spectrometry; 5β-cholest-24-en-26-oates; E/Z isomers; bile acid precursors

Introduction

Hydroxylated 5β-cholest-24-en-26-oic acids are important intermediates in the biosynthesis of C_{24} bile acids from cholesterol.[1-3] A few of these C_{27} acids have been obtained in small quantities from various animals and fish.[3,4] To study details of conversion of these C_{27} acids to C_{24} bile acids, it was necessary to acquire the substrates by synthesis. Une et al.[2] have reported a synthesis of the diastereoisomers at C-24 and C-25 of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid in which the E and Z isomers of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acids were intermediates for their desired products. This report includes the syntheses of the above 5β-cholest-24-en-26-oic acids by two methods. Their analogs, namely, the 3α-hydroxy, the 3α,7α-dihydroxy, and 3α,12α-dihydroxy acids, were also prepared. The E and Z isomers were distinguished by fragmentation patterns in mass spectrometry as well as by appropriate nuclear magnetic resonance (NMR) signals.

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Experimental

Lithocholic acid and 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) were purchased from Sigma Chemical Company (St. Louis, MO,USA). Other bile acids used in these studies were available from stock in this laboratory. The formates of each of the four acids prepared for this study (**Ia–d**) exhibited melting points comparable to those reported.[5] Methyl α-bromopropionate, triphenylphosphine, trimethyl phosphite, lithium tri-*tert*-butoxyaluminohydride, *N,N*-carbonyldiimidazole, and silica gel (100 to 200 mesh) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Potassium hexamethyldisilazane (KN $[Si(CH_3)_3]_2$) (15% in toluene) was supplied by Callery Chemical Co. (Callery, PA, USA). Tetrahydrofuran (THF) from Fisher Scientific (St. Louis, MO, USA) was freshly distilled from $LiAlH_4$. All other solvents were distilled before use.

Analyses by thin-layer chromatography (TLC) were done on aluminum plates coated with silica gel 60F (E. Merck, supplied by VWR Scientific, Chicago, IL, USA). After development, the plates were dried, sprayed with 10% phosphomolybdic acid, and warmed in an oven or on a hot plate to locate the bile acids.

Gas chromatography (GC) was carried out with a Hewlett-Packard Model 5890A instrument using a coiled glass column (0.25 in × 6 ft) packed with 3%

OV-1 at 260 C oven temperature with helium as the carrier gas. Methyl esters of the samples were converted to their trimethylsilyl (TMS) derivatives prior to injection.[4] The TMS derivative of methyl deoxycholate was used as an internal standard and coinjected with the samples to determine relative retention times (RRT).

Mass spectra of methyl esters of samples were obtained via a direct inlet probe (DIP) or by GC/mass spectrometry (MS) with an LKB model 9000 mass spectrometer. In later experiments, a VG Trio 2 quadrupole spectrometer (VG Instruments, Mass Lab Ltd., Altrinham, Cheshire, England) capable of electron impact (EI)/MS, CI/MS, fast atom bombardment (FAB)/MS, and DIP was used. Conditions for analysis of TMS derivatives for GC/EI$^+$/MS with the VG Trio 2 were as follows: column (an OV-5 column provided by Ohio Valley Specialty Co., Marietta, OH, USA, is comparable to a DB-5 column coated internally with 5% phenyl methyl polysiloxane), 30 m × 0.5 mm internal diameter; film thickness, 0.32 mm; temperature of the injector, 280 C; temperature of the column, 265 C; temperature of the interface, 280 C; temperature of the source, 290 C; and helium flow, 3.5 ml/min.

Nuclear magnetic resonance spectra were obtained with a Varian HA 100 MHZ or a Jeol 90 MHZ spectrometer using pyridine-d_5 as solvent and are reported in δ units as parts per million. Infrared (IR) spectra were recorded from KBr disks using a Model 21 Perkin Elmer spectrophotometer.[6] High-performance liquid chromatography (HPLC) was carried out with a Waters ALC-201 System; melting points were determined on a Fisher-Johns apparatus and are uncorrected.

3α,7α,12α-Triformoxy-5β-cholan-24-al (IIa)

To a solution of 4.92 g (10 mmol) of 3α,7α,12α-triformoxy-5β-cholanic acid **Ia**[5] in 50 ml of dry THF was added 4.86 g (30 mmol) of *N,N*-carbonyldiimidazole. The solution was refluxed under a nitrogen atmosphere with stirring for 45 minutes. After cooling to room temperature, the mixture was poured with stirring into 400 ml of cold water. The precipitated solid was filtered, washed twice with 100 ml of water, and dried overnight in vacuum to provide 5.05 g of the imidazolide. This material was dissolved without further purification in 40 ml of dry THF and a solution of 2.74 g (10.8 mmol) of lithium tri-*tert*-butoxyaluminohydride (LTBAH) in 50 ml of dry THF was added dropwise with stirring over a period of 1 hour. After stirring for an additional 30 minutes, most of the solvent was removed under vacuum and the residue was poured into 100 ml of 1 N HCl. The gummy product was taken up in ether, washed with $NaHCO_3$ and saturated salt solutions, and the solvent was evaporated to afford 3.0 g of product. Thin-layer chromatography on silica gel (hexane/acetone 3 : 1) of an aliquot of the product showed a very small amount of the imidazolide and two principal compounds. The mixture was separated on a silica gel column (100 to 200 mesh, 50 g) using acetone/hexane (1 : 2) to provide 2.1 g (44%) of an oil identified as 3α,7α,12α-triformoxy-5β-cholan-24-al **(IIa)**: IR (KBr) 1,720 cm^{-1}, MS 476 (M$^+$). The second compound in the mixture was identified by MS as 3α,7α,12α-triformoxy-5β-cholan-24-ol, a reduction product of the aldehyde **(IIa)** produced in the reaction.

Methyl E/Z 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oates (IIIa)

Method A. A total of 1.904 g (4 mmol) 3α,7α,12α-Triformoxy-5β-cholan-24-al **(IIa)** and 1.5 g (approximately 4.4 mmol) of carbomethoxymethyltriphenylphosphine ylide were refluxed in 50 ml of dry benzene for 6 hours. After cooling to room temperature, benzene was evaporated under vacuum and the reaction mixture was chromatographed over silica with acetone/hexane (1 : 3) to give 2.15 g (93%) of the product. It was refluxed in 25 ml of 5% methanolic KOH for 2 hours. After cooling, most of the methanol was evaporated under vacuum, and the remaining solution was acidified with 6 N HCl. The precipitated acid was extracted with ether, washed with water and saturated NaCl, and the ether was removed to give 1.6 g of the acid mixture. Attempts to purify the acid by recrystallization from ethyl acetate, as reported,[2] gave a product that was only 88% pure according to GC. The acid was redissolved in 20 ml of methanol to which 1 ml of concentrated HCl was added followed by 5 ml of 2,2-dimethoxypropane.[7] After stirring overnight, enough solid Na_2CO_3 was added to neutralize the acid and the solvent was evaporated. The resulting product was refluxed with 2 × 25 ml of acetone, filtered, and the solvent was evaporated to give 1.63 g (88%) of the ester **IIIa.** Gas chromatography showed the presence of two isomers, Z (RRT = 1.89, 29%) and E (RRT = 2.41, 71%). The E isomer exhibited a melting point (mp) of 170 to 172 C (from synthesis and after HPLC and crystallization from benzene/hexane). Physical properties of the final products **(IIIa–d)** are given in Table 1. Important fragment ions in MS of these isomers are presented in Table 2.

Method B. A total of 450 mg (1.5 mmol)[8] bis(trifluoroethyl)phosphonopropionate and 460 mg (1.5 mmol) of the 18-crown-6/CH_3CN adduct[9] were dissolved in 20 ml of dry THF and cooled to −78 C. Via syringe, 2.1 ml of 15% $KN(TMS)_2$ in toluene was added and the solution was stirred for 10 minutes, followed by the addition of a solution of 476 mg (1 mmol) of 3α,7α,12α-triformoxy-5β-cholan-24-al **(IIa)** in 5 ml THF, also via a syringe. The stirring was continued for 30 minutes, after which TLC analysis showed that a very small amount of the starting material was left. Cooling was discontinued and a saturated solution of NH_4Cl was added. The organic phase was separated and the aqueous phase was extracted with 3 × 20 ml of ether. The combined organic phases were washed successively with water and saturated NaCl solution; after drying over $MgSO_4$, the solvent was evaporated to yield 470 mg of reaction mixture. A portion (100 mg) of this mixture was purified by preparative-layer chromatog-

Table 1 Physical and chromatographic properties of methyl esters of hydroxylated 5β-cholest-24-en-26-oates

Method	Isomer	3α,7α,12α-triol (% yield)	3α,7α-diol (% yield)	3α,12α-diol (% yield)	3α-ol (% yield)
GLC					
A	E	71	73	77	75
	Z	29	27	23	25
B	E	56	—	62	—
	Z	44	—	38	—
GLC (RRT)					
A	E	2.41	2.35	2.20	1.90
	Z	1.89	1.69	1.64	1.34
B	E	2.38	—	2.20	—
	Z	1.88	—	1.65	—
HPLC (peak elution in minutes)					
A	E	49.5[a]	29.7[b,c]	27.5[b]	52.9[b]
	Z	47.8[a]	26.5[b,d]	27.3[b]	46.4[b]
Ratio		(76 : 24)	(78 : 22)	—	(79 : 21)
B	E	—	58.8[e]	64.5[a]	—
	Z	—	55.2[e]	62.7[a]	—
Ratio		—	(74 : 26)	—	—
B	E	50.4[a]	—	64.5[a]	—
	Z	47.9[a]	—	62.7[a]	—
Ratio		(60 : 40)	—	—	—
NMR (δ for 24 H)	E	7.13[d]	6.95[c]	—	6.94[c]
	Z	5.85[d]	5.65[c]	—	5.69[c]

[a] HPLC system $CH_3OH/CH_3CN/H_2O$, 225 : 45 : 60; two cartridges in tandem.
[b] C_{18} silica cartridge, 5 μm particles; solvent: $CH_3OH/CH_3CN/H_2O$, 230 : 30 : 40.
[c] Reported by Elliott, Iqbal, and Patrick (1986; *Fed Proc* **45**, 1885).
[d] Reported by Une et al.[2]
[e] Silica cartridge, 5 μm particles; solvent: hexane/CH_2Cl_2/2-propanol, 30 : 10 : 1.

Table 2 Fragment ions via direct inlet probe/mass spectrometry of methyl hydroxy-5β-cholest-24-en-26-oates[a]

	IIIa 3α,7α,12α-triol			**IIIb** 3α,7α-diol			**IIIc** 3α,12α-diol			**IIId** 3α,ol		
Fragment ion	E	m/z	Z	E	m/z	Z	E	m/z	Z	E	m/z	Z
M^+	(6%)	462	(5%)	*	446	*	(45)	446	(37)	*	430	*
M-(H_2O)	(27)	444	(17)	(10)	428	(12)	(74)	428	(33)	(53)	412	(18)
M-(H_2O + CH_3)	(5)	429	*	(7)	413	(10)	(8)	413	(*)	(29)	397	(12)
M-(2H_2O)	(80)	426	(44)	(40)	410	(64)	(47)	410	(15)	—	—	—
M-(2H_2O + CH_3)	(21)	411	(16)	(30)	395	(34)	(17)	395	(5)	—	—	—
M-(3H_2O)	(51)	408	(19)	—	—	—	—	—	—	—	—	—
M-(3H_2O + CH_3)	(21)	393	(11)	—	—	—	—	—	—	—	—	—
M-(127)	*	335	*	—	319	(11)	*	319	(16)	(5)	303	(33)
M-(H_2O + 127)	(5)	317	(13)	*	301	(77)	(27)	301	(80)	(18)	285	100
M-(H_2O + 127 + 2H)	*	315	*	(12)	299	(9)	(20)	299	(11)	(71)	283	(16)
M-(SC + 2H)	(14)	305	(27)	(6)	289	(23)	(22)	289	(37)	(48)	273	(74)
M-(2H_2O + 127)	(32)	299	(93)	(6)	293	100	(30)	283	100	—	—	—
M-(2H_2O + 127 + 2H)	(15)	297	(12)	(24)	281	(11)	(24)	281	(14)	—	—	—
M-(H_2O + SC)	(20)	289	*	(13)	273	*	(51)	273	(24)	(65)	257	(13)
M-(H_2O + SC + 2H)	(12)	287	(10)	100	271	(34)	(38)	271	(19)	100	255	(43)
M-(3H_2O) + 127)	(39)	281	100	—	—	—	—	—	—	—	—	—
M-(3H_2O + 127 + 2H)	(23)	279	(12)	—	—	—	—	—	—	—	—	—
M-(2H_2O + SC)	(75)	271	(70)	(34)	255	(16)	(87)	255	(34)	—	—	—
M-(2H_2O + SC + 2H)	(37)	269	(27)	(95)	253	(36)	(47)	253	(18)	—	—	—
M-(3H_2O + SC)	(96)	253	(65)	—	—	—	—	—	—	—	—	—
M-(3H_2O + SC + 2H)	(46)	251	(20)	—	—	—	—	—	—	—	—	—

[a] Fragment ions of the group m/z 217–211 derived from further degradation of the steroid nucleus were present in spectra for each of the eight substances. Since this information probably has no bearing on the original structures of these esters, the data were not added to this table. These data were acquired with the LKB 9000 mass spectrometer.
*, Less than 5%.
—, 1% or less.

raphy to give 85 mg of a mixture of esters. Hydrolysis and re-esterification as above gave 80 mg of a mixture of the methyl esters. According to GC analysis, the two isomers E and Z were present as esters in the ratios of 56% and 44%, respectively, but with RRTs comparable to those found in method A. High-performance liquid chromatography analysis showed peaks at 47.9 minutes (15.8%), 50.4 minutes (23.3%), and 52.3 minutes (60.9%) with a mixture of $CH_3OH/CH_3CN/H_2O$ (225 : 45 : 60 v/v/v), indicating the presence of three instead of two products in the mixture. The first two peaks were identified by comparison of the retention factors as the Z and E isomers of compound **IIIa** with a ratio of 40 : 60. The third component provided a mass spectrum comparable to that of the E isomer of compound **IIIa.** Apparently, the adduct **IVa,** produced as an intermediate in the reaction, did not decompose completely at room temperature, but afforded the E isomer **(IIIa)** within the heated ion source of the MS, and in the column of the GC.

3α,7α-Diformoxy-5β-cholan-24-al **(IIb)**

A total of 3.584 g (8 mmol)[5] 3α,7α-diformoxy-5β-cholanic acid **(Ib)** was reacted with 3.888 g (24 mmol) of *N,N*-carbonyldiimidazole according to the method above. The resulting imidazolide was reduced with 2.3 g (9 mmol) of LTBAH. The resulting mixture (3.1 g) was separated over silica gel with acetone/hexane (1 : 3) to yield 1.446 g (42%) of compound **IIb**: IR (KBr) 1,725 cm^{-1}, MS 432 (M^+).

Methyl E/Z 3α,7α-dihydroxy-5β-cholest-24-en-26-oates **(IIIb)**

A total of 800 mg (1.85 mmol) 3α,7α-diformoxy-5β-cholan-24-al **(IIb)** and 663 mg (approximately 1.86 mmol) of ylide were converted via method A to a mixture that consisted of 27% of the Z (RRT = 1.69) and 73% of the E (RRT = 2.35) isomers by GC. The isomers of compound **IIIb** were separated by HPLC using $CH_3OH/CH_3CN/H_2O$ (230 : 30 : 40 v/v/v) as the Z isomer (RT = 26.5 minutes) and the E isomer (RT = 29.7 minutes): E isomer, mp 72 to 73 C from benzene/hexane (mp 68 to 70 C from aqueous methanol); Z isomer, mp 60 to 62 C from aqueous methanol (mp 58 to 59 C from HPLC). The NMR spectrum (pyridine-d_5) of the E isomer showed a triplet at 6.95 ppm (1H, t, J = 6 Hz, 24-H), whereas the Z isomer showed absorption characteristic of an olefinic proton at 5.65 ppm (1H, m, 24-H). Characteristic absorption in the ultraviolet was shown at λ_{max} 220 nm for the E form and λ_{max} 210 nm for the Z form. Important fragmentation peaks in the MS of these isomers are presented in Table 2.

3α,12α-Diformoxy-5β-cholan-24-al **(IIc)**

3α,12α-Diformoxy-5β-cholanic acid **(Ic),** 2.24 g (5 mmol), was reacted with 2.43 g (15 mmol) of *N,N*-carbonyldiimidazole, and the resulting imidazolide was reduced with 2.15 g of LTBAH as described above. Column chromatography on silica gel using acetone/hexane (1 : 2) yielded 1.17 g (54%) of the desired material **IIc**: IR (KBr) 1,720 cm^{-1}, MS 432 (M^+).

Methyl E/Z 3α,12α-dihydroxy-5β-cholest-24-en-26-oates **(IIIc)**

Method A. A total of 1.17 g (2.7 mmol) 3α,12α-diformoxy-5β-cholan-24-al **(IIc)** and 970 mg of the ylide were reacted to give 1.2 g (ca 90%) of the reaction mixture. This was hydrolyzed and re-esterified, analogous to the procedure above, to give 970 mg (81%) of compound **IIIc.** According to GC analysis, the mixture consists of 23% Z isomer (RRT = 1.64) and 77% E isomer (RRT = 2.20). In HPLC, the two isomers have very close retention times, the Z isomer appearing as a shoulder on the peak of the E isomer. The compositions of the mixtures of solvents used for HPLC are given in Table 1, and a list of fragment ions and their abundances obtained by MS is given in Table 2.

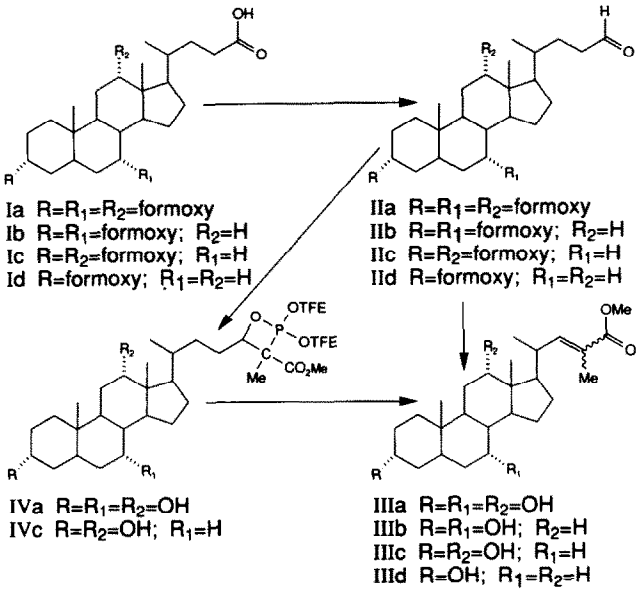
Method B. A total of 2.16 g (5 mmol) 3α,12α-Diformoxy-5β-cholan-24-al **(IIc)** and 1.8 g (6 mmol) *bis*(trifluoroethyl)phosphonopropionate were reacted as above. Column chromatography on silica gel with acetone/hexane (1 : 2) yielded 2.05 g of a heavy oil that was hydrolyzed and re-esterified as described above to give 1.6 g (72%) of an isomeric mixture. Analysis by GC showed the presence of 38% Z isomer (RRT = 1.65) and 62% E isomer (RRT = 2.20). High-performance liquid chromatography analysis of the mixture with $CH_3OH/CH_3CN/H_2O$ (225 : 45 : 60 v/v/v) showed three peaks at 62.7, 64.5, and 70.1 minutes. Small quantities of the first two components were collected for MS analysis and were identified as the Z and E isomers, respectively. The third peak gave a mass spectrum identical to that of the E isomer. As in the case of **IIIa,** this component must be the adduct **(IVc)** formed during the reaction.

3α-Formoxy-5β-cholan-24-al **(IId)**

A total of 4.04 g (10.4 mmol) 3α-formoxy-5β-cholanic acid **(Id)** and 4.86 g (30 mmol) *N,N*-carbonyldiimidazole were reacted as before and the resulting imidazolide was reduced with 4.88 g of LTBAH. Column chromatography on silica gel with acetone/hexane (1 : 4) gave 2.80 g (72%) of the compound **IId**: IR (KBr), 1,720 cm^{-1}, MS 388 (M^+).

Methyl E/Z 3α-hydroxycholest-24-en-26-oates **(IIId)**

A total of 2.8 g (7.3 mmol) 3α-formoxy-5β-cholan-24-al **(IId)** was refluxed with 2.6 g (7.5 mmol) of the ylide and was converted to compound **IIId** according to method A to provide, after column chromatography over silica gel with acetone/hexane (1 : 3), 2.51 g (80%) of a mixture that remained a heavy oil after the removal of solvent. The mixture was hydrolyzed and re-esterified as before to give 2.2 g (70.5%) of the isomeric mixture **IIId.** Gas chromatography analysis indicated the presence of 25% Z isomer (RRT = 1.34) and 75% E



Scheme 1 General pathway of synthesis of isomeric E/Z esters (**IIIa–d**).

isomer (RRT = 1.90). These were separated by HPLC with a mixture of $CH_3OH/CH_3CN/H_2O$ (230 : 30 : 40 v/v/v). The E isomer exhibited a double mp 75 to 76 C (from benzene/hexane) and 98 to 100 C (from aqueous methanol). The Z isomer provided mp 107 to 108 C (from aqueous methanol) and mp 104 to 105 C (from benzene/hexane). The NMR spectrum of the E isomer exhibited a triplet at 6.94 ppm (1H, t, J = 6 Hz, 24H), whereas the Z isomer showed absorption at 5.69 ppm (1H, m, 24-H). The UV spectra showed λ_{max} = 219 nm for the E isomer and λ_{max} = 212 nm for the Z isomer.

Results

The general pathway of synthesis of the isomeric E/Z esters of bile acids **IIIa–d** is shown in Scheme 1. The formylated bile acids **Ia–d**[5] were preferred over the acetates[2] in these studies because of greater ease in crystallization, and were converted to the corresponding imidazolides by the modified procedure of Staab and Braeunling.[8] These products were then reduced to their respective aldehydes with LTBAH in THF.[10]

The aldehydes **IIa–d** were purified by chromatography over silica gel using acetone/hexane mixtures and were converted either by a Wittig reaction using carbomethoxymethyl-triphenylphosphorane[11] or by a modified Horner-Emmons reaction using bis(trifluoroethoxy)phosphonopropionate[12] to give a mixture of the formylated methyl E/Z 5β-cholest-24-en-26-oates. These were hydrolyzed to remove the formoxy groups and were re-esterified to the methyl esters IIIa–d.

Gas chromatography analysis showed that the E and Z isomers were present in a ratio of 70 : 30 when chain lengthening was achieved by the Wittig reaction,[11] but slightly higher amounts of the Z isomer were obtained by the other method[12] (Table 1). Attempts to purify the E/Z mixture of compound **IIIa**, both as free acid[2] and as its methyl ester, resulted in fractions that consisted of 92% E according to HPLC analysis. The mixtures **IIIa, IIIb,** and **IIId** were separated by HPLC using a solvent system of $CH_3OH/CH_3CN/H_2O$ (225 : 45 : 60 v/v/v) to provide the E and Z isomers in a pure state. The mixture from **IIIc** was more difficult to separate because the two peaks overlapped considerably. Hence, only small amounts of the E and Z isomers of the 3α,12α-dihydroxy compound could be isolated for MS analysis. The realization of poorer yields of desired final products via method B led to preparation of the 3α,7α-dihydroxy-C_{27} and 3α-hydroxy-C_{27} products only via method A.

Table 1 summarizes the yields of products and physical properties of the synthetic Δ^{24}-C_{27} derivatives. Une et al.[2] reported the preparation of the 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acids as intermediates for the 24-hydroxy C_{27} acids. They identified by NMR the E isomer of this Δ^{24} acid by the 24-H signal at 7.13 ppm, and the 24-Z isomer at 5.85 ppm (Table 1) in a ratio of 7 : 3. These data were confirmed and extended to include the 3α,7α-dihydroxy and 3α-hydroxy analogs (Table 1) by the 24-H signals at 6.95 and 6.94 ppm for the E isomers, respectively, and 5.65 and 5.69 ppm for the Z isomers, respectively. Similar data for the 3α,12α-dihydroxy-5β-cholest-24-en-26-oate esters were not obtained because of the paucity of purified products.

Discussion

Mass spectrometry provided supporting data (Table 2) to confirm assignment of structures, and was especially helpful in the GC/MS mode in identifying mixtures of the E and Z isomers as the methyl esters (Table 1) and methyl ester TMS derivatives, particularly of the incompletely separated 3α,12α-dihydroxy analogs. Mass spectra of many saturated bile acids and sterols exhibit fragment ions in the region of m/z 253 to 257 resulting from loss of the side chain after or with the nuclear hydroxyl group(s) as molecules of water.[13–16] The exposure of hydroxylated substances to elevated temperatures of a heated inlet system prior to bombardment of the substrate in the ion chamber (generally of a lower temperature) to produce ions has long been of concern because of the probable generation of unsaturated products by thermal loss of the elements of water.[13,17] Correlation of results from several methods of MS used in these studies shows consistency of collection of similar data (e.g., GC/MS of the methyl esters, methyl ester TMS derivatives, and the usage of the DIP for the methyl esters with an LKB 9000 magnetic instrument and the VG Trio 2 quadrupole). Spectra acquired by FAB^+/MS (VG Trio 2) provided the fragment ions of particular interest, but were generally less abundant, probably because the energy of the fast uncharged xenon atoms was too high.

Data in Table 2 show that the most abundant ions (100%) exhibited in the spectra of this family of four esters were found among the Z isomers as m/z 281,

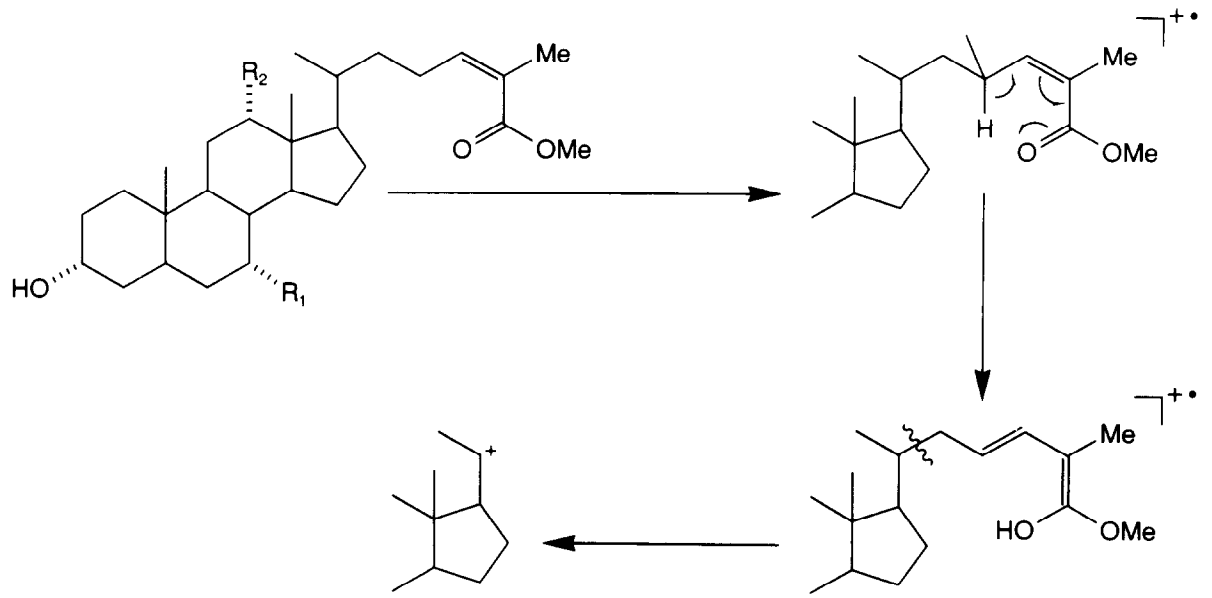


Figure 1 Mechanism of formation of the ion (M-127) from Z isomers **(IIIa–d)**.

283, 283, and 285, representative of fragment ions of M-(x · H_2O + 127). Ions representing M-127 from the E isomers were detected in abundances of 5% or less, whereas intensities of ions of the Z series ranged from less than 5% (the triol) to 33% for the 3α-ol. The relative intensities of these ions [M-(x · H_2O + 127)] increased with successive loss of each molecule of water up to 100% and loss of *all* hydroxyl groups. Abundances of these ions from the methyl ester TMS ethers of this Z series were quite small for the ion M-(90 + 127), except for that from the 3α-ol, which was 100% (m/z 283). These spectra compare resonably well with earlier reports of methyl ester TMS ethers of C_{27} bile acids obtained from Varanus monitor[4] and of minor bile acids of the toad,[18] although the separation of E and Z isomers was not reported.

A mechanism for elimination of the fragment ion m/z 127 from the side chain at position 20–22 is presented in Figure 1. The shift of a hydrogen from position 23 to protonate the oxo moiety at position 26 facilitates rupture of the 20–22 bond with liberation of the polar end of the side chain and generation of the ion with a charge at C-20. The Z epimers of this family are the substrates for the ions m/z 281, 283, 283, and 285 (100%, Table 2) from the methyl esters whether or not the nuclear hydroxyl groups exist as TMS ethers.

Related to the above ions is another family of fragment ions representing the loss of the entire side chain and *all* of the nuclear hydroxyl groups, e.g., M-(x · H_2O + S.C.) providing the ions m/z 253, 255, 255, and 257 for compounds **IIIa** through **IIId.** These ions are commonly seen in EI^+/MS fragmentation of C_{24} bile acids and their derivatives[13,14] (ion A iii in Figure 2A). Data in Table 2 show that these ions are derived preferentially from the E isomers. The intensities (96% and 87%) of the ions m/z 253 from the triol and m/z 255 from the 3α,12α-diol contrast with intensities of 34% and 65% for the ions m/z 255 (3α,7α-diol) and m/z 257 (3α-ol), respectively. Although there is probably some contribution to the intensities of these ions from the ions m/z 251, 253, 253, and 255 (the "isotope effect"), the greater magnitude of the intensities from the two 12α-hydroxylated derivatives may be associated with the ease of loss of the 12α-hydroxyl group through dehydration and association with loss of the entire side chain, as noted with 12α-hydroxylated bile acids.[13,14]

A second major fragmentation is associated with production of the ions m/z 251, 253, 253, and 255, M-(x · H_2O + S.C. + 2H), wherein the abundances of ions from the E isomer are again larger than those from the Z form (Table 2). The genesis of ions of this type has been explained in earlier studies of fragmentation of 3-hydroxy-Δ^{24}-sterols by EI^+/MS by Wyllie, Massey, and Djerassi[19,20] (Figure 2B). These researchers concluded that Δ^{24}-sterols may undergo a McLafferty rearrangement in the area of the Δ^{24} side chain involving a shift of the 17α-proton to C-24 followed by migration of the C-18 methyl group to the electron-deficient site at C-17. Abstraction of a second proton to the side chain and homolysis of the 17–20 bond would provide the product shown in Figure 2B vii. Using deuterium at selected sites, these investigators demonstrated that the first proton to shift in this sequence was exclusively the C-17 proton, and the second proton in this sequence was derived from C-16, C14, and C-12.

Within this family of four compounds **(IIIa–d),** the intensities of the ions m/z 251 (46%) and m/z 253 (47%) for the E isomers of the two 12α-hydroxylated compounds are markedly different from the relative intensities of the ions m/z 253 (95%) and m/z 255 (100%) for the 3α,7α-diol and 3α-ol, respectively. The 3α,7α,12α-triol and 3α,12α-diol likely have been dehydrated as

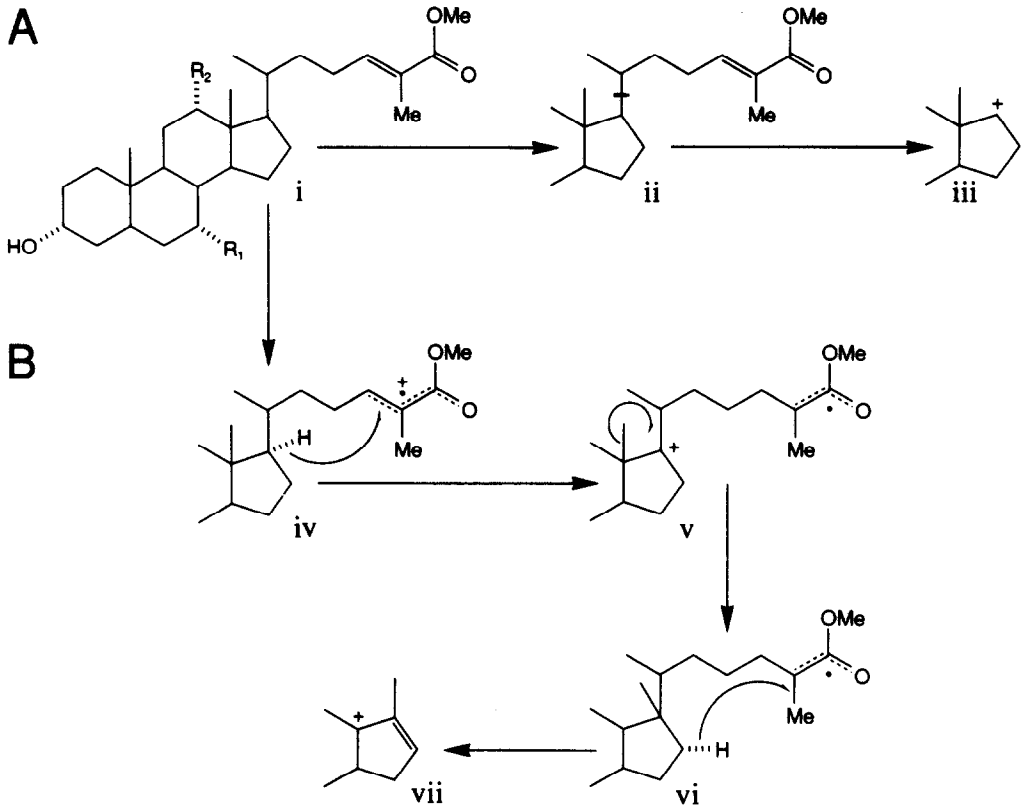


Figure 2 (A) Mechanism for formation of the ion (M-side chain) (iii) from E isomers **(IIIa–d)**. (B) Mechanism for formation of the ion (M-side chain + 2H) (vii) from E isomers **(IIIa–d)**.

mentioned above. The 3α,7α-diol and 3α-ol each have a pair of C-12 protons, one of which is involved as the *second* proton in completion of the elimination of the side chain from C-17 (Figure 2B). Supporting evidence was acquired with the TMS ethers via the VG quadrupole; e.g., the intensities of ions of the E series were 100% for both the 3α,7α-diol (m/z 253) and the 3α-ol (m/z 255), contrasting with the comparable ions m/z 253 (23%) from the 3α,12α-diol and m/z 251 (17%) from the 3α,7α,12α-triol (data not tabulated). These data support the pathway via M-(x · H_2O + S.C. + 2H). Thus, in the E series, two pathways of fragmentation appear to remove functional groups (hydroxyl groups) from the ring system and the side chain, namely M-(x · H_2O + S.C.) and M-(x · H_2O + S.C. + 2H). The 12-hydroxylated substrates favor the former system and the 12-deoxy samples appear to favor the later mechanism. Studies with a larger number of Δ^{24}-steroids or C_{27} analogs of bile acids are clearly desirable before generalizing.

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