Microbial synthesis of 1β- and 15β-hydroxylated bile acids

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Abstract Lithocholic, deoxycholic, and cholic acids were incubated with the molds *Absidia coerulea* Bain (CBS 105.08, I), three strains of *Rhizoctonia solani* Kühn (CBS 130.14 (II), CBS 341.36 (III), CBS 325.47 (IV)), and *Penicillium* species ATCC 12556 (V). The products were analyzed by gas–liquid chromatography and partially characterized by gas–liquid chromatography–mass spectrometry. Lithocholic acid was most extensively hydroxylated while cholic acid yielded only traces of hydroxylated metabolites. I, II, III, and V hydroxylated at position C-1; I at C-6; I and II at C-7; III at C-12; and I, II, IV, and V at C-15. Hydroxylations at unidentified positions were observed for all molds, and oxidoreductions at C-7 and C-12 were carried out by I, II, and III. The two major trihydroxycholanoates formed by V from deoxycholic acid were characterized by chemical, mass spectrometric, and nuclear magnetic resonance techniques as 1β,3α,12α- and 3α,12α,15β-trihydroxy-5β-cholanoic acids. The former bile acid was identical with the 1,3,12-trihydroxycholanoic acid present in human urine.—**Carlström, K., D. N. Kirk, and J. Sjövall.** Microbial synthesis of 1β- and 15β-hydroxylated bile acids. *J. Lipid Res.* 1981. **22:** 1225–1234.

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In a recent study of bile acids in urine, compounds were found which carried hydroxyl groups in positions other than 3, 6, 7, and 12 (1). Mass spectrometric data showed that one type of bile acids was hydroxylated at C-1. The most commonly occurring bile acid of this type was tentatively identified as 1,3,12-trihydroxycholanoic acid. It was desirable to obtain larger amounts of this and similar bile acids for further chemical characterization, and a synthesis by microbial hydroxylation appeared to be the most convenient method. As far as we know, microbial 1-hydroxylation of bile acids has not been described. However, 1-hydroxylation of steroids and cardiac steroid aglycones with C_{18}, C_{19}, C_{21}, and C_{23} skeletons has been reported for about 40 different microorganisms, most of them molds (2–6). From these data we selected a number of molds that were tested for their ability to 1-hydroxylate bile acids. The present study describes the results of incubations of lithocholic, deoxycholic, chenodeoxycholic, and cholic acids with the selected molds.

MATERIAL AND METHODS

Growth of microorganisms

Absidia coerulea Bain (CBS 105.08), and three strains of *Rhizoctonia solani* Kühn (CBS 130.14, CBS 341.36, and CBS 325.47) were obtained from Centraalbureau voor Schimmelcultures, Baarn, Holland. *Penicillium* species ATCC 12556 was obtained from the American Type Culture Collection, Rockville, MD.

A. coerulea has been reported to hydroxylate C_{23} cardiac steroid aglycones in positions 1β,5β, and 7β, to carry out oxidoreduction in the 3-position, and to introduce a double bond in position 4 (6). The three strains of *R. solani* have been reported to hydroxylate C_{19} and C_{21} steroids in positions 1ξ, 2β, 6β, 11α, 11β, 15ξ, and to carry out oxidoreductions at positions 11 and 20 (6). Finally, *Penicillium* sp. has been reported to hydroxylate C_{19} and C_{21} steroids in positions 1α, 2β, 6β, 7β, and 15β, to carry out oxidoreduction at positions 3 and 15, to isomerize a 5,6 double bond to a 4,5 position, and to saturate the 4,5 double bond (6).

The following media were used: *A. coerulea:* 35 g

Abbreviations and trivial names: chenodeoxycholic, 3α,7α-dihydroxy-5β-cholanoic; deoxycholic, 3α,12α-dihydroxy-5β-cholanoic; lithocholic, 3α-hydroxy-5β-cholanoic; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance, TMS, trimethylsilyl; t_R, retention time relative to that of the TMS ether of methyl cholate.

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glucose, 3 g peptone, 10 g malt extract, 2.5 g yeast extract, and 2.5 g prune agar in 1 litre of distilled water was adjusted to pH 7.0 and autoclaved at 120°C for 45 min. After cooling, 100 ml of a sterile solution of 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7 H_2O$, 0.5 g KCl, and 0.01 g $FeSO_4 \cdot 7 H_2O$ per litre was added aseptically. *Rhizoctonia* strains: as for *A. coerulea*, but 2.5 g soytone and 0.29 g $CaCO_3$ were added before pH adjustment. *Penicillium* sp.: 28 g glucose, 2.4 g AC Broth, 8 g malt extract, 2 g yeast extract, 2 g soytone, and 1 g prune agar per litre was adjusted to pH 7.0 and autoclaved at 120°C for 45 min.

Growth of the organisms as well as the bile acid transformations were carried out in cotton-plugged conical flasks on a shaking table at 28°C. Inoculum was prepared by growing the organism on 50 ml of medium for 6 days. Biomass for the bile acid transformations was prepared by growing in 150–300-ml portions of medium for time intervals given below for the individual transformations. The mycelia were harvested by centrifugation, washed three times with 0.1 M potassium phosphate buffer, pH 7.0, and resuspended to original density in this buffer. The washed cells were immediately used for the bile acid transformations.

Transformations of bile acids

Bile acids dissolved in methanol were added to the cell suspension, and aliquots were withdrawn at intervals. The samples were adjusted to pH 3.0 and centrifuged. The clear supernatant was extracted with 2 × 1 volume of ethyl acetate. The cell mass was extracted by soaking with acetone. In one experiment using *Penicillium* sp., the cell mass was soaked with 1 M NaOH. In this case, the alkaline extract was added to the clear fermentation liquid, and the pH was adjusted to 3. Bile acids were obtained as a precipitate and by adsorption on Amberlite XAD-2 from the supernatant (7).

Separation of bile acids

Solvents were of reagent grade and were redistilled. The extracts containing bile acids were methylated with diazomethane in methanol–diethyl ether 1:10 (v/v). The methyl esters were separated by two methods. For analytical purposes a group separation was performed on small columns of Unisil using increasing concentrations of ethyl acetate in benzene to elute mono-, di-, and trihydroxycholanoates as groups (8). For preparative and analytical purposes, columns (120 × 10 mm) containing 4 g of Lipidex 5000 were packed in hexane–chloroform 8:2 (9). The bile acid methyl esters were applied to the column in this solvent (a maximum of 50 mg for a 4-g column) and the column was eluted (0.4 ml × cm^{-2} × min^{-1}) with 50 or 25 ml each of hexane–chloroform 8:2, 7:3, 6:4, and 5:5 (v/v). Fractions of 10 or 5 ml were collected and analyzed by GLC after preparation of TMS ethers. When 50 ml of each solvent was used, the approximate peak elution volumes of some representative methyl cholanoates were: lithocholate, 20–30 ml; deoxycholate, 70–80 ml; 1-hydroxylithocholate, 80–90 ml; 15-hydroxydeoxycholate, 110–120 ml; and 1-hydroxydeoxycholate, 170–180 ml. The column was finally eluted with 50 ml of methanol.

Analysis and identification of bile acids

The methylated bile acids from the incubation mixtures or chromatographic fractions were converted into TMS ethers with pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol) (10). GLC analyses were made using 1.5% SE-30 on 80–100 mesh Chromosorb WHP (see 1). The same column was used in a modified LKB 9000 instrument for analysis by gas–liquid chromatography–mass spectrometry (GLC/MS) (1, 11). Repetitive magnetic scanning was used and spectra were recorded on magnetic tape. Data were evaluated on an IBM 1800 computer and presented as conventional mass spectra or fragment ion current chromatograms (11). Ions for the latter purpose were selected to be representative both for general bile acid structures (e.g., mono-, di-, and trihydroxy) and for specific structural features (e.g., 1,3-dihydroxy-, 15-hydroxy-, 12-keto-) as previously known (1, 12) or based on the spectra of the new microbial metabolites. In this way a directed search for the TMS ethers of the desired 1-hydroxylated metabolites could be made (*m/z* 217 (base peak), 142, 143, 182, 195) with a high degree of sensitivity.

Identifications of bile acids were based either on comparison with reference compounds or on the mass spectra of the metabolite and products of microchemical reactions. The latter reactions included methylation (see above), chromic acid oxidation in acetone under N_2 (see 1), reduction with $NaBH_4$ (1 mg in 1 ml of methanol, 15 min, 20°C) (13) formation of O-methyloximes (5 mg methoxyamine hydrochloride in 50 μl of pyridine, 3 hr, 60°C (14)), treatment with methylboronic acid (0.4 μmol in 40 μl ethyl acetate, 5 min, 20°C (15). In all reactions, 20–40 μg of bile acid was used. At the end of the reactions (except in the case of methylboronates) excess reagent (CrO_3 and $NaBH_4$) was destroyed with a suitable solvent. The mixture was diluted with water, acidified and extracted with ethyl acetate. The extract was washed with water until neutral and the solvent was evaporated. In reactions with methylboronic acid,

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solvent was evaporated directly. When appropriate, the products were methylated and/or converted into TMS ethers for GLC/MS analysis.

The 1β- and 15β-configurations of the hydroxy groups in the new bile acids were confirmed by nuclear magnetic resonance (NMR) studies. ^{1}H spectra were recorded in the Fourier transform mode for deuteriochloroform solutions at 100 MHz on a Jeol FX100 instrument. Lanthanide-shift experiments in the case of 1β,3α,12α-trihydroxy-5β-cholanoic acid were performed with the addition of tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato)europium ($Eu(fod)_3$) (16) to the solution. Chemical shifts (δ) are reported in p.p.m. relative to tetramethylsilane as internal standard.

RESULTS

Pattern of hydroxylations with different microorganisms

All strains were screened for their ability to hydroxylate lithocholic, deoxycholic, chenodeoxycholic and cholic acids. The mixture of bile acids formed after different times of incubation was analyzed by GLC either directly or after preliminary group separation on Unisil columns. A rough estimate of the relative distribution of metabolites was obtained from the peak areas in the chromatograms. Only compounds appearing in the retention time range for bile acid derivatives were studied. Representative samples from incubations with each microorganism were analyzed by GLC/MS, and a search for formation of 1-hydroxylated metabolites was performed based on the appearance of peaks in the chromatogram of the m/z 217 fragment ion current. Mass spectra were plotted of each major metabolite and an attempt was made to locate the hydroxyl groups from the fragmentation patterns. In addition, samples of particular interest were analyzed by GLC/MS following subfractionation of the bile acid methyl esters on Lipidex 5000.

Transformations with R. solani *Kühn (CBS 130.14).* Fifteen mg of bile acid in 3 ml of methanol was added to 200 ml of cell suspension. Samples, 65 ml, were withdrawn after 20, 48, and 96 h. The extracts from the clean supernatant and the cells were combined and analyzed by GLC, in some cases after fractionation on Lipidex 5000. The results are summarized in **Table 1.** Hydroxylation was observed in positions 1, 7, and 15 and in unidentified positions. Oxidoreduction occurred at C-7 and at an unidentified position. Lithocholic acid was extensively metabolized, and after 96 hr the mixture was extremely complex and not further processed. Deoxycholic and chenodeoxycholic acids were transformed to a rather limited degree while cholic acid yielded sizeable amounts of metabolites after 96 hr.

Transformations with R. solani *Kühn (CBS 341.36).* Ten mg of bile acid in 2 ml of methanol was added to 200 ml of cell suspension. Samples, 65 ml, were withdrawn at 10, 58, and 200 hr and processed as described for *R. solani* Kühn (CBS 130.14). The results are given in Table 1. Hydroxylation was observed in positions 1 and 12 and in unidentified positions. Oxidoreduction occurred at C-7. Lithocholic acid yielded sizeable amounts of metabolites after 10 and 58 hr while minor amounts of metabolites were formed from the other acids up to 58 hr of fermentation. After 200 hr, however, all four acids were completely metabolized and only traces of bile acids could be demonstrated in the mixture.

Transformations with R. solani *Kühn (CBS 325.47).* Five mg of bile acid in 1 ml of methanol was added to 100 ml of cell suspension. Samples, 50 ml, were withdrawn after 11 and 115 hr and processed as described for *R. solani* Kühn (CBS 130.14). The results are given in Table 1. Hydroxylation was observed in position 15. Deoxycholic and chenodeoxycholic acids were completely metabolized after 115 hr, while lithocholic and cholic acids underwent a more limited degree of transformation.

Transformations with A. coerulea. Ten mg of bile acid in 2 ml of methanol was added to 200 ml of cell suspension. Samples, 65 ml, were withdrawn after 9, 81, and 177 hr. The extracts from the clear supernatant and from the cells were combined and analyzed by GLC after group fractionation on silicic acid. The results are summarized in **Table 2.** Hydroxylation was observed in positions 1, 6α, 7β, 15ξ, and in unidentified positions. In some cases oxidoreduction occurred at C-7 and C-12. Lithocholic, deoxycholic and chenodeoxycholic acids were completely metabolized after 9 hr. After 177 hr only minor compounds of polar character were left in the incubations with lithocholic and chenodeoxycholic acids. In contrast, only insignificant amounts of metabolites were formed from cholic acid.

Transformations with Pencillium *sp.* Twenty mg of bile acid in 4 ml of methanol was added to 200 ml of cell suspension. Aliquots of 100 ml were withdrawn after 16 and 112 hr. The samples were treated and analyzed as described for *R. solani* (Kühn (CBS 130.14)); however, for the samples from the incubations with deoxycholic and cholic acids, the extracts from the supernatants and the cells were analyzed separately. The results are given in Table 2. Hydroxylation was observed in positions 1 and 15 and in

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TABLE 1. Retention times, tentative sites of hydroxylation, and relative amounts of bile acid metabolites formed by different species of *R. solani* Kühn (compounds with transformed carbon skeleton were not analyzed)

	R. solani Kühn (CBS 130.14)					*R. solani* Kühn (CBS 341.36)					*R. solani* Kühn (CBS 325.47)			
		Site of Additional	% of Total at				Site of Additional	% of Total at				Site of Additional	% of Total at	
Substrate	t_R[a]	Hydroxyl[b]	20 hr	48 hr	96 hr	t_R[a]	Hydroxyl[b]	10 hr	58 hr	200 hr	t_R[a]	Hydroxyl[b]	11 hr	115 hr
Lithocholic acid	0.78	–[c]	25	15	–[d]	0.78	–[c]	70	30	–	0.78	–[c]	94	75
	0.90	15ξ	tr	tr	–	0.86	12β	10	30	–	0.91	U	4	15
	1.08	U	tr	tr	–	0.90	U	5	10	–	1.00	U	2	10
	1.16	1ξ	65	75	–	0.95	U	5	3	–				
	Others		10	10	(+)[d]	1.00	U	10	15	–				
						1.15	1ξ	–	10	–				
						1.24	1ξ	–	2	–				
Deoxycholic acid	0.90	–[c]	90	90	80	0.90	–[c]	96	85	–	0.90	–[c]	>95	<5
	0.99	7α	1	1	1	1.00	U	1	2	–	1.00	15ξ	<1	>90
	1.15	1ξ	<1	1	1	1.16	1ξ	2	9	–	Others		<5	<5
	1.31	U[e]	–	–	10	Others		1	4	+				
	Others		8	8	8									
Chenodeoxy-cholic acid	0.95	–[c]	>95	90	80	0.95	–[c]	95	86	10	0.95	–[c]	94	–
	1.18	–[f]	<1	–	–	1.11	U	2	2	60	1.10	U	1	–
	1.36	1ξ	<1	–	<1	1.18	–[f]	1	8	1	1.23	U	5	–
	Others		–	10	20	1.23	U	3	4	5				
						Others		–	–	24				
Cholic acid	1.00	–[c]	>95	90	30	1.00	–[c]	95	95	–	1.00	–[c]	>90	>95
	1.37	U	–	5	10	0.90	U	3	2	–	0.90	U }		
	1.59	U	–	–	50	1.16	U	1	2	–	1.14	U }	<10	<5
	Others		<5	5	10	1.33	U	1	1	–	1.32	U }		

[a] Retention time relative to that of the cholic acid derivative, variable by about ±2%.
[b] U, site of hydroxylation unknown.
[c] Substrate bile acid.
[d] The + signs indicate approximate relative amounts; –, not detected.
[e] Two hydroxyl groups oxidized to ketones.
[f] Oxidized to ketone at C-7.

unidentified positions. Oxidoreduction occurred in one unidentified position. Lithocholic, deoxycholic, and chenodeoxycholic acids were extensively metabolized after 112 hr, while cholic acid yielded only traces of metabolites.

In order to obtain more material, a second experiment was made where 15, 100, and 300 mg of bile acid were added to 300 ml of cell suspension. After incubation for 96 hr, the cultures were centrifuged and the cells were soaked with 1 M NaOH. The alkaline extract was combined with the clear fermentation liquid and acidified to pH 3. Bile acids precipitated in some samples from the 100- and 300-mg incubations but not in that from the 15-mg incubation. The precipitate was collected and the clear supernatant was extracted with Amberlite XAD-2. The bile acids in this extract and those in the precipitate were analyzed separately. The precipitates contained almost pure substrate bile acid and the precipitation technique could thus be used to enrich more polar bile acid metabolites. Lithocholic, deoxycholic, and chenodeoxycholic acids were extensively metabolized (**Table 3**), while cholic acid yielded minor amounts of metabolites including one 1-hydroxylated compound.

In the fermentation with *Penicillium* sp., deoxycholic acid yielded the least complex mixture of metabolites and substantial amounts of the physiologically interesting 1-hydroxylated metabolite as well as a 15-hydroxy compound. Thus, these samples were selected for preparative purposes. Supernatants after precipitation of bile acids at pH 3 from the 100-mg and 300-mg fermentations were extracted with XAD-2, and part of the extract was chromatographed on Lipidex 5000. In this way, 11 mg of 1-hydroxydeoxycholic acid and 13 mg of 15-hydroxydeoxycholic acid were obtained.

Identification of 1β,3α,12α-trihydroxy-5β-cholanoic acid

GLC/MS analysis. The mass spectrum of the methyl ester TMS ether showed a molecular ion of very low intensity at *m/z* 638 and a prominent peak at *m/z* 217 indicative of a 1,3-bistrimethylsiloxy structure (**Fig. 1**). Peaks at *m/z* 316 (loss of A-ring with substituents) and at *m/z* 142 and 143 (A-ring fragments) also support the presence of two TMS ether groups in the A-ring.

Reaction of the methyl ester with dimethoxypro-

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TABLE 2. Retention times, tentative sites of hydroxylation, and relative amounts of bile acid metabolites formed by *A. coerulea* and *Penicillium* sp. (compounds with transformed carbon skeleton were not analyzed)

	A. coerulea					*Penicillium* sp.			
			% of Total at					% of Total at	
Substrate	t_R[a]	Site of Additional Hydroxyl[b]	9 hr	81 hr	177 hr	t_R[a]	Site of Additional Hydroxyl[b]	16 hr	112 hr
Lithocholic acid	0.79	–[c]	–	–	–	0.78	–[c]	95	15
	0.90	15ξ	++++[d]	–[d]	–	0.91	15ξ	4	15
	1.00	U	+++	(+)	–	1.02	U	–	+
	1.06	7β	(+)	(+)	–	1.14	1ξ[f]	1	20
	1.13	U	+++	–	–	1.21	15ξ, U	–	20
	1.17	1ξ, 15ξ	(+)	+	–	1.28	1ξ, 15ξ	–	20
	1.29	U, U	+	+++	–	1.42	1ξ, U	–	5
	Others		(+)	(+)	(+)	1.55	1ξ, U	–	+
						1.65	1ξ, U, U, U[e]	–	5
Deoxycholic acid	0.90	—[c]	<1	<1	<1	0.90	—[c]	80	30
	0.86	U[e]	6	4	20	0.84	U	2	–
	1.02	15ξ	62	81	64	1.00	15ξ		30
	1.38	15ξ[g]	19	11	7	1.09	U	10	–
	Others		13	4	9	1.17	1ξ		30
						Others		–	10
Chenodeoxycholic acid	0.95	—[c]	(+)	(+)	–	0.95	—[c]	>95	–
	0.96	U[f]	++++	++++	++	0.93	U		30
	1.12	U	+	+	–	1.14	U		60
	1.21	U[f]	+++	+++	+	1.33	1ξ	<5	<2
	1.20	–[h]	–	(+)	–	1.56	U		<8
	1.30	6α	–	(+)	–	1.65	1ξ		1
	Others		(+)	(+)	(+)				

[a] Retention time relative to that of the cholic acid derivative, variable by about ±2%.
[b] U, site of hydroxylation unknown.
[c] Substrate bile acid.
[d] The + signs indicate approximate relative amounts; –, not detected.
[e] Possibly dehydroxylated.
[f] Mixture of at least two bile acids.
[g] Oxidized to ketone at C-12.
[h] Oxidized to ketone at C-7.

pane did not yield an acetonide, and a cyclic boronate was not formed upon treatment with methyl boronic acid. Oxidation of the methyl ester with CrO_3 in acetic acid yielded a complex mixture of products. The mass spectra of the methyl ester TMS derivatives indicated partial oxidation of the hydroxyl groups as well as oxidation to a 1,3-seco-2-nor-1,3-dioic acid. Attempts were made to obtain a more homogenous product by oxidation with chromic acid in acetone at low temperature and with different reaction times. The products were derivatized in different ways prior to GLC/MS analysis (**Fig. 2**). Brief oxidation at 0°C gave the best yields of a triketocholanoate. This compound could not be analyzed without derivatization, in agreement with the facile enolization of an expected β-diketo structure (see 17). Treatment with diazomethane yielded two products with t_R 2.07 and 2.39, respectively, roughly in proportion 2:1. Both gave molecular ions at *m/z* 430 (**Fig. 3**) indicative of mono-enol ethers expected to be formed from the two possible enol forms of a 1,3-dione (17). The loss of 155 mass units from the molecular ion is typical of a 12-ketocholanoate (12). Diagnostically important ions are seen at *m/z* 139 and 152 (both isomers) and at *m/z* 98 (the isomer with t_R 2.07). The two former ions are assumed to arise by fragmentation through the B-ring and to contain the entire A-ring with (*m/z* 152) and without (*m/z* 139) C-6 (see 18). The ion at *m/z* 98 probably contains C-1–C-4 with substituents and is assumed to arise only from the isomer with a 2,3-double bond (see 19). Based on these spectra the two compounds are identified as the 1-enol (t_R 2.39) and 3-enol (t_R 2.07) methyl ethers of 1,3,12-triketo-5β-cholanote. The predominance of the 3-methoxy isomer is in agreement with previous studies of the methylation of other steroid 1,3-diones (17, 20).

When the mixture of the two enol ethers was treated with methoxyamine hydrochloride, only one peak (t_R 1.52) was seen in the GLC/MS analysis. The mass spectrum indicated formation of a bis-O-methyloxime ($M^+ = 488$), and *m/z* (M-155) was a pronounced base peak. Apparently the two expected methoximes did not separate on the SE-30 column.

Reduction of the two enol ethers with $NaBH_4$

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TABLE 3. Formation of hydroxylated bile acid metabolites at different substrate concentrations in incubations with *Penicillium* sp.

Substrate	t_R[a]	Site of Additional Hydroxyl[b]	% of Total from 15 mg	100 mg	300 mg
Lithocholic acid	0.78	–[c]	–	–	10
	0.90	15ξ	–	–	20
	1.01	U, U[d]	8	3	–
	1.11	U, U	4	–	–
	1.22	15ξ, U	44	63	50
	1.30	1ξ, 15ξ	31	22	–
	1.46	1ξ, U	13	12	20
	1.61	1ξ, U	+	–	–
Deoxycholic acid	0.90	–[c]	22	50	60
	1.00	15ξ	46	29	25
	1.15	U	4	5	3
	1.20	1ξ	23	16	12
	Others		5	(+)	(+)
Chenodeoxycholic acid	0.95	–[c]	–	–	90
	0.94	U	48	42	8
	1.15	U	11	17	1
	1.20	15ξ[e]	31	18	1
	1.37	1ξ	+	+	–
	1.63	U	7	6	+
	1.69	1ξ	–	+	–
	1.72	U[f]	2	–	–

All columns are under the heading *Penicillium* sp.

[a] Retention time relative to that of the cholic acid derivative.
[b] U, site of hydroxylation unknown.
[c] Substrate bile acid.
[d] Possibly dehydroxylated.
[e] Oxidized to ketone at C-7.
[f] Probably ketonic.

yielded four products. The mass spectra of the TMS ethers showed that only the 12-keto group was reduced. The relative amounts of the two main products (t_R 1.39 and 1.63, respectively) were the same as for the two enol ethers in the starting material. Only the compound with t_R 1.39 gave an ion at *m/z* 98. Since $NaBH_4$ reduction is expected to give predominately 12α isomers, the two major compounds were identified as methyl 3,12α-dihydroxy-1-keto-5β-chol-2-enoate (t_R 1.39) and its 1-enol isomer, respectively.

The 1,3,12-triketocholanote could also be converted into a tris-O-methyloxime. This derivative gave a poor response in the GLC/MS analysis, possibly due to losses on the GLC column. The mass spectrum showed a molecular ion at *m/z* 503 and expected losses of 31 and 155 mass units. Plots of fragment ion current chromatograms showed that the GLC peak was not homogenous, probably due to formation of *syn/anti* isomers of the methoxime.

NMR analysis. The NMR spectrum of methyl deoxycholate, used for reference, included sharp singlets at 0.67 and 0.90 ppm due to the 18- and 19-methyl protons, respectively, a singlet at 3.65 ppm resulting from the methyl group of the ester which partly obscured a broad multiplet (21) due to the 3β (axial) methine proton, and a narrow, poorly resolved multiplet ($W_{1/2}$ ca 6 Hz) from the 12 β (equatorial) methine proton. The 3β-proton signal was more clearly revealed in the spectrum of free deoxycholic acid, as a poorly resolved heptuplet of width ca 26 Hz (21, 22).

The spectrum of methyl 1β, 3α,12α-trihydroxy-5β-cholan-24-oate was very similar in regard to the methyl proton signals (0.68, 18-CH_3; 1.04, 19-CH_3; 3.65, CO_2CH_3). The three methine protons adjacent to hydroxy groups appeared as a group of overlapping signals in the region 3.8–4.2 ppm. The profile of the composite signal suggested that it contained a broad multiplet (ca 4.08 ppm) and two narrower multiplets (ca 3.94 and 3.87 ppm), but it seemed unsafe to base an assignment of configuration at C-1 on this impression alone. The methine signals were therefore separated by stepwise addition of small quantities of $Eu(fod)_3$, which shifted the three multiplets down-field at different rates, revealing them as a broad signal ($W_{1/2}$ ≈ 25 Hz) and two narrow bands ($W_{1/2}$ ≈ 10 Hz and 6 Hz). These are attributed respectively to the 3β-, 1α-, and 12β-protons, from a comparison with band widths of the corresponding signals in the spectrum of methyl deoxycholate, and from the relative magnitudes of the shifts induced by $Eu(fod)_3$ which were in the ratio 3.4:1.7:0.6. The shifts correspond to the order of increasing steric hindrance to complexation between $Eu(fod)_3$ and the respective hydroxy groups. The signal due to the proton at C-1 had the narrow width characteristic of an equatorial conformation (21),

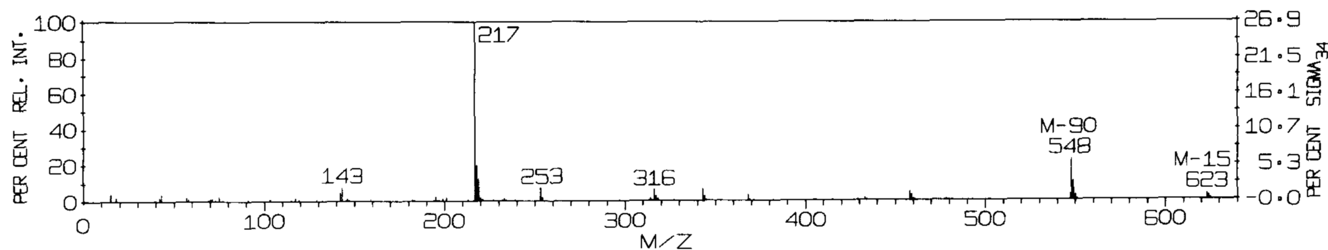

Fig. 1. Mass spectrum of the methyl ester trimethylsilyl ether of 1β,3α,12α-trihydroxy-5β-cholanoic acid formed from deoxycholic acid by *Penicillium* sp.

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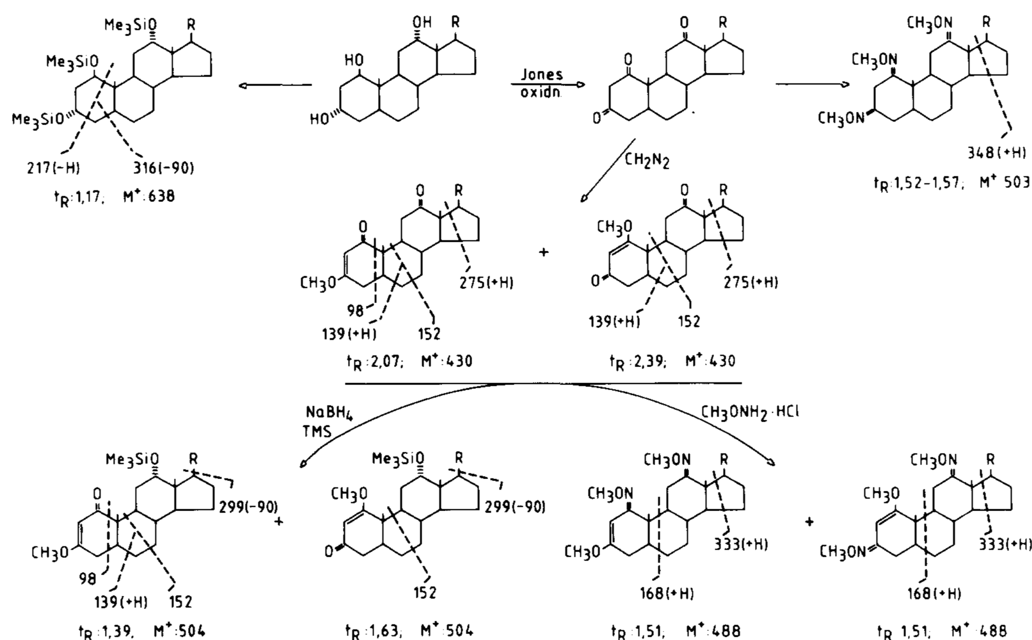

Fig. 2. Microchemical reactions used in the identification of the 1-hydroxylated metabolite formed from deoxycholic acid by *Penicillium* sp.

which at C-1 in a 5β-steroid implies that the hydroxy group has the 1β (axial) configuration.

The available lists of "Zürcher" shifts of the angular methyl proton resonances by substituents (22–25) do not include increments for a 1β-hydroxy group in a steroid of 5β-configuration. From the present data these shifts are found to be: for 19-CH_3,+0.14 ppm; for 18-CH_3,+0.01 ppm.

Identification of 3α,12α,15β-trihydroxy-5β-cholanoic acid

GLC/MS analysis. The mass spectrum of the TMS ether (t_R 1.0) (**Fig. 4**) showed a molecular ion of low intensity at *m/z* 638 and an ABCD-ring fragment ion at *m/z* 253 indicating the presence of three nuclear trimethylsiloxy groups. Diagnostically significant peaks were present at *m/z* 243 and 393 (M-245). Although *m/z* 243 is typical of a 3,7-bistrimethylsiloxy structure (12), the simultaneous occurrence of an ion formed by loss of 245 mass units indicated the presence of a trimethylsiloxy group in the D-ring (cf. loss of 157 amu in methyl cholanoates without D-ring substituents (12)). Supporting this interpretation was the mass spectrum of the TMS ether of a metabolite formed by *A. coerulea* (t_R 1.35). A molecular ion at *m/z* 564 indicated a bistrimethylsiloxy-monoketo structure. The base peak was at *m/z* 243 and peaks were present at *m/z* 321 (M-243) and 231 (M-243-90). The latter ion corresponds to that formed from the TMS ether of methyl 3α-hydroxy-12-keto-5β-cholanoate by loss of side chain, D-ring, and trimethylsilanol (M-155-90) (12). Thus, the simultaneous occurrence of peaks at *m/z* 243, 231, and 321 indicates the presence of a trimethylsiloxy group in the D-ring. The prominent fragmentation between C-13 and C-15 supported a 15-trimethylsiloxy group as do the results of previous comparisons between mass spectra of 15- and 16-hydroxylated C_{21} steroid derivatives (26).

Partial oxidations of the trihydroxycholanoate were performed with chromic acid in acetone at low temperatures and short reaction times. The major product formed at −70°C was identical with the ketonic bile acid formed by *A. coerulea*. The O-methyloxime TMS ether derivative (t_R 1.05) gave further support for a 12-keto-15-trimethylsiloxy structure by giving a very prominent base peak ion at *m/z* 350 (M-243). Two monohydroxy-diketocholanoates were also formed after partial oxidation. Their O-methyloxime TMS ether derivatives (M^+ = 548) had very similar retention times (t_R 1.28). One compound gave a peak at *m/z* 350 (M-198) corresponding to loss of the side chain and D-ring with an O-methyloxime group; the other gave a base peak ion at *m/z* 305 formed by loss of 243 amu. These mass spectra further support substitution at C-15. The O-methyloxime of the fully oxidized compound gave an expected molecular ion at *m/z* 503 and a diagnostically important ion at *m/z* 305 (M-198).

Based on these results the compound was identified as 3α,12α,15ξ-trihydroxy-5β-cholanoic acid. The 15α isomer of this comound has been synthesized by Kimura et al. (27) and a sample was kindly provided

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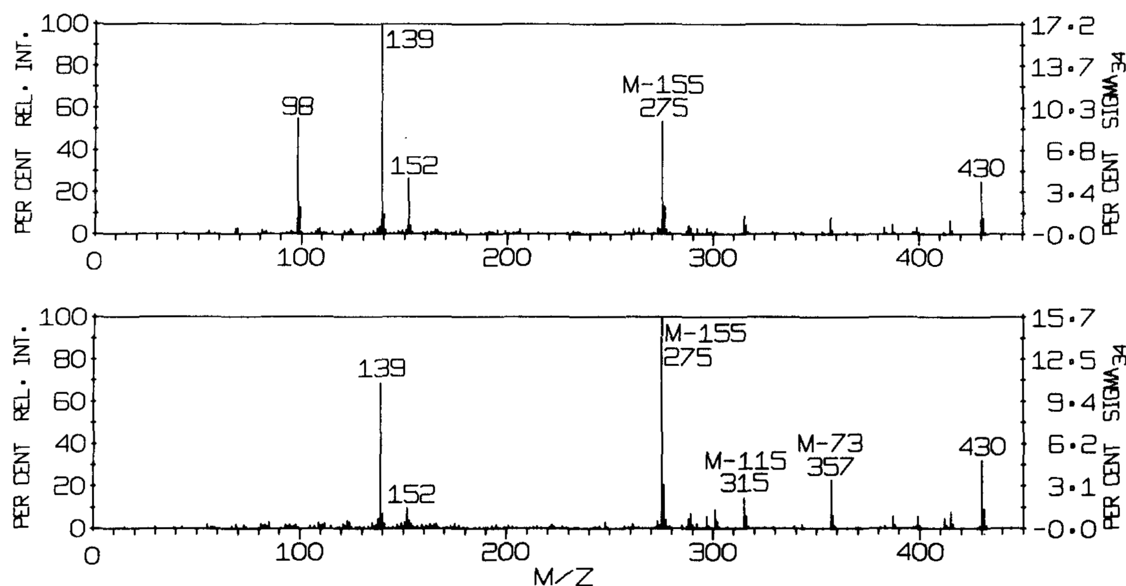

Fig. 3. Mass spectra of the two enol ethers formed by treatment with diazomethane of the oxidation product of methyl 1β,3α,12α-trihydroxy-5β-cholanoate. Upper spectrum: main product, t_R2.07; lower spectrum: minor product, t_R 2.39 (cf. Fig. 2).

by Dr. Kimura. The TMS ether had a t_R of 0.91, i.e., shorter than that of the microbial product. Oxidation to the triketocholanoate and reaction with methoxyamine hydrochloride yielded a product which had the same t_R and mass spectrum as the product from the biological compound. Based on these comparisons, the fermentation product was assigned the structure 3α,12α,15β-trihydroxy-5β-cholanoic acid.

NMR analysis. Interpretation of the NMR spectrum of methyl 3α,12α,15β-trihydroxy-5β-cholanoate was straightforward, and did not require the addition of a shift reagent. By comparison with the spectrum of methyl deoxycholate, an additional methine proton was seen to resonate at 4.18 ppm. The signal was a triplet of width 13 Hz, characteristic of the 15α-proton in a 15β-alcohol (22). A 15α-hydroxysteroid normally gives a broader triplet of doublets due to its 15β-proton (22). The angular methyl proton resonances confirmed the 15β-hydroxy assignment. The signals were superimposed as a 6-proton singlet at 0.94 ppm. Chemical shifts calculated on the basis of "Zürcher" increments coincided at 0.95 ppm for the 15β-hydroxy configuration, but required distinct signals at 0.925 ($19\text{-}CH_3$) and 0.725 ppm ($18\text{-}CH_3$) for a 15α-hydroxy derivative.

DISCUSSION

The results of this investigation indicate that microbial tranformation is a reasonably convenient method for the preparation of certain 1β- and 15β-hydroxylated bile acids. A chemical method has been described for synthesis of 15α-hydroxydeoxycholic acid (27), and chemical procedures are being developed for synthesis of 1-hydroxylated bile acids.[4] A drawback of the microbial technique, probably shared by chemical methods, is the influence of other substituents on the yield of transformation products. Less polar substrates (e.g., lithocholic acid) are more efficiently metabolized than more polar substrates (cholic acid). This might indicate a role for hydrophobic binding as has been shown previously for the hepatic hydroxylation of steroids and steroid sul-

[4] Herz, J. Personal communication.

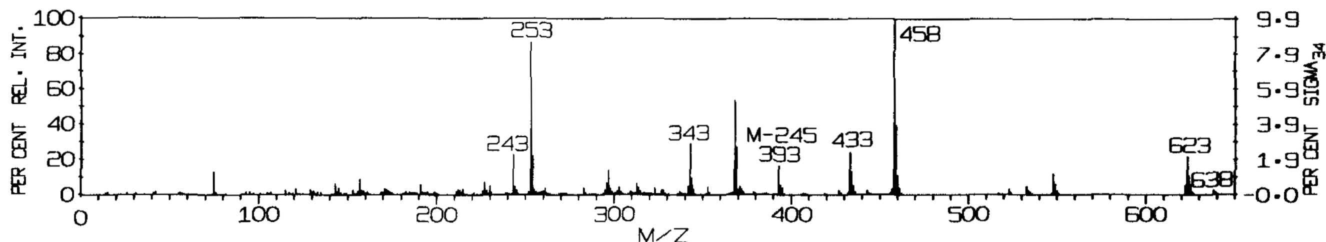

Fig. 4. Mass spectrum of the methyl ester trimethylsilyl ether of 3α,12α,15β-trihydroxy-5β-cholanoic acid formed from deoxycholic acid by *Penicillium* sp.

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phates (28–30). The polarity of the substrate might also influence the transport into the fungal cell, so that less polar substrates enter the cells more readily and are subjected to enzymatic transformations, while polar substrates remain in the aqueous fermentation medium (31).

While polar substituents in the nucleus obviously influence the transformations, the nature of the 17β-side chain seems to be of minor importance, at least for 1-hydroxylation in general. Thus, besides bile acids, *A. coerulea* can use C_{23} steroids, and the three *R. solani* strains and *Penicillium* sp. can use C_{19} and C_{21} steroids as substrates (6). The same lack of structural specificity seems to be valid for the A/B ring fusion. Thus, saturated as well as 3-oxo-4-ene and 3β-hydroxy-5-ene steroids are 1-hydroxylated by the same microorganism (6). In general, microbial hydroxylations depend less on the structure of the steroid than do other microbial steroid transformations (4, 6). An exception is constituted by the cytochrome P-450-catalyzed 15β-hydroxylating system in *Bacillus megaterium* ATCC 13368, which seems to have an absolute specificity for 3-oxo-4-ene steroids (32).

The hydroxylation of a steroid by a microorganism is usually not restricted to a single position in the molecule (6). Thus, microbial 1-hydroxylation is often accompanied by formation of metabolites hydroxylated in positions 6 and 7 and, less frequently, in positions 2, 11, 12, and 15 (2–6). In the present study, 1-hydroxylation was most frequently accompanied by 15-hydroxylation. Introduction of hydroxyl groups in 6α, 7α, 7β, 12β, and unidentified positions was also observed. It is tempting to speculate that a common enzyme carries out the 1β- and 15β-hydroxylations and perhaps also hydroxylations in other positions (4). The distances from carbon atoms 11, 12, and 15 to the C-18 methyl group are similar to those from carbon atoms 1, 6, 7, and 11 to the C-19 methyl group. If one assumes that the substrate is bound to the enzyme with its nonpolar β-side, several of these positions could conceivably be hydroxylated by the same enzyme.

Most enzymes catalyzing hydroxylations of steroids at unactivated positions have been shown to be cytochrome P-450-dependent. This is true both of microbial and mammalian systems (33, 34). The broad specificity shown by the microbes used in this study is compatible with cytochrome P-450-containing systems (4, 30). However, it is not yet possible to draw any conclusions regarding the nature of the enzymes responsible for the hydroxylations observed.

Bile acids in urine. The 1β,3α,12α-trihydroxy-5β-cholanoic acid prepared and identified in the present study has the same gas–liquid chromatographic and mass spectrometric characteristics as the 1,3,12-trihydroxycholanoic acid previously found in human urine (1). This establishes the stereochemistry of the urinary bile acid which is formed by hydroxylation of deoxycholic acid, presumably in the liver (35).

Hydroxylation in the 1-position is particularly marked in cholestasis and in the neonatal period, and 1-hydroxylated bile acids and steroids have been found in urine and meconium (1, 36–43). An accompanying 15-hydroxylation has not been observed with certainty in man. The 1-hydroxylation of cholic acid is relatively more efficient in humans than in the microorganisms. This may be due to a better uptake of cholic acid in the hepatocyte than in the microorganisms. The stereochemistry of 1-hydroxylated cholic acid (and 1,3,7-trihydroxycholanoic acid (36)) in urine has not been determined. Since deoxycholic acid and polar corticosteroid metabolites are hydroxylated in the 1β-position (present study, 35, 40, 43), it is reasonable to assume that the stereochemistry in 1-hydroxylated bile acids in urine is 1β. It is of interest to note that all saturated 1-hydroxylated steroids so far identified in man have a 3α-hydroxy-5β configuration. It is possible that this is a particularly favorable structure for a substrate of a 1β-hydroxylase.

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