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High-performance liquid chromatographic analysis of human erythrocyte oxysterols as Δ^4-3-ketone derivatives

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Abstract

Following oxidation by cholesterol oxidase human erythrocyte oxysterols were analyzed as the corresponding hydroxy-Δ^4-3-ketones by HPLC with effluent monitoring at 235 nm. Variable amounts of epimeric cholest-5-ene-3β,7-diols, 3β-hydroxycholest-5-en-7-one, cholest-5-ene-3β,19-diol, cholest-5-ene-3β,20-diol, cholest-5-ene-3β,25-diol and cholest-5-ene-3β,26-diol were detected in erythrocyte membranes of patients with sickle cell anemia or sickle cell trait. Only 3β-hydroxycholest-5-en-7-one was detected in erythrocyte membranes of healthy donors.

1. Introduction

Proper analysis of tissue oxysterols, simple sterol oxidation products, requires resolution of up to ten or so components by thin-layer (TLC), gas or high-performance liquid (HPLC) chromatography. Of the most frequently encountered oxysterols, the B-ring oxidized sterols cholest-5-ene-3β,7α-diol (**1**), cholest-5-ene-3β,7β-diol (**2**), 3β-hydroxycholest-5-en-7-one (**3**) constitute one set, the isomeric 5,6-epoxides 5,6α-epoxy-5α-cholestan-3β-ol (**4**) and 5,6β-epoxy-5β-cholestan-3β-ol (**5**) and their common hydration product 5α-cholestane-3β,5,6β-triol (**6**) another, and the cholesterol metabolites oxidized in the side-chain (20*S*)-cholest-5-ene-3β,20-diol (**7**), (24*S*)-cholest-5-ene-3β,24-diol (**8**), cholest-5-ene-3β,25-diol (**9**), and (25*R*)-cholest-5-ene-3β,26-diol (**10**) yet another.

Each oxysterol set has individual analysis requirements, and despite the many analysis schemes devised analysis of all ten oxysterols yet poses problems of resolution, detection and estimation. Recovery of individual oxysterols for characterization and other work is best served by HPLC, and the Δ^5-oxysterols **1–3** and **7–10** have been resolved in a variety of HPLC procedures that generally rely on effluent monitoring at 205–215 nm [1,2]. The presence of confounding non-sterol tissue components limits these means to relatively pure tissue sterol samples.

In seeking to devise improved yet simplified means of resolution and recovery of common tissue oxysterols in complex tissue matrices we explored analysis of oxysterols **1**, **2** and **7–10** that are substrates for microbial cholesterol oxidases (cholesterol: O_2 oxidoreductase, EC 1.1.3.6) [3–7]. The 7-ketone **3** is not a substrate [7,8], but as a hydroxy-α,β-unsaturated ketone the 7-ketone **3** and the enzyme product hydroxy-Δ^4-3-ketones from **1** and **2**, and **7**, **9** and **10** are resolved by HPLC and detected at 235–240 nm together as a set. The longer monitoring wavelength eliminates interferences from non-sterol tissue components, and the enzymic alteration of oxysterol samples prior to HPLC ensures that only

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genuine oxysterol Δ^5-3β-alcohols will be observed at the improved sensitivity.

Application of this approach to analysis of Δ^5-sterols and oxysterols has been previously described [9–14], and we record here exploration of the oxysterol composition of human erythrocyte membranes by these means.

2. Experimental

2.1. Materials

Oxysterols **1–10** are from our extensive sterol collection and from Steraloids (Wilton, NH, USA). Cholest-5-3β,19-diol (**11**) and cholesterol oxidase from *Nocardia erythropolis* were obtained from Sigma (St. Louis, MO, USA). Reference samples of oxysterol Δ^4-3-ketones were prepared by incubation of parent oxysterols **1**, **2**, **7** and **9–11** with cholesterol oxidase, with isolation of the product by TLC. Solvents for HPLC are from Burdick & Jackson Labs. (Muskegon, MI, USA).

2.2. Erythrocyte sample preparation

Erythrocytes from fresh blood samples obtained from hospitalized patients with sickle cell trait or sickle cell anemia (1.0–2.0 ml) and from healthy volunteers (4.0–6.0 ml) were washed three times with two volumes each of 0.001 *M* phosphate–saline pH 7.5 buffer and centrifuged at 1500 *g* for 15 min at 4°C. The erythrocytes were then lysed with three volumes of 0.01 *M* pH 8.6 phosphate buffer, centrifuged at 4000 *g* for 15 min, and transferred to a 100 × 13 mm glass tube. Lysed erythrocyte membranes were mixed with an equal volume of methanol (alternatively 2-propanol) on a vortex shaker to avoid aggregation, and total lipids were extracted by mixing in a vortex shaker with two volumes chloroform (1.5 volumes if 2-propanol used). Following centrifugation the chloroform phase was removed and the extraction process repeated twice with premixed chloroform–methanol (2:1, v/v) or chloroform–2-propanol (11:7, v/v). The chloroform–methanol extraction gave a yellow-colored extract whereas the chloroform–2-propanol solvent gave a colorless extract but with slightly less cholesterol and phospholipids recovered. Chloroform–methanol was the preferred extraction solvent.

Pooled chloroform extracts were evaporated under nitrogen, redissolved in 0.5–1.0 ml chloroform, and applied as a zone no more than 10 cm long to 20 × 20 cm Uniplate HLF silica gel chromatoplates 0.25 mm thick (Analtech, Newark, DE, USA), together with reference oxysterols 3β,7α-diol **1** and 3β,20-diol **7** well away from the applied test sample. The applied material was concentrated into a compact, thin zone by ascending irrigation with chloroform–methanol (2:1, v/v), and then irrigated with hexane–diethyl ether–acetic acid (65:35:1, v/v/v) in ascending fashion to a height of 15 cm two times. The sample region was covered with a 20 × 10 cm glass plate and the chromatogram exposed to iodine vapors to visualize the reference oxysterols **1** and **7** as guides for recovery of the oxysterols in the test sample. The test sample zone limited by oxysterols **1** and **7** was excised, packed into a 2-ml ASTM Pyrex filter funnel, and eluted with three 2.0-ml portions of chloroform–methanol (3:1, v/v). The combined oxysterols eluates were evaporated under nitrogen.

2.3. Cholesterol oxidase incubations

Incubation procedures of Goh et al. [9] were modified. To the total oxysterols dissolved in 50 μl 2-propanol in a 75 × 12 mm culture tube were added 250 μl saline, the mixture mixed by vortex shaker briefly, and 500 μl 20 m*M* pH 7.5 phosphate buffer containing 0.1% sodium cholate and 0.3% (w/v) Tween 20 were added. After gentle mixing by vortex shaker a clear dispersion was obtained. Samples of cholesterol (200 μg) so treated may be cloudy at this point.

To the clear dispersion was added 100 μl 0.1 *M* pH 7.8 Tris buffer containing 60 units catal-

ase, and oxidation was initiated at 37°C by adding 100 μl of the same buffer containing 0.4 unit cholesterol oxidase. After 60 min 300 μl methanol were added to stop the reaction, after which products were extracted with three 2-ml portions of light petroleum (b.p. 35–60°C).

2.4. Chromatography

TLC was conducted with 20 × 20 cm chromatoplates 0.25 mm thick irrigated twice in ascending fashion with hexane–diethyl ether (2:3, v/v). Steroid Δ^4-3-ketones were detected by their 254 nm light absorption; Δ^5-sterols were detected with iodine vapors.

HPLC was conducted using Beckman Instruments equipment consisting of system controller Model 421A, solvent-delivery module Model 114M, and organizer Model 340 with universal sample injection valve Model 210 with 20-μl sample loop, variable-wavelength detector Model 165 with 5-mm micro flow cell (2.2 ml illuminated volume, set at 0.01 AUFS), all connected to two Waters Model 740 data modules (attenuation setting 16).

Samples (0.005–5.0 μg in 10 μl chloroform) were injected through a Perisorb A precolumn onto the analysis column: system A, 250 mm × 4.6 mm Ultrasphere SIL 5 μm particle size (Beckman Instruments), or system B, 300 × 3.9 mm μPorasil 10 μm particle size (Waters), irrigated with hexane–2-propanol (50:1, v/v) at 1 ml/min flow-rate. Effluent was monitored at 210 nm for oxysterols and at 235 nm for the corresponding Δ^4-3-ketosteroids.

3. Results

Oxysterols **1**, **2**, **7** and **9–11** were smoothly transformed by cholesterol oxidase to the corresponding hydroxy-Δ^4-3-ketones. Resolution of oxysterols **1–3**, **7** and **9–11** and of the corresponding hydroxy-Δ^4-3-ketones and hydroxy-Δ^5-7-ketone **3** by TLC and by HPLC is accomplished with our systems (Table 1, Fig. 1) (with the exception of the 3β,25-diol **9** and 7α-hydroxy-Δ^4-3-ketone pair), thus according means of analysis of oxysterol and oxidized oxysterol mixtures.

Comparison of detection sensitivity of the Δ^4-3-ketones (235 nm) versus their parent Δ^5-3β-alcohols (210 nm) (Table 2) shows improved sensitivity ranging from 3.1–24-fold by peak height, 1.8–20-fold by peak area. Linear relationships between steroid mass and absorbance (peak height) were obtained over the range 0.1–5.0 μg for the oxysterols, but linear responses were had for the Δ^4-3-ketones at lower levels: 0.04–1.0 μg (100 pmol–2.5 nmol) for the 7α-, 20-, 25- and 26-hydroxy-Δ^4-3-ketones; 0.04–2.0 μg (100 pmol–5 nmol) for the 19-hydroxy-Δ^4-3-ketone; and 0.16–4.0 μg (400 pmol–10 nmol) for the 7β-hydroxy-Δ^4-3-ketone. Absorbance responses (AU/μg steroid) were very similar for the 7α-hydroxy- (0.025), 19-hydroxy- (0.026), 20-hydroxy- (0.026), 25-hydroxy- (0.024) and 26-hydroxy- (0.025) Δ^4-3-ketones but less for the 7β-hydroxy-Δ^4-3-ketone (0.011). A detection limit of 0.005–0.05 μg (12–125 pmol) is suggested, somewhat greater than 10 pmol suggested in other work [9,10].

Recoveries of 100-μg samples of the hydroxy-Δ^4-3-ketone derivatives taken through the full procedure of TLC, elution, concentration, and HPLC averaged 90 ± 5% for the 20-, 25- and 26-hydroxyketones, 92 ± 5% for the 7α- and 19-hydroxyketones, and 88 ± 5% for the 7β-hydroxyketone.

Human blood samples from four healthy subjects, from four sickle cell trait patients, and from nine patients with sickle cell anemia or sickle cell trait (uncertain which) were analyzed by our procedure. In those cases where blood sample size permitted analysis of both oxysterol and hydroxy-Δ^4-3-ketone mixtures, there was agreement between the analyses with regard to the number and identity of oxysterols detected.

Oxysterols detected in erythrocyte membranes of ill patients as the corresponding Δ^4-3-ketones included the epimeric 3β,7-diols **1** and **2**, 3β,19-

Table 1
Chromatographic properties of oxysterols and their Δ^4-3-ketones

	Relative mobility[a]		
	TLC	HPLC system A	HPLC system B
Cholesterol	1.98	0.16	–
Cholest-4-en-3-one	2.31	0.10	0.11
Cholest-5-ene-3β,7α-diol (**1**)	1.02	2.70	–
7α-Hydroxycholest-4-en-3-one	1.19	0.53	0.51
Cholest-5-ene-3β,7β-diol (**2**)	1.08	2.55	–
7β-Hydroxycholest-4-en-3-one	1.05	1.35	1.00
3β-Hydroxycholest-5-en-7-one (**3**)	1.00	1.00	1.00
Cholest-5-ene-3β,19-diol (**11**)	0.64	2.07	–
19-Hydroxycholest-4-en-3-one	0.93	0.93	0.93
(20*S*)-Cholest-5-ene-3β,20-diol (**7**)	1.66	0.26	–
20-Hydroxy-(20*S*)-cholest-4-en-3-one	1.83	0.39	0.15
Cholest-5-ene-3β,23-diol	–	0.41	–
23-Hydroxycholest-4-en-3-one	–	0.43	–
(24*S*)-Cholest-5-ene-3β,24-diol (**8**)	–	0.35	–
24-Hydroxy-(24*S*)-cholest-4-en-3-one	–	–	–
Cholest-5-ene-3β,25-diol (**9**)	1.24	0.54	–
25-Hydroxycholest-4-en-3-one	1.37	0.46	0.27
(25*R*)-Cholest-5-ene-3β,26-diol (**10**)	1.20	0.76	–
26-Hydroxy-(25*R*)-cholest-4-en-3-one	1.37	0.57	0.39

[a] Mobility data are calculated with 3β-hydroxycholest-5-en-7-one (**3**) (unaltered by cholesterol oxidase) as unit mobility, with R_F 0.40 in TLC system hexane–diethyl ether (2:3, v/v), retention time 50 min in HPLC system A: Ultrasphere SIL, hexane–2-propanol (50:1, v/v), and retention time 58 min in HPLC system B: μPorasil, hexane–2-propanol (50:1, v/v).

diol **11**, 3β,20-diol **7**, 3β,25-diol **9**, 3β,26-diol **10**, the 7-ketone **3** and an unidentified component. Moreover, oxysterols were not detected in erythrocyte membranes from four healthy donors; only a low level (30 ng/ml) of **3** was detected in one sample (Table 3).

In membrane extracts where cholesterol was not excluded by prior TLC low levels of 20-hydroxycholest-4-en-3-one and 25-hydroxycholest-4-en-3-one were detected following cholesterol oxidase incubations where the corresponding oxysterol 3β,20-diol **7** and 3β,25-diol **9** had not been detected in unoxidized samples. Repeated incubations of 200 μg cholesterol with 0.4 units cholesterol oxidase even in the presence of 60 units catalase established that both 20- and 25-hydroxy-Δ^4-3-ketone products were regularly formed. Also, some cholesterol autoxidation products 3β,7α-diol **1** and 3β,7β-diol **2** (detected as the corresponding 7α- and 7β-hydroxy-Δ^4-3-ketones) and 7-ketone **3** were detected following cholesterol oxidase treatment.

4. Discussion

The indicated increased sensitivity, linear responses, good analyte recoveries and resolution of the hydroxy-Δ^4-3-ketosteroids of interest provides an improved means of their analysis in complex mixtures. The procedure applied to analysis of these oxysterols in human erythrocytes also gives additional insight into the enzymic oxidation of cholesterol by cholesterol oxidase and into the nature of erythrocyte membrane oxysterols of patients suffering from sickle cell anemia and sickle cell trait.

The adventitious autoxidation of cholesterol to

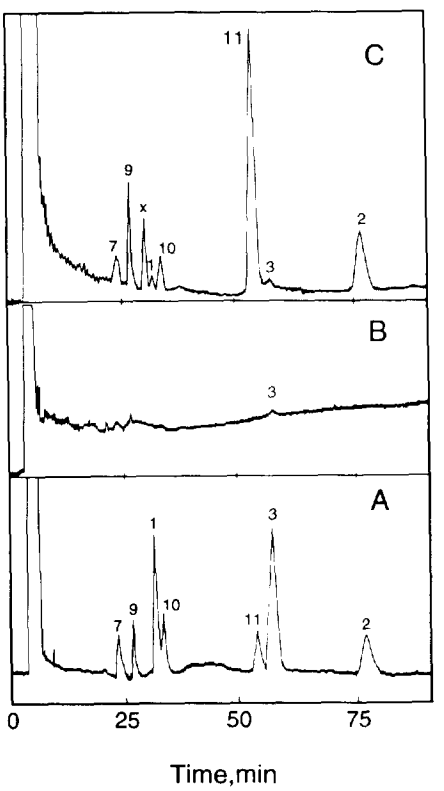

Fig. 1. HPLC elution profiles of oxysterol α,β-unsaturated ketones in system A [Ultrasphere SIL with hexane–2-propanol (50:1, v/v) monitored at 235 nm for absorbance]. The ordinate scale is 0.01 absorbance unit for the initial pen excursion from start to solvent peak height. Components: (A) reference Δ^5-7-ketone **3** and Δ^4-3-ketone derivatives of oxysterols **1**, **2**, **7**, **9**–**11**; (B) absorbing components obtained following cholesterol oxidase treatment of total oxysterols from erythrocyte membranes of a healthy donor; only Δ^5-7-ketone **3** is evident; (C) Δ^5-7-ketone **3** and Δ^4-3-ketones of oxysterols **1**, **2**, **7**, **9**–**11** formed by cholesterol oxidase treatment of total oxysterols from erythrocyte membranes of a sickle cell trait patient; peak x is an unidentified absorbing component.

3β,7α-diol **1**, 3β,7β-diol **2** and 7-ketone **3** during cholesterol oxidase incubations represents yet another example of this insidious process. However, the additional oxidation of cholesterol at the tertiary C-20 and C-25 carbon atoms yielding 20- and 25-hydroxy-Δ^4-3-ketones poses new concern about the nature of the enzymic oxidation. The oxidation of cholesterol at the C-20 and C-25 sites by cholesterol oxidase of *Pseudomonas fluorescens* has previously been observed [15]. This complication necessarily affects the analysis of tissue oxysterols, and both autoxidation and C-20 and C-25 oxidations must be avoided rigorously. Accordingly, only the more polar erythrocyte membrane oxysterols are taken for analysis, and the bulk membrane cholesterol is excluded carefully in our procedure.

Whereas the oxysterol composition of selected human tissues, plasma and serum has been extensively examined, analysis of human erythrocyte oxysterols has received very limited attention. Our early gas chromatography study [16] of human erythrocyte sterols suggested the presence of low levels of a cholest-5-ene-3β,26-diol, and later TLC examination of human erythrocytes sterols by others failed to detect oxysterols [17–19]. More recently the epimeric 3β,7-diols **1** and **2**, 7-ketone **3**, 5α,6α-epoxide **4**, 3β,5α,6β-triol **6**, 3β,20-diol **7** and 3β,25-diol **9**, but also the 3β,19-diol **11** have been detected as oxysterol components of human sickle cell erythrocyte membranes [20].

Our present study confirms some of these results but differs in other aspects. Compounds **1**, **2**, **3**, **11**, **7** and **9** were found in both studies, and we also detect the 3β,26-diol **10** but not the 5α,6α-epoxide **4** and 3β,5α,6β-triol **6** that are undetectable by our methods. Although the 5,6-epoxides **4** and **5** may be poor substrates [11], their oxidized products are not Δ^4-3-ketones, and the 3β,5α,6β-triol **6** is not a substrate [7].

The individual oxysterol patterns and levels (Table 3) vary from case to case. Although **3** is present in all samples and **1**, **2**, **7** and **9** in most, **11** and **10** were found in but a few instances. Moreover, oxysterol levels range from undetected to traces to high levels (**1**, 19.2 μg/dl; **2**, 18.2 μg/dl; **3**, 75.0 μg/dl; **7**, 20.2 μg/dl; **9**, 25.2 μg/dl; **10**, 9.0 μg/dl; **11**, 30.6 μg/dl) comparable to levels previously reported for sickle cell erythrocytes and for human plasma and serum. For the ubiquitous 7-ketone **3** we estimate 1.0–75.0 μg/dl whole blood, Küçük et al. [20] report 900 ng/ml erythrocytes, and human plasma and serum levels of 0.4–373 ng/ml occur [21].

The B-ring oxysterols **1**–**6** are generally regarded as arising from in vivo interception by

Table 2
Increased sensitivity of detection of Δ^4-3-ketosteroids in comparison with parent Δ^5-3β-hydroxysterols

Parent Oxysterol	Peak height (cm)			Peak area (cm^2)		
	Δ^5-3β-Alcohol	Δ^4-3-Ketone	Ratio	Δ^5-3β-Alcohol	Δ^4-3-Ketone	Ratio
3β,7α-Diol **1**	0.2	4.8	24.0	0.025	0.500	20.0
3β,7β-Diol **2**	0.4	1.9	4.8	0.200	0.475	2.4
3β,19-Diol **11**	0.3	4.1	13.6	0.125	0.820	6.6
3β,20-Diol **7**	1.0	3.1	3.1	0.100	0.310	3.1
3β,25-Diol **9**	1.5	5.5	3.7	0.220	0.600	2.7
3β,26-Diol **10**	1.7	6.3	3.7	0.340	0.620	1.8

Data are from elution charts obtained with 0.2 μg parent oxysterol or Δ^4-3-ketone derivative in 10 μl chloroform injected on column, eluate measured at 210 nm for Δ^5-3β-hydroxysteroids, at 235 nm for Δ^4-3-ketosteroids.

cholesterol of reactive oxygen species, whereas the side-chain hydroxylated sterols **7–10** (and 3β,7α-diol **1**) are cholesterol metabolites [21]. The 3β,19-diol **11** has not previously been recognized as a component of human tissues, although the oxysterol **11** is suggested present in meat

Table 3
Oxysterol levels in sickle cell erythrocytes

Patient[a]	Oxysterols found (μg/dl)[b]						
	3β,7α-Diol **1**	3β,7β-Diol **2**	7-Ketone **3**	3β,20-Diol **7**	3β,25-Diol **9**	3β,26-Diol **10**	3β,19-Diol **11**
Sickle cell trait							
No. 1	19.2	18.2	5.4	9.0	25.2	9.0	2.4
	–	–	35.8	12.0	–	–	–
No. 2	1.2	7.2	4.2	2.4	12.6	3.6	1.2
	1.6	0.8	1.2	2.4	4.8	2.4	–
	2.5	tr	1.0	tr	tr	–	30.6
No. 3	1.2	tr	1.0	tr	tr	–	3.8
No. 4	tr	–	4.0	–	tr	–	–
Sickle cell anemia and/or sickle cell trait							
No. 5	7.8	–	36.8	16.8	–	–	–
No. 6	tr	–	13.8	16.8	–	–	–
No. 7	4.8	–	75.0	19.8	tr	–	–
No. 8	8.0	tr	4.0	11.0	tr	–	–
No. 9	8.0	tr	2.0	2.0	3.0	–	tr
No. 10	–	–	2.5	5.0	–	–	–
No. 11	–	–	1.0	–	11.0	–	–
	4.0	tr	1.0	tr	20.0	–	–
No. 12	–	–	6.0	20.2	–	–	–
No. 13	6.0	–	60.0	2.4	tr	tr	–

[a] Patients: No. 1 = male pediatric; No. 2 = female pediatric; No. 3 = male adult; No. 4 = female adult, with diagnosed sickle cell trait. Diagnoses and personal data for patients Nos. 5–13 not available.
[b] Calculated for whole blood. Where there is no entry, the component was not detected; unmeasured trace amounts abbreviated tr.

products [22] and is implicated in sterol metabolism of the sponge *Axinella polypoides* [23].

Thus, the two oxysterol sets **7**, **9**, **10** and **1–4**, **6** found indicate that sickle cell and sickle cell trait erythrocytes accumulate cholesterol metabolites, perhaps from plasma, and also products of cholesterol oxidation by reactive oxygen species formed via in vivo oxygen metabolism. As sickle cell erythrocytes appear to generate substantial amounts of superoxide, peroxide, and hydroxyl radical [24], the B-ring oxidized sterols **1–4** and **6** may be formed within the cell as well as accumulated from the plasma. In any event, these items suggest that increased oxidative stress be a component of the disease.

Blood samples for two pediatric sickle cell trait patients, Nos. 1 and 2 (Table 3) were analyzed at different times. Considerable variations appear among data for patient No. 1, with high levels of 3β,7-diols **1** and **2** and 3β,25-diol **9** at one time but lacking in a sample two months later, at which time increased level of 7-ketone **3** is evident. For patient No. 2 a high level of 3β,19-diol **11** occurs three months after an analysis in which the oxysterol was undetected. Data for adult patients Nos. 3 and 4 suggest similar variable erythrocyte membrane composition.

Data for patients Nos. 5–13 for whom diagnoses and personal data are unavailable exhibit similar variability but with greater absence of 3β,7β-diol **2**, 3β,19-diol **11** and 3β,26-diol **10**. Two samples from patient No. 11 demonstrate variation from time to time in the same patient.

In view of the limited diagnosis and personal data it is not possible to conclude whether patient age or sex are factors, and whether sickle cell anemic or sickle cell trait affect results. However, the variations in data do not appear to represent experimental error. Rather, the variable presence and levels of oxysterols may reflect the periodic crisis nature of the disease in which variable non-enzymic oxidation processes occur.

However, other factors (liver function, bile acid biosynthesis) influence fluctuations in plasma levels of oxysterols and may also affect erythrocyte membrane levels. The presence in human plasma of 3β,7α-diol **1** and its oxidation product 7α-hydroxycholest-4-en-3-one (3–40 ng/ml [25,26]) may arise in part from hepatic biosynthesis of bile acids subject to various health states.

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