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CONCERNING THE THERMOLABILITY OF CHOLESTA-5,8-DIEN-3β-OL, A STEROL THAT ACCUMULATES IN BLOOD AND TISSUES IN A HUMAN GENETIC DEVELOPMENTAL DISORDER

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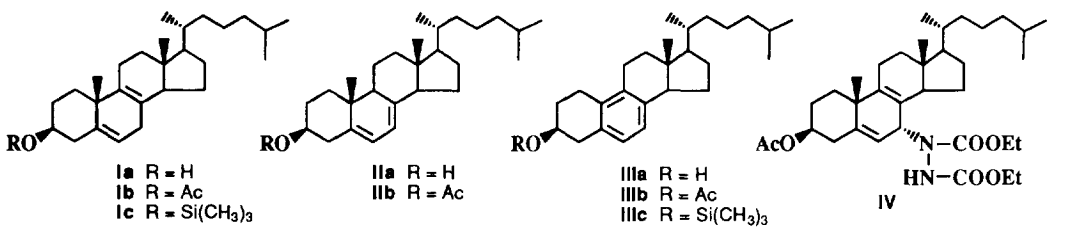
Abstract: Cholesta-5,8-dien-3β-ol, a sterol of current biomedical interest, undergoes facile thermal decomposition as its acetate and TMS derivatives to the corresponding derivatives of 19-norcholesta-5,7,9-trien-3β-ol. This finding may be relevant to the reported occurrence of the nortriene in blood of patients with Smith-Lemli-Opitz syndrome.

Cholesta-5,8-dien-3β-ol (**Ia**) has been reported to be present at very low levels in blood of patients with cerebrotendinous xanthomatosis[1] and from normal subjects.[2] The same sterol, along with 7-dehydrocholesterol (**IIa**), has been reported[3,4] to accumulate at substantial levels in blood and tissues of individuals with the Smith-Lemli-Opitz syndrome (SLOS), a hereditary disorder affecting the development of multiple organ systems.[5] With the exception of NMR studies[3] of **Ia** from feces, the identification of the $\Delta^{5,8}$-sterol from human subjects has been based largely on GC–MS studies. **Ia** has also been reported[6] to accumulate in livers of newborn rats of mothers treated in late pregnancy with AY-9944, an inhibitor of the enzymatic conversion of **IIa** to cholesterol. Very recently, the catalysis, by rat liver microsomes, of the conversion of **IIa** to **Ia** has been demonstrated.[7]



Ia R = H
Ib R = Ac
Ic R = $Si(CH_3)_3$

IIa R = H
IIb R = Ac

IIIa R = H
IIIb R = Ac
IIIc R = $Si(CH_3)_3$

IV

Batta et al.[4] recently reported the presence of substantial levels of 19-norcholesta-5,7,9-trien-3β-ol (**IIIa**), along with **Ia** and **IIa**, in blood plasma and feces from SLOS patients and speculated that **IIIa** might be partially responsible for some developmental abnormalities in SLOS patients. Whereas we know of no other reports of the occurrence of such a 19-norsterol with an aromatic ring B from mammalian sources, it is interesting to note early reports[8,9] of the occurrence of a 3-hydroxy-$\Delta^{5,7,9}$-estratrien-17-one in pregnant mare urine. Despite the reported[4] occurrence of **IIIa** in plasma and dried feces at significant levels (~3.6 and ~1.4 percent of total sterols, respectively), it is important to note that the identification of **IIIa** was based solely upon GC–MS properties of its TMS derivative **IIIc** in comparison with those of an authentic sample.

In ongoing studies of the sterols in plasma and erythrocytes of SLOS patients, we have also noted the presence of material with the expected properties of **IIIa**, as its TMS derivative, upon GC or GC–MS analysis of crude nonsaponifiable lipids, crude C_{27} sterols, or fractions derived therefrom containing **Ia**. However, ^1H NMR and Ag^+-HPLC analyses of the same samples did not indicate the presence of **IIIa**. These observations indicating the formation of material with the expected properties of **IIIa** only upon GC or GC–MS analyses prompted investigations of the thermolability of **Ia**.

For studies of this matter, authentic samples of **Ia**,[10a] **Ib**,[10a] **IIIb**,[11a] and **IIIa**[12] were required. In addition, a labeled analog of **Ia**, [3α-^{3}H]-**Ia**,[13] was prepared to facilitate quantitative studies of the thermolability of the TMS derivative of **Ia**.

Although the synthetic samples of **Ia** and **Ib** were of very high purity, GC–MS[14] analyses of **Ib** (or **Ic**) showed the presence of significant amounts of material with the GC–MS properties of authentic **IIIb** (or **IIIc**). The formation of the latter material varied directly with the amount of material injected and inversely with the injector head pressure with a falling-needle injector system (Table 1). Similar relationships were also observed with a split-splitless injector[15] (data not shown).

Table 1. Thermolability of **Ic** upon GC-MS analysis: formation of **IIIc**.[a]

Mass Injected (μg)	Injector Head Pressure (psi)	Sterol Composition	
		Ic	**IIIc**
0.2	18	100.0	0.0
0.7	18	96.6	3.4
1.2	18	95.8	4.2
2.5	18	93.7	6.3
0.2	10	98.7	1.3
0.7	10	91.7	8.3
1.2	10	89.6	10.4
2.5	10	87.2	12.8
0.2	5	94.3	5.7
0.7	5	91.4	8.6
1.2	5	82.7	17.3
2.5	5	77.5	22.5

[a] GC-MS conditions described in note 14. Relative amounts of **Ic** and **IIIc** were estimated by integration of peaks in the total ion chromatogram. These peaks showed baseline resolution at higher injection head pressures and lower injection amounts. No other components were observed.

Heating of the TMS derivative of [3α-^{3}H]-**Ia** (0.1 mg) in a sealed glass ampule under argon at 150 °C or above in an oven[15] for 5 min gave varying amounts of material with the properties of **IIIc** as indicated by the results of GC-MS analyses[16] and, without exposure to heat of the GC, by Ag^+-HPLC analyses[17] of the free sterols obtained after hydrolysis of the TMS ethers[18] or by Ag^+-HPLC analyses[19] of the acetate derivatives[20] (Table 2). Heating at 100 °C for 5 min did not lead to the formation of **IIIc**, whereas heating at 150 °C or

higher for 5 min gave substantial amounts of the 19-nor-$\Delta^{5,7,9}$-triene. Heating at 200 °C (or higher) for 5 min gave lower recoveries of the ^3H label in **Ic** or **IIIc**, presumably due to substantial further decomposition of the sterol(s).

Table 2. Thermal induction of the conversion of the TMS derivative of [3α-^{3}H]-**Ia** to the TMS ether of **IIIa** by heating in an oven for 5 min.

		Sterol Distribution					
Temperature (°C)	Recovery of ^3H (%)[a]	Ag^+-HPLC of free sterol		Ag^+-HPLC of acetate		GC-MS (TMS)	
		% of Recovered ^3H in:				% Recovered in:	
		Ia	**IIIa**	**Ib**	**IIIb**	**Ic**	**IIIc**
100	97	100	0	96	0	98	2
150	91	82	11	88	10	73	8
200	88	40	49	36	45	37	45
250	61	21	55	16	72	22	63
300	44	4	54	1	71	0	78

[a] Recovery of toluene-soluble products, including **Ic**, **IIIc**, and non-volatile material.

In separate experiments, the TMS derivative of **Ia** (1 mg) was heated in a sealed glass ampule at 200 °C[21] or 250 °C[22] for 10 min in an oven. The free sterols, obtained by treatment with tetrabutylammonium fluoride in THF,[18] were subjected to semi-preparative Ag^+-HPLC[23] to give **IIIa**. Both samples of **IIIa** (i.e., from heating at 200 °C and 250 °C) showed GC-MS and ^1H NMR spectra[24] essentially identical to those of authentic **IIIa**.

The results of the present study demonstrate the facile, thermally induced conversion of cholesta-5,8-dien-3β-ol, as its TMS or acetate derivatives, to the corresponding derivatives of 19-norcholesta-5,7,9-trien-3β-ol. These findings have obvious relevance to the reported occurrence of substantial levels of the 19-nortriene in samples of blood and feces from patients with SLOS[4,25,26] and indicate the requirement for careful attention to experimental conditions in GC and GC–MS analyses of samples containing the $\Delta^{5,8}$-sterol so as to avoid the artifactual generation of the 19-nortriene.

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10. (a) Authentic **Ia** and **Ib** of high purity (>99%) were prepared and characterized as described previously.[10b] (b) Wilson, W. K.; Sumpter, R. M; Warren, J. J.; Rogers, P. S.; Ruan, B.; Schroepfer, G. J., Jr. *J. Lipid Res.* **1996**, *37*, 1529.
11. (a) Pyrolysis of the $\Delta^{5,8}$-adduct (**IV**) of **IIb** and diethyl azodicarboxylate[10b] under the conditions described by van der Gen et al.[11b] gave, after purification, an authentic sample of **IIIb**, which showed a single component (>99%) on Ag^+-HPLC[7] and was characterized by GC-MS and ^1H and ^{13}C NMR.
(b) van der Gen, A; Lakeman, J.; Gras, M. A. M. P.; Huisman, H. O. *Tetrahedron* **1964**, *20*, 2521.
12. Saponification of **IIIb** gave **IIIa**, mp 118.0-118.5 °C which was characterized by ^1H and ^{13}C NMR, high resolution MS, and by GC-MS of its TMS derivative.
13. [3α-^{3}H]-**Ia** was prepared from **Ia** by oxidation to the corresponding 3-ketone followed by reduction with sodium borotritide to give, after chromatographic purification, the desired labeled sterol (11.3 μCi/μmol), which was characterized by its chromatographic behavior on normal and reversed phase HPLC, Ag^+-HPLC,[7] and capillary GC (60 m DB-5 column; TMS derivative) and by GC-MS (TMS derivative). The radiopurity was >99% as judged by normal and reversed phase HPLC and Ag^+-HPLC.
14. Varian 3400 GC, helium carrier gas, falling needle injector, 30-m DB-5MS column (0.25 mm id, 0.1 mm film thickness), injector and column at 250 °C, GC-MS transfer line at 260 °C, Extrel ELQ-400 quadrupole mass spectrometer, 70 eV.
15. Hewlett Packard 5890 GC.
16. Same GC-MS conditions as in note 14, with injector head pressure of 18 psi.
17. Ag^+-HPLC column (300 mm × 2.5 mm), prepared as described previously,[7] with 9.1% acetone in hexane as eluting solvent and sample application with hexane. Under these conditions **Ia** and **IIIa** showed retention times of ~31 min and ~15 min, respectively).
18. Tetrabutylammonium fluoride (1 M) in THF (200 μL) for 30 min at rt. Water was added and the mixture was extracted with MTBE. The free sterols were recovered after passage through a short silica gel column (5 cm × 0.5 cm) using 5% acetone in hexane (20 mL) prior to Ag^+-HPLC.[17]
19. Ag^+-HPLC column (250 mm × 4.6 mm), prepared as described previously;[7] elution with 3% acetone in hexane. Under these conditions, **Ib** and **IIIb** showed retention times of 37 min and 17.5 min, respectively.
20. Prepared from the free sterols obtained above[18] with acetic anhydride and pyridine (1:1) for 24 h at rt. After addition of water, the acetates were recovered by extraction with MTBE and passed through a silica gel column[18] prior to analysis by Ag^+-HPLC.[19]
21. Placed in oven[15] at 150 °C. The oven temperature was increased from 150 °C to 200 °C over 0.5 min and then maintained at 200 °C for 10 min.
22. Placed in oven[15] at 200 °C. The oven temperature was increased from 200 °C to 250 °C over 0.5 min and then maintained at 250 °C for 10 min.
23. Ag^+-HPLC[7] column (30 cm × 1 cm) using 15% acetone in hexane.
24. ^1H NMR chemical shifts, obtained as described previously,[10b] matched those of an authentic standard of **IIIa** to within 0.001 ppm (methyl and olefinic signals) or 0.002 ppm (other signals).
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