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NEW TRIHYDROXY BILE ACIDS PRODUCED BY *CUNNINGHAMELLA BLAKESLEEANA* ST-22 FROM LITHOCHOLIC ACID

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Abstract - The microbiological transformation of lithocholic acid (3α-hydroxy-5β-cholanic acid) (1) by a fungus *Cunninghamella blakesleeana* ST-22 was investigated. Three new trihydroxy bile acids, 3 - 5, were produced via a dihydroxy bile acid, 2. These new compounds all possess a unique 15β-hydroxy group. The structure assignments of these new compounds were provided by interpretation of spectral data, especially two-dimensional J-correlated ^{1}H-NMR spectral data.

For several decades, a great deal of attention has been given to the study of the microbiological transformation of steroidal compounds, particularly of the corticosteroid type[1,2]. During the same period, however, the structurally related bile acids have received much less attention. More recently, interests in the bile acids have increased considerably, principally due to the finding that two naturally occurring bile acids (ursodeoxycholic acid and chenodeoxycholic acid) possess the therapeutic properties to solubilize gallstones[3 - 6]. Because both compounds are the dihydroxy derivatives of 5β-cholanic acid, hydroxylation of bile acids seems a promising field for obtaining useful chemotherapeutic agents. However, little is known on the hydroxylation of bile acids, because most studies on bile acids have been done with respect to cholesterol metabolism.

As a part of our studies on the microbial transformation of bile acids, in a previous paper[7], we

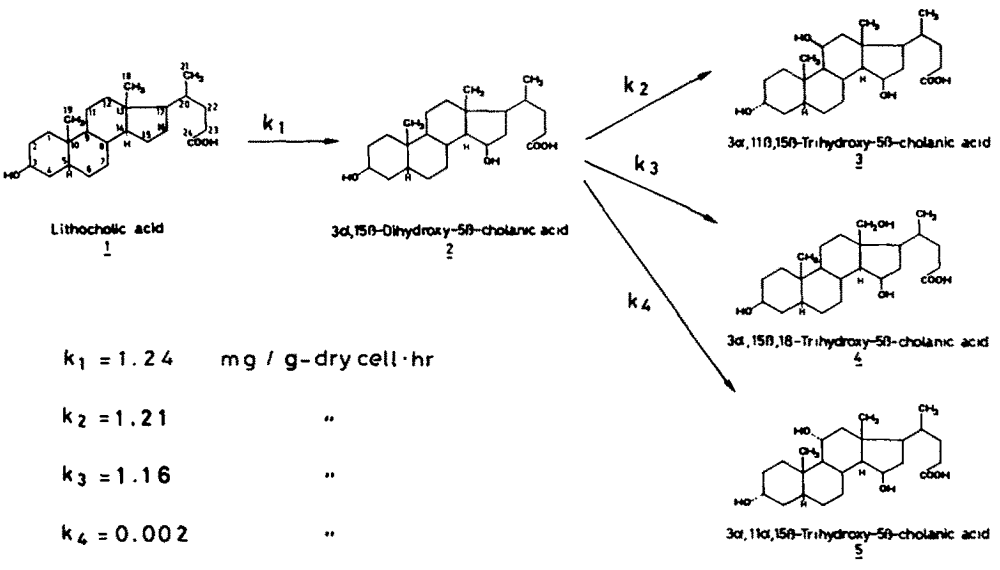


Figure 1. Lithocholic acid metabolism by *Cunninghamella blakesleeana* ST-22.

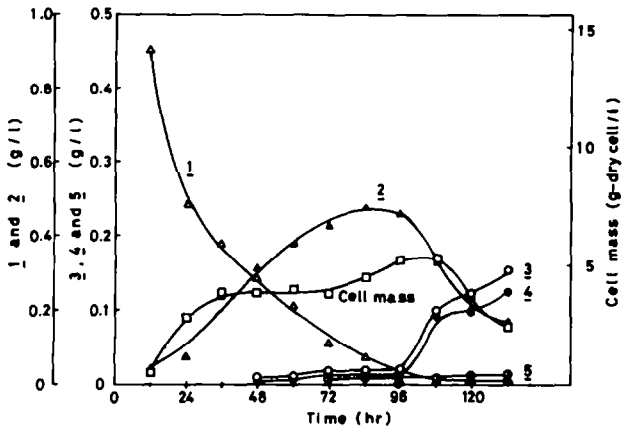


Figure 2. Cultivation of *C. blakesleeana* ST-22 in a 10-l jar fermentor.

isolated a fungus *Cunninghamella blakesleeana* ST-22 which can hydroxylate lithocholic acid (3α-hydroxy-5β-cholanic acid) (1) at the 15β position and produce a new dihydroxy bile acid, 2. During the fermentation of *C. blakesleeana* ST-22, we noticed that 2 was further metabolized to three more hydrophilic compounds. To learn more about the ability of *C. blakesleeana* ST-22 to transform bile acids, we isolated the metabolites, and these compounds were found to be new bile acids, 3 - 5, containing the unique 15β-hydroxyl group. In this paper, we describe the structure elucidation of the three compounds 3α,11β,15β-trihydroxy-5β-cholanic acid (3), 3α,15β,18-trihydroxy-5β-cholanic acid (4) and 3α,11α,15β-trihydroxy-5β-cholanic acid (5), (Figure 1).

Results and Discussion

The microbiological transformation was carried out in submerged cultures of *Cunninghamella blakesleeana* ST-22 containing 1 g lithocholic acid, 40 g dextrin, 12 g asparagine, 3 g KCl, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract and 0.1 g each of $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $MnSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ per 1 of the culture. A typical example using a 10-l fermentor is shown in Figure 2. Analyses with TLC[8] and gas chromatography revealed that three metabolites, 3, 4 and 5, were formed (Rf=0.68, 0.58, 0.49, 0.43, 0.32, $CHCl_3$-Acetone-Acetic acid,40:40:0.6; retention time in GC=3.24, 5.83, 11.64, 10.57 and 9.68 min, for 1, 2, 3, 4 and 5, respectively). As shown in Figure 2, 2 was the main product from 1 until 96-h of cultivation. After 96-h of cultivation, 2 decreased with concomitant increase of the three metabolites, especially 3 and 4. From this phenomena, it seemed that 2 rather than 1 was converted to 3, 4 and 5. To confirm this metabolic pathway, we isolated these metabolites from the culture broth of *C. blakesleeana* ST-22 with final yields of 15, 4 and 9 %, respectively (3 and 5 as a free acid form, 4 as a methyl ester form).

By molecular secondary ion mass spectrometry[9], 3, 4 and 5 were shown to have a molecular weight of 408 [3, $(M+Li)^+$ m/z 415, $(M+H+glycerol)^+$ m/z 501 and $(M+Li+glycerol)^+$ m/z 507; 4 methyl ester, $(M+H)^+$ m/z 423; 5, $(M+Na)^+$ m/z 431, and $(M+Li)^+$ m/z 415], which suggested that these compounds were trihydroxy derivatives of 5β-cholanic acid assuming that 2 (M.W.=392) was converted to them. The IR spectra[10] indicating the presence of -OH and C=O groups as the main functional groups also supported the above estimation. For further structural elucidation, we performed ^{13}C-NMR (Table 1), one-dimensional ^1H-NMR and two-dimensional J-correlated ^1H-NMR spectroscopy (Figures 3 - 5).

As shown in Table 1, 3 was found to contain 24 carbonds, of which 3 carbonds were present as methyl, 9 carbons as methylene, 9 carbons as methine and 3 carbons as quaternary carbons by the DEPT method. Furthermore, three of the methine carbons showed CHOH resonances (67.2, 69.2 and 71.3 ppm), and one quaternary carbon showed COOH resonance (174.5 ppm). Comparing with the ^{13}C-NMR data of 2, it became apparent that a triplet signal of C-11 (21 ppm) disappeared with the appearance of a new doublet signal at 67.2 ppm. Therefore, in addition to the 3α-hydroxy (71.3 ppm) and 15β-hydroxy groups (69.2 ppm), 3 seemed to contain one more hydroxy group at C-11. In the 300 MHz ^1H-NMR spectrum (in CD_3OD) following resonances were observed; 4.18 (1H, td, J=5.8 and 0.8 Hz, CHOH), 4.10 (1H, td, J=4.1 and 2.5 Hz, CHOH), 3.55 (1H, m, 3β-H), 1.20 (3H, s, 19-CH_3), 1.13 (3H, s, 18-CH_3),

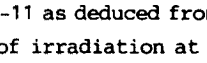
Table 1. ^{13}C-NMR data[a] for 2, 3, 4 methyl ester and 5 in pyridine-d_5

^{13}C	2	3	4 methyl ester	5[b]	^{13}C	2	3	4 methyl ester	5[b]
1	36.2 (t)	35.9 (t)	36.1 (t)	39.2	13	42.8 (s)	42.4 (s)	47.3 (s)	–
2	31.6 (t)[c]	32.2 (t)	31.2 (t)[f]	33.0	14	61.5 (d)	63.4 (d)	60.6 (d)	60.7
3	71.2 (d)	71.3 (d)	71.1 (d)	71.7	15	69.1 (d)	69.2 (d)	69.1 (d)	69.0
4	37.5 (t)	37.6 (t)	37.5 (t)	38.2	16	41.9 (t)[d]	41.9 (t)	42.7 (t)	42.4
5	42.7 (d)	44.8 (d)[e]	42.6 (d)	44.5	17	56.8 (d)	57.5 (d)	56.4 (d)	56.9
6	27.8 (t)	27.2 (t)	27.7 (t)	28.4	18	15.1 (q)	17.9 (q)	61.8 (t)	16.4
7	26.1 (t)	27.2 (t)	25.8 (t)	25.9	19	23.7 (q)	27.2 (q)	23.5 (q)	24.4
8	32.4 (d)	28.5 (d)	32.3 (d)	31.5	20	35.7 (d)	35.9 (d)	34.9 (d)	35.9
9	41.2 (d)	45.0 (d)[e]	41.4 (d)	47.9	21	18.8 (q)	18.8 (q)	19.5 (q)	18.8
10	35.1 (s)	35.7 (s)	35.1 (s)	–	22	31.9 (t)[c]	32.2 (t)	31.5 (t)[f]	31.5
11	21.2 (t)	67.2 (d)	21.6 (t)	68.6	23	31.9 (t)[c]	32.2 (t)	31.7 (t)[f]	31.5
12	42.2 (t)[d]	51.1 (t)	38.9 (t)	54.0	24	176.5 (s)	174.5 (s)	174.5 (s)	–
					25	–	–	51.3 (q)	–

a) δ in ppm ( ); multiplicity in off-resonance spectra.

b) Because we could observe only the spectra of protonated carbons, each signal was tentatively assigned based only on chemical shifts.

c), d), e) and f) Each assignment may be exchanged.

0.97 ppm (3H, d, J=6.3 Hz, 21-C<u>H</u>$_3$). These two C<u>H</u>OH signals (4.18 and 4.10 ppm) correspond to 15-C<u>H</u>OH and a newly formed C<u>H</u>OH. To confirm the position of the two hydroxy groups other than 3α, we investigated the two-dimensional J-correlated ^{1}H-NMR (Figure 3). Signal at 4.18 ppm exhibited a typical coupling pattern with C-16 methylene protons (2.40 and 1.39 ppm) and C-14 methine proton (0.93 ppm), indicating that 3 has a 15β-hydroxy group. By irradiating the 0.93 ppm signal, coupling between 0.93 ppm proton and 2.17 ppm proton became evident from the coupling difference spectra (Figure 3, left side), allowing the assignment of the 2.17 ppm proton as 8-H. This 8-H should show coupling with C-7 methylene protons and C-9 methine proton. Indeed, there were a set of signals (1.89 and 1.23 ppm) which showed strong coupling with each other, and one more methine proton (1.54 ppm, 9-H). This 1.54 ppm signal was one of the protons with which the newly formed C<u>H</u>OH signal (4.10 ppm) showed coupling. This coupling pattern can be well explained by locating the newly formed hydroxy group at C-11 as deduced from ^{13}C-NMR data. To confirm this position further, we investigated the effect of irradiation at 4.10 ppm (Figure 3, left side). From the decoupling difference spectrum, it was evident that both the methylene (2.16 and 1.36 ppm, J=13.5 Hz) and the methine (1.54 ppm) signals changed from the double doublets to doublets. Based on these coupling patterns, the following partial structure,  was determined, indicating that the position of the newly formed CHOH was at C-11. As for the configuration of the hydroxy group, we estimated as β-configuration due to the down field shifts of both the 19-C<u>H</u>$_3$ (from 1.00 in 2 to 1.20 ppm) and 18-C<u>H</u>$_3$ (from 0.92 in 2 to 1.13 ppm) signals[11]. Consequently, the structure of 3 was determined as 3α,11β,15β-trihydroxy-5β-cholanic acid.

4 was isolated as its methyl ester by diazomethane treatment. As shown in Table 1, 4 exhibited 25 signals in the ^{13}C-NMR. By the DEPT method, we detected 3 methyl, 11 methylene, 8 methine and 3 quaternary carbons. As expected from the structure of a methyl ester of a trihydroxy bile acid, three C-OH resonances were detected in addition to one O-C<u>H</u>$_3$ resonance (51.3 ppm, methyl carbon) and one C<u>O</u>O resonance (174.5 ppm, quaternary carbon). Two of the C-OH were methine carbons (69.1 and 71.1 ppm), and the third one was a methylene carbon (61.8 ppm). Comparison with 2 indicated that, in 4 methyl ester, the quartet signal of 18-C<u>H</u>$_3$ disappeared and a new triplet signal appeared as C<u>H</u>$_2$OH (61.8 ppm). Therefore, 4 seemed to contain the 18-hydroxymethyl group in addition to 3α- and 15β-hydroxy groups. Actually, in 300 MHz two-dimensional ^{1}H-NMR (in $CDCl_3$, Figure 4), 0.92 ppm signal of 18-C<u>H</u>$_3$ originally present in 2 disappeared and new peaks appeared at 3.94 and 3.59 ppm (1H each, d, J=11.6Hz, C<u>H</u>$_2$OH). Other characteristic signals were as follows: 4.24 (1H, td, J=6.5 and 1.4 Hz, 15α-H), 3.65 (3H, s, 24-OC<u>H</u>$_3$), 3.63 (1H, m, 3β-H), 0.98 (3H, d, J=6.3 Hz, 21-C<u>H</u>$_3$) and 0.93 ppm

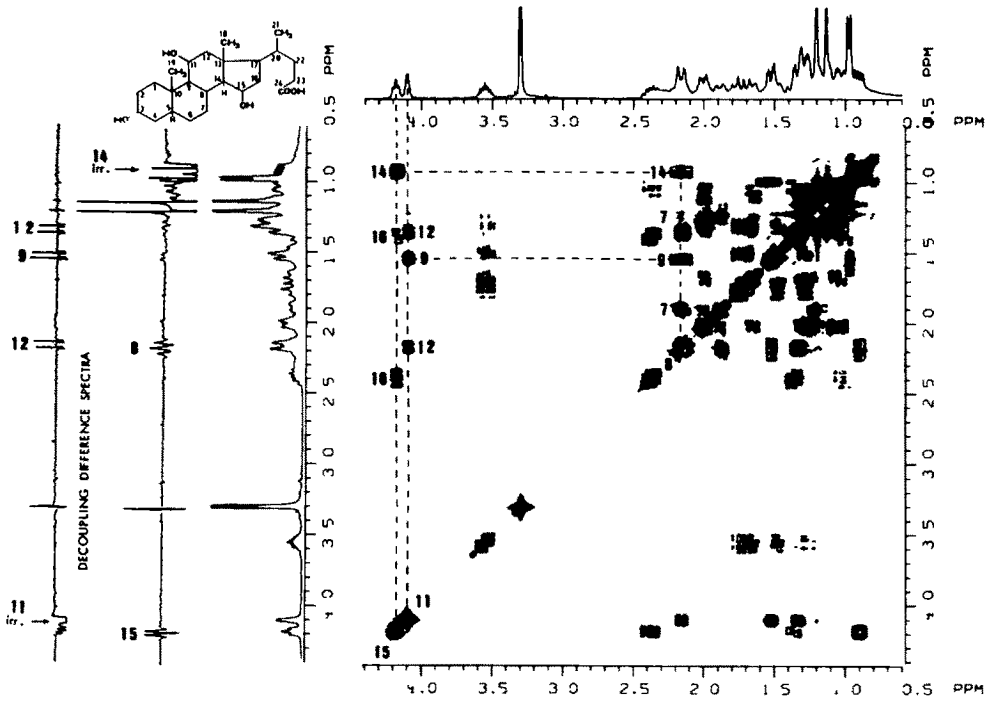
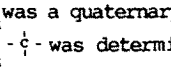


Figure 3. Two-dimensional ^{1}H-NMR of 3.

(3H, s, 19-C$\underline{H}_3$). From the coupling pattern of the 4.24 ppm signal with C-16 methylene protons (2.40 and 1.51 ppm) and C-14 methine proton (1.18 ppm), it was assigned as 15α-H. Therefore, we concluded that 4 was 3α,15β,18-trihydroxy-5β-cholanic acid.

For 5, because of the insufficient sample, we could detect only signals from protonated carbons and could not assign each resonance by the DEPT method. However, preliminary data indicated that at around 20 ppm only 2 resonances (16.4 and 18.8 ppm, 18-C$\underline{H}_3$ and 21-C$\underline{H}_3$) were present in 5 instead of the three resonances in 2 (15.1 ppm, 18-C$\underline{H}_3$; 18.8 ppm, 21-C$\underline{H}_3$; 21.2 ppm, 11-C$\underline{H}_2$). Furthermore, the presence of tree signals at C$\underline{H}$OH region (68.6, 69.0 and 71.7 ppm) suggested that 5 may be a C-11 epimer of 3, i.e., 3α,11α,15β-trihydroxy-5β-cholanic acid. To elucidate this structure, we peformed two-dimensional ^{1}H-NMR spectroscopy of 5 (in CD_3OD, Figure 5), and observed the following characteristic signals: 4.14 (1H,td, J=5.6 and 0.9 Hz, C$\underline{H}$OH), 3.79 (1H, td, J=10.7 and 4.2 Hz, C$\underline{H}$OH), 3.57 (1H, m, 3β-H), 1.08 (3H, s, 19-C$\underline{H}_3$), 0.98 (3H, d, J=9.4 Hz, 21-C$\underline{H}_3$) and 0.95 ppm (3H, s, 18-C$\underline{H}_3$). The signal at 4.14 ppm showed a typical coupling pattern with C-16 methylene protons (2.47 and 1.42 ppm) and C-14 methine proton (1.02 ppm), indicating that the 4.14 ppm signal was 15-C$\underline{H}$OH signal. The new C$\underline{H}$OH signal (3.79 ppm) showed couplings with 2.18, 1.60 and 1.24 ppm protons, of which 2.18 and 1.24 ppm protons were geminal protons of a methylene carbon deduced from their strong couplings with each other. When the 2.18 ppm proton was irradiated, from decoupling difference spectrum (Figure 5, left side), the 1.24 ppm signal changed from a triplet to a doublet, and the 3.79 ppm signal from a triplet of doublets to a triplet, indicating that the carbon atom adjacent to the methylene carbon was a quaternary carbon. Based on these coupling patterns, the following partial structure,  was determined. This partial structure indicated that the new hydroxy group should exist either at C-11 or 2. Because the signal from 3β proton (3.57 ppm) in 5 showed no down field shift (3.52 ppm) in 2, the presence of hydroxy group at C-2 seemed unlikely. To further confirm the position of the newly introduced hydroxy group at C-11 , we observed nuclear Overhauser effect. By irradiating 18-C$\underline{H}_3$ (0.95 ppm) or 19-C$\underline{H}_3$ (1.08 ppm), enhancement of 3.79 ppm signal was 6.21 and 9.21 %, respectively, indicating that the hydroxy group should locate at C-11. As for the configuration of the hydroxy group, chemical shift pattern of 18-C$\underline{H}_3$ and 19-C$\underline{H}_3$, i.e., down field shift of 19-C$\underline{H}_3$ (from 1.00 in 2 to 1.08 ppm) and almost no shift of 18-C$\underline{H}_3$ signal (from 0.92 in 2 to 0.95 ppm), supported our estimated α-configuration[11].

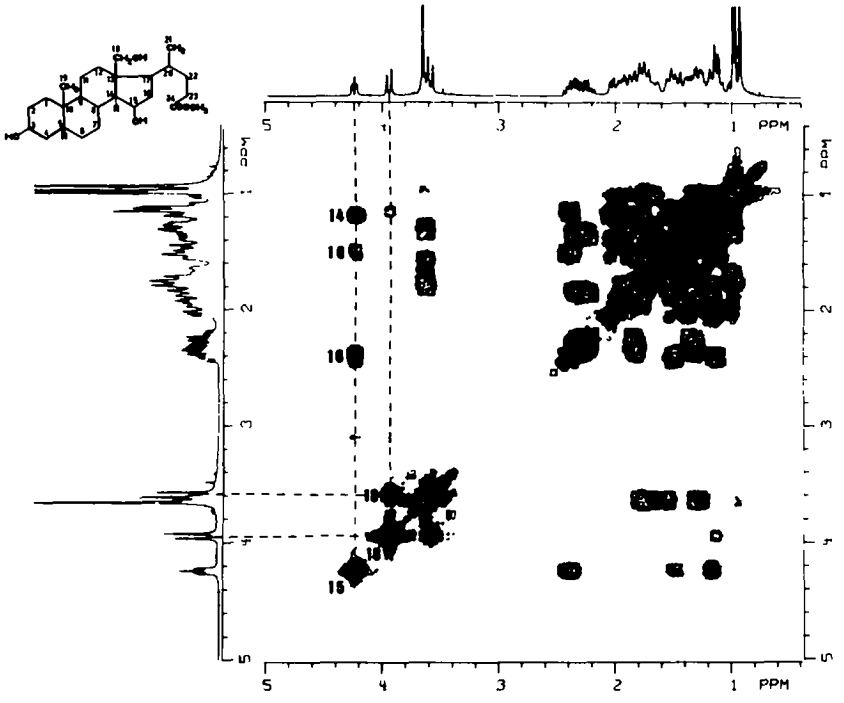


Figure 4. Two-dimensional ^{1}H-NMR of 4 methyl ester.

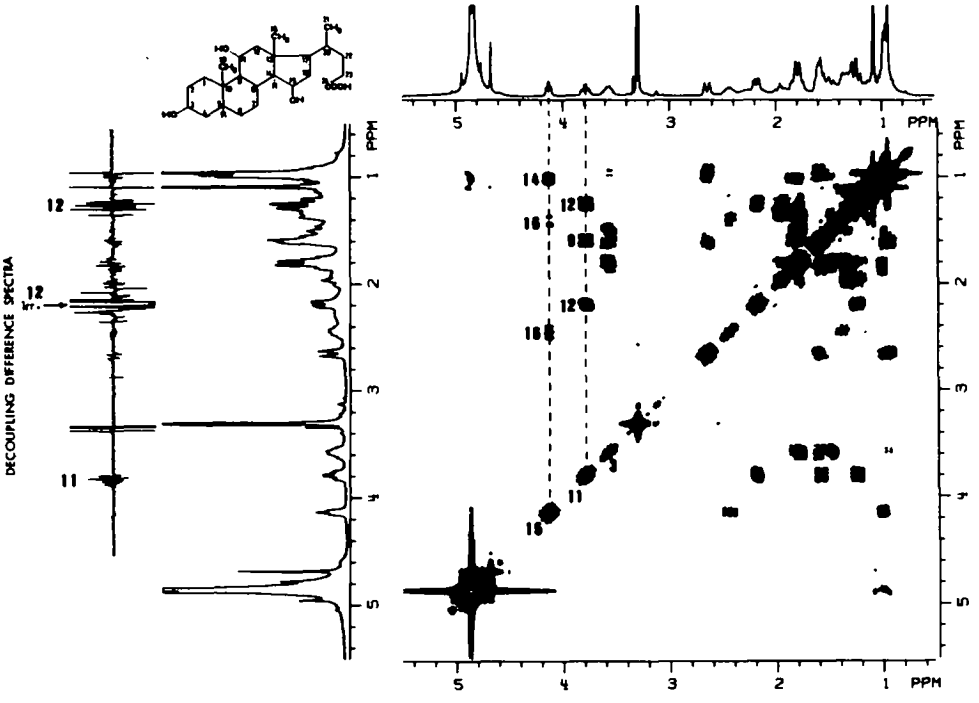


Figure 5, Two-dimensional ^{1}H-NMR of 5.

In conclusion, *Cunninghamella blakesleeana* ST-22 has the ability to hydroxylate bile acids at positions 15β, 11β, 11α, and 18. The conversion rates, calculated from the data in Figure 2, were 1.24, 1.21, 1.16 and 0.002 mg/g-dry cell·hr for 15β-hydroxylation, 11β-hydroxylation, 18-hydroxylation and 11α-hydroxylation, respectively. Thus, the ability to hydroxylate at 15β, 11β, and 18 are almost the same, while 11α-hydroxylation occurs very slowly.

EXPERIMENTAL

General. Infrared (IR) spectra were recorded by a film method on a Nicolet-7119 FT-IR spectrometer. One-dimensional and two-dimensional ^{1}H-NMR data were obtained with a Nicolet NT-300 spectrometer at 300 MHz. ^{13}C-NMR spectra were obtained at 25 MHz using a JEOL FX-100 spectrometer. Chemical shifts were expressed in parts per million from internal tetramethylsilane (δ) unless otherwise noted. Coupling constants are in Hz and splitting pattern abbreviations are: s, singlet; d, doublet; t, triplet; q, quartet; td, triplet of doublets; m, multiplet. Mass spectra (MS) were obtained on a Hitachi M-80 mass spectrometer. Optical rotations were determined with a DIP-181 polarimeter (Japan Spectroscopic Co. Ltd.). High performance liquid chromatography (HPLC) were performed with a Trirotar II (Japan spectroscopic Co. Ltd.) equipped with a Zorbax-ODS column (Dupont Instruments). Gas chromatography was performed with a Hitachi 163 equipped with FID. A glass column 3 mm x 1 m packed with 2 % Silicone DC-QF-1 on Uniport HP 80/100 mesh was used; col. temp. and inj. temp. were 220°C and 240°C, respectively. Samples for GC were derivatized with hexafluoro isopropanol and trifluoroacetic anhydride according to the method of Imai and Tamura[12]. Analytical thin-layer chromatography (TLC) was conducted on precoated HPTLC glass sheets (silica gel 60 F_{254}) manufactred by E. Merck. Preparative silica gel thick layer chromatography was performed on 20 x 20 cm glass plates coated with silica gel 60 (E. Merck, Darmstadt). Silica gel column for chromatography were prepared with Wako gel C-300 (Wako Pure Chemical Industries, Ltd. 200-300 mesh). For other chromatography, Amberlite XAD-4 (Rohm & Haas Co., Philadelphia), DEAE-cellulose (Brown company), active carbon (Nakarai Chemicals, LTD. Kyoto) were used. All solvents used were purchased from commercial sources and were used without further purification.

Culitivation of Cunninghamella blakesleeana ST-22 The cultivation was performed either with a 10-l (Type MD-500; L.E. Marubishi) or with a 50-l jar fermentor (type MSJ-VC 501; L.E.Marubishi). The pH during cultivation was kept at 8.0, the temperature at 33°C, the aeration rate at 1.0 vvm, and dissolved oxygen tension between 40-60 % saturation by changing automatically the agitation speed.

Purification of the compounds, 3, 4 methyl ester and 5. After 132-h of cultivation, culture broth (30-l) was filtrated with suction through cheese cloth, followed by centrifugation. After adjusting the pH of the supernatant to 3.0, a white precipitate was formed, and was separated by centrifugation. The clear supernatant containing 950 mg of 3 was adsorbed to an Amberlite XAD-4 column (6 x 65 cm), and the adsorbed compounds were eluted with methanol. Fractions containing 3 were concentrated yielding 6.1 g of oily material, followed by silica gel column chromatography using 2,2,4-trimethylpentane-isopropanol-acetic acid (60:20:0.6, v/v/v). Crystallization from isopropanol/hexane yielded 250 mg of crude crystals in which 180 mg of 3 was present. A part of the crystals (30 mg) was further purified by reversed phase HPLC with methanol-water (62.5:37.5, v/v) as eluent, and pure crystals of 3 (14 mg) were obtained from isopropanol/hexane (total yield 15 %).

For the purification of 4, we found that 4 was present mainly in the white precipitate. The precipitate (403 g-wet containing 1.96 g of 4) was extracted with ethyl acetate. After concentrating the ethyl acetate layer, a precipitate consisting mainly of 2 was removed by filtration, and the filtrate was adsorbed on a DEAE-cellulose column (5 x 35 cm) followed by elution with 80 % ethanol. After adsorption on a active charcoal column (5 x 31 cm), elution with ethanol and concentration, a white powder (11.5 g) was obtained. A part of the powder (2.5 g containing 49 mg of 4) was methylated with diazomethane, followed by extraction with CH_2Cl_2, and concentration. The yellow oily material thus obtained was chromatographed on a silica gel column using CH_2Cl_2-acetone (85:15, v/v), and 39 mg of crude crystals were obtained from ethyl acetate/hexane. After further purification by reversed phase HPLC (methanol-water, 7:3, v/v), and preparative thick layer chromatography (2,2,4-trimethylpentane-ethyl acetate-acetic acid, 7:35:0.1, v/v/v) at 4°C, pure crystals of 4 methyl ester (19 mg) were obtained (total yield 4 %).

For the purification of 5, 3-l of culture broth was extracted with ethyl acetate after acidified to pH 3.0. A brown oily material (17 g containing 46 mg of 5) obtained after concentration of the ethyl acetate layer was chromatographed on a silica gel column using cyclohexane-ethyl acetate-acetic acid (7:23:3, v/v/v) yielding 180 mg of yellow powder (38 mg of 5). Crude crystals obtained after crystalization from ethyl acetate three times, was further purified by reversed phase HPLC (methanol-water, 7:3, v/v) yielding pure crystals of 5 (4 mg, total yield 9 %).

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8. Rf value of 3, 4 methyl ester and 5 in several solvent systems are as follows: Rf=0.33, 0.46, 0.25, TMeP:AcOEt:AcOH=5:25:0.2; Rf=0.23, 0.44, 0.20, Benzene:Dioxane:AcOH=75:20:2; Rf=0.68, 0.70, 0.61, Cyclohxane:AcOEt:AcOH=7:23:3; Rf=0.67, 0.82, 0.72, Benzene:i-PrOH:AcOH=30:10:1.
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10. data of IR. νmax (cm^{-1}):3, 3422, 3402, 2929, 2863, 1711, mp, 236-237°C, $[\alpha]_D^{25}$ =+20.8° (c 0.31, methanol); 4 methyl ester, 3283, 3251, 2936, 2863, 1739, mp, 191-192°C, $[\alpha]_D^{25}$ =+10.2° (c 0.46, methanol); 5, 3408, 3380, 2934, 2863, 1723, mp, 153-155°C.
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