CHAPTER 3

Transformation of Lithocholic Acid to a New Bile Acid, 3α,15β-Dihydroxy-5β-Cholanic Acid, by *Cunninghamella blakesleeana* ST22

Part 1 Isolation, Screening and Identification of the Strain ST22

Introduction

As mentioned, there have been several reports on the conversion of steroidal compounds by microorganisms. For the hydroxylation of steroids, most microorganisms with the necessary activity were mycelial molds. Likewise, the search for a transformer of lithocholic acid to the dihydroxyl bile acid aimed to isolate mycelial molds rather than other groups of microorganisms.

In the course of screening, a fungal strain, ST22, was found which formed a new bile acid (3α,15β-dihydroxy-5β-cholanic acid; 3α,15β-DHC) from lithocholic acid (49). This part describes the isolation, screening and identification of strain ST22.

Materials and Methods

Isolation and screening of microorganisms Fungi were isolated by spreading soil suspensions on agar plates containing 10 g of glucose, 5 g of peptone, 0.5 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 33 mg of rose bengal and

15 g of agar (pH 6.5) in 1 ℓ. After incubation for 2 - 5 days at 30°C, fungal colonies were transferred and reisolated until pure cultures were obtained. Potato dextrose agar was used for maintaining the pure isolates. In screening for an LCA-transforming strain, pure isolates were cultivated for 7 days at 30°C on a rotary shaker (250 rpm) in 250-ml Erlenmeyer flasks containing 50 ml of cultivation medium. The medium contained 1 g of LCA, 50 g of glucose, 10 g of $NaNO_3$, 3 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g each of $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ in 1 ℓ. The pH of the medium was adjusted to 7 before sterilization. The products were extracted with ethyl acetate, and analyzed by thin layer chromatography as described in Chapter 1 (54).

Results and Discussion

1. Screening of LCA Transformers

Of 110 fungal isolates, only 5 isolates (ST07, 15, 22, 67 and 91) could convert LCA into more hydrophilic compounds which seemed to be dihydroxy derivatives of 5β-cholanic acid by thin layer chromatographic analysis (chloroform-acetone-acetic acid, 100:100:1, by vol.). The R_f values of the products from these strains and of standards were as follows: 0.40 and 0.53 (ST07), 0.43 and 0.53 (ST15), 0.43 and 0.53 (ST22), 0.46 and 0.53 (ST67), 0.43 and 0.55 (ST91), 0.69 (LCA), 0.53 (ursodeoxycholic acid) and 0.43 (chenodeoxychoic acid).

Because strain ST22 formed 10 times higher amounts of products than the other strains, it was selected for further study.

2. Identification of strain ST22

The main axis and terminal branches of strain ST22 terminated in

spherical heads furnished with small swellings which were the insertion points for conidia. Colonies on beerwort-agar grew rapidly at 25°C up to 2 cm high. Other morphological characteristics were: permanently white; mycelium smooth and hyaline, hyphae up to 15 μ in diameter; rhizoid present; conidia globose to subglobose (9 - 13 μm) with long echinulations. The strain grew well at 25 - 37°C, while no growth was observed at 47°C. No zygospore formation was observed. From these characteristics, strain ST22 was identified as *Cunninghamella blakesleeana* (Lender) according to Zycha *et al*. (1966) and Samson (1969). It has been reported that *Cunninghamella blakesleeana* is capable of hydroxylating steroidal compounds at several positions: 1β, 6β, 9α, 11α, 11β, 14α, 16β (63), and 15β (79). But none of these steroidal compounds were lithocholic acid, a bile acid, or its related compounds. So this is the first study of the hydroxylation of lithocholic acid to a dihydroxy-5β-cholanic acid by the newly isolated strain of *C. blakesleeana*.

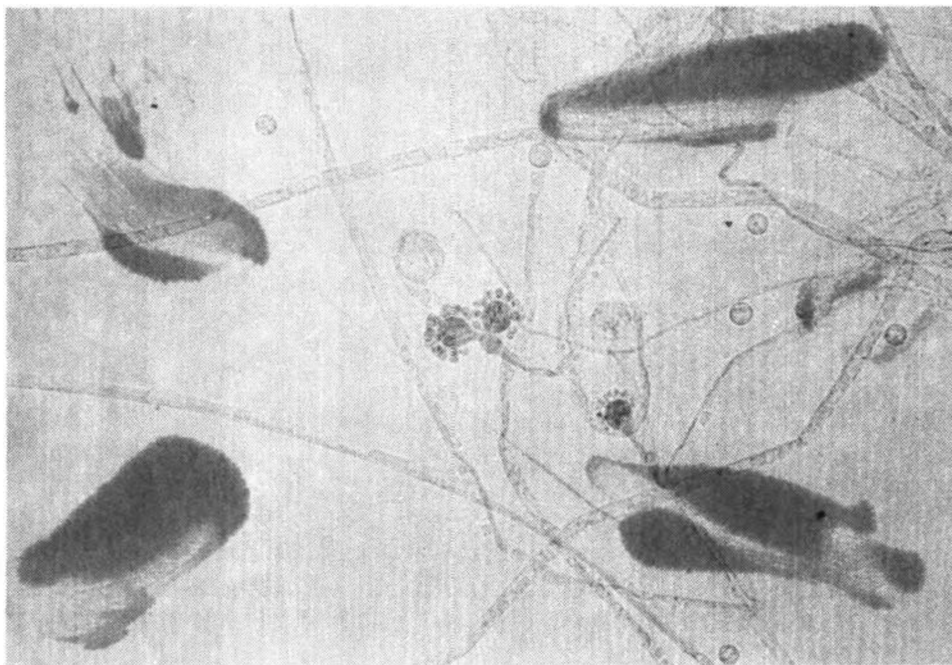


Fig. 1. Photomicrograph of the strain ST22 (× 200).

Summary

Out of 110 fungal isolates, strain ST22 was found to convert lithocholic acid, a monohydroxy bile acid, into a new dihydroxy bile acid. Strain ST22 was identified as *Cunninghamella blakesleeana* (Lender).

Part 2 Structural Elucidation of the New Dihydroxy-Bile Acid Produced by *Cunninghamella blakesleeana* ST22

Introduction

Since Danzinger *et al.* (37) and Makino *et al.* (43) reported that ursodeoxycholic acid and chenodeoxycholic acid are effective in dissolving cholesterol gallstones and these compounds have been used successfully for treating patients with gallstone disease, more attention has been given to the hydroxylation of bile acid. In the course of this study on the biotransformation of bile acids by fungi (54), *Cunninghamella blakesleeana* ST22 was isolated from acid soil in Thailand in 1982 and selected for its ability to convert lithocholic acid into more hydrophilic compounds. This part describes the structural elucidation of the main product, 3α,15β-dihydroxy-5β-cholanic acid (Fig. 1).

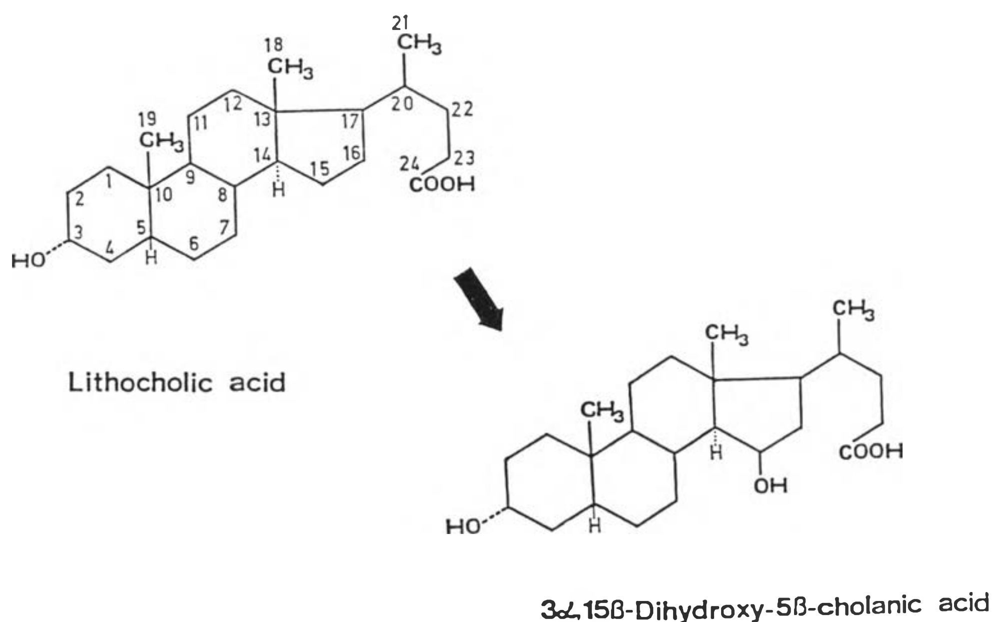


Fig. 1. Conversion of lithocholic acid to 3α,15β-dihydroxy-5β-cholanic acid by *C. blakesleeana* ST22.

Materials and Methods

Cultivation of strain ST22 The cultivation was performed either in 2-ℓ Sakaguchi flasks on a reciprocal shaker (120 spm) or in a 10-ℓ jar fermentor (type MD-500; L.E. Marubishi) at 30°C. For cultivation in a fermentor, the pH was maintained at 7.0 with 3 N HCl and 3 N NaOH, the temperature at 30°C, the aeration rate at 1.0 vvm and the agitation speed at 300 rpm. The cultivation medium contained 1 g of lithocholic acid, 40 g of dextrin, 12 g of asparagine, 3 g of KCl, 2 g of KH_2PO_4, 0.5 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of yeast extract and 0.01 g each of $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $MnSO_4 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$ in 1 ℓ. This composition is same as that used for the production of UDCA.

Isolation and purification of the product For silica gel chromatography of the product, Wakogel C-300 (Wako Pure Chemicals Industries, Ltd.) was used. All solvents were used without further purification. The product was purified by adjusting the pH of 9 liters of culture broth to pH 3 with 3 N HCl, then extracting with 19 liters of ethyl acetate with vigorous mixing for 2 - 3 hr at room temperature. After removing the water from ethyl acetate layer with anhydrous Na_2SO_4, the extract was concentrated in a rotary evaporator at 50°C. About 1.53 g of the crude product was recovered as oily material at this step. The oily material was dissolved in a minimum amount of a mixture of ethyl acetate, 2,2,4-trimethylpentane and acetic acid (10:10:2, by vol.) and chromatographed on a silica gel column (3.0 × 96 cm) using the same solvent mixture as the eluent. Fractions of 10 ml were collected and analysed by gas chromatography and by TLC using the same solvent system. Fractions containing the product were pooled, concentrated in a rotary evaporator at 50°C, and subjected to silica gel chromatography (column size 3.0 × 96 cm) with chloroform-acetone-acetic acid (50:10:0.6,

by vol.) as a solvent system. Fractions containing the product were pooled, evaporated in a rotary evaporator at 50°C, and crystallized twice with ethyl acetate/hexane. At this step, 475 mg of purified product was recovered, which is equivalent to 31% yield. The purity of the product was confirmed by high performance liquid chromatography.

Analysis of the product   IR spectra were recorded as nujol mulls on a Hitachi 215 spectrophotometer. Mass spectrometrical measurements were performed using a Hitachi RMU-6E or Hitachi M-80 mass spectrometer. Optical rotations were determined with a DIP-181 polarimeter (Japan Spectroscopic Co. Ltd.). Elemental analysis was performed with a C, H, N, O analyzer. ^{13}C-NMR spectra were obtained at 25 MHz using a JEOL FX-100 spectrometer. One-dimensional and two-dimensional ^{1}H-NMR data were obtained with Nicolet NT-300 spectrometer at 300 MHz. High performance liquid chromatographic (HPLC) analyses of the product were carried out with a Trirotar II (Japan Spectroscopic Co. Ltd.) equipped with Zorbax-ODS column (DuPont Instruments). The elution solvent used was methanol-water (73:27, by vol., pH 3 with H_3PO_4) with detection at 210 nm. Gas chromatography (GC) was performed with a Hitachi 163 equipped with FID. A glass column 3 mm × 1 m, packed with 2% Silicone DC-QF-1 on Uniport HP 80/100 mesh, was used with column and injection port temperatures of 220°C and 240°C, respectively. Samples for GC were derivatized with hexafluoroisopropanol and trifluoroacetic anhydride according to the method of Imai and Tamura (59).

Results and Discussion

Structure analysis of the product

The crystalline product dissolved in methanol showed a single peak on

HPLC. The product, m.p. 185.5 - 186.5°C and $[\alpha]_D^{25}$ + 3.4 (C 1, EtOH), was shown to have molecular formula of $C_{24}H_{40}O_4$ by elemental analysis (Found: C, 73.38%; H, 10.30%; Calcd. for $C_{24}H_{40}O_4$: C, 73.42%, H, 10.27%) and mass spectral data of molecular secondary ion mass spectrometry (80) [$(M + Na)^+$ at m/z 415 and $(M + H + glycerol)^+$ at m/z 485] (Fig. 2). In that the

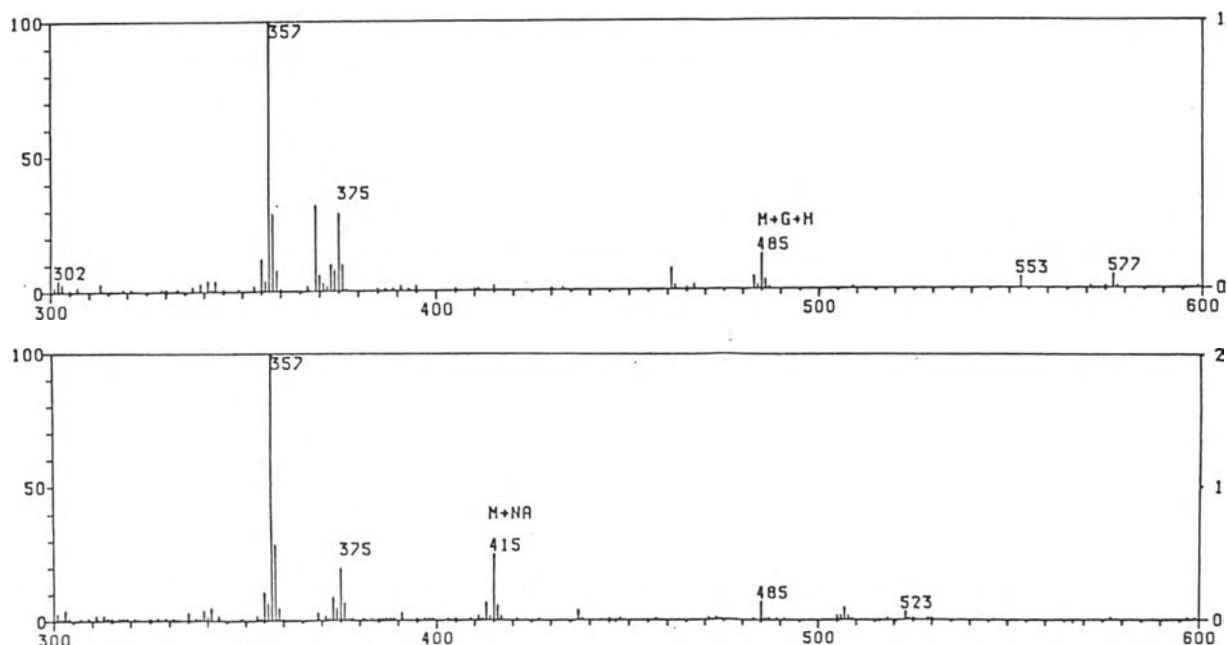


Fig. 2. Mass spectrum of the product.

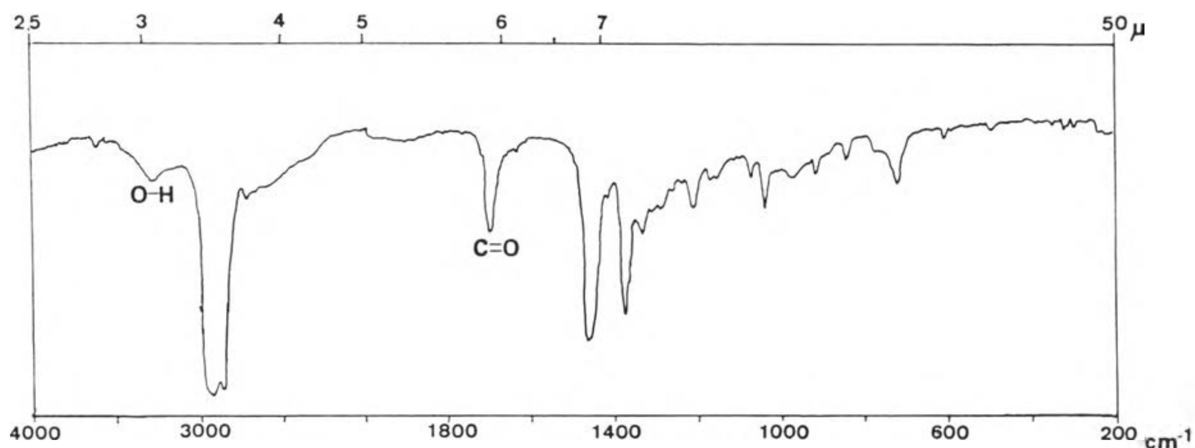


Fig. 3. IR spectrum (in nujol) of the product.

starting material was lithocholic acid ($C_{24}H_{40}O_3$), the product was assumed to be a dihydroxy derivative of 5β-cholanic acid. The IR spectrum of the

product, which exhibited absorption at ν_{max} (nujol) 3280 (bonded OH) and 1700 cm^{-1} (C = 0 dimer), supported this assumption (Fig. 3). Further confirmation of the structure came from the ^{13}C-NMR of the product (Table 1 and Fig. 4), which showed 24 signals. By INEPT ^{13}C-NMR (81), the product was found to contain 3 methyl carbons, 10 methylene carbons, 8 methine carbons and 3 quaternary carbons. Two methine carbons showed <u>C</u>HOH resonances (70.6 and 72.7 ppm) and one quaternary carbon showed <u>C</u>OOH resonance (178.0 ppm). From these data, the product was concluded to be a dihydroxy derivative of 5ß-cholanic acid.

Table 1. ^{13}C-NMR data[a)] for the product in CD_3OD.

1-$\underline{C}H_2$	37.3	(t)	13-$\underline{C}$	43.6	(s)
2-$\underline{C}H_2$	32.3	(t)	14-$\underline{C}H$	62.4	(d)[d)]
3-$\underline{C}HOH$	72.7	(d)	15-$\underline{C}HOH$	70.6	(d)
4-$\underline{C}H_2$	42.1	(t)[c)]	16-$\underline{C}H_2$	31.2	(t)
5-$\underline{C}H$	43.6	(d)	17-$\underline{C}H$	61.5	(d)[d)]
6-$\underline{C}H_2$	28.3	(t)	18-$\underline{C}H_3$	15.2	(q)
7-$\underline{C}H_2$	26.8	(t)	19-$\underline{C}H_3$	24.0	(q)
8-$\underline{C}H$	36.4	(d)[b)]	20-$\underline{C}H$	33.0	(d)[b)]
9-$\underline{C}H$	42.2	(d)	21-$\underline{C}H_3$	18.9	(q)
10-$\underline{C}$	35.9	(s)	22-CH_2	36.6	(t)
11-$\underline{C}H_2$	21.8	(t)	23-CH_2	31.9	(t)
12-$\underline{C}H_2$	42.9	(t)[c)]	24-$\underline{C}OOH$	178.0	(s)

a) δ in ppm; ( ), multiplicity in off-resonance spectra.
b), c) and d), each assignment may be exchanged.

Although several dihydroxy derivatives have been reported (82), comparison of several properties of the product, i.e. m.p., $[\alpha]_D^{25}$, retention time on GC or HPLC, and R_f values in 9 solvents, with those of known dihydroxy derivatives (83-85) indicated that the product was a new compound. Therefore, the ^1H-NMR spectrum shown in Fig. 5 was examined to assign the position of the second hydroxy group besides that at the 3α position. The

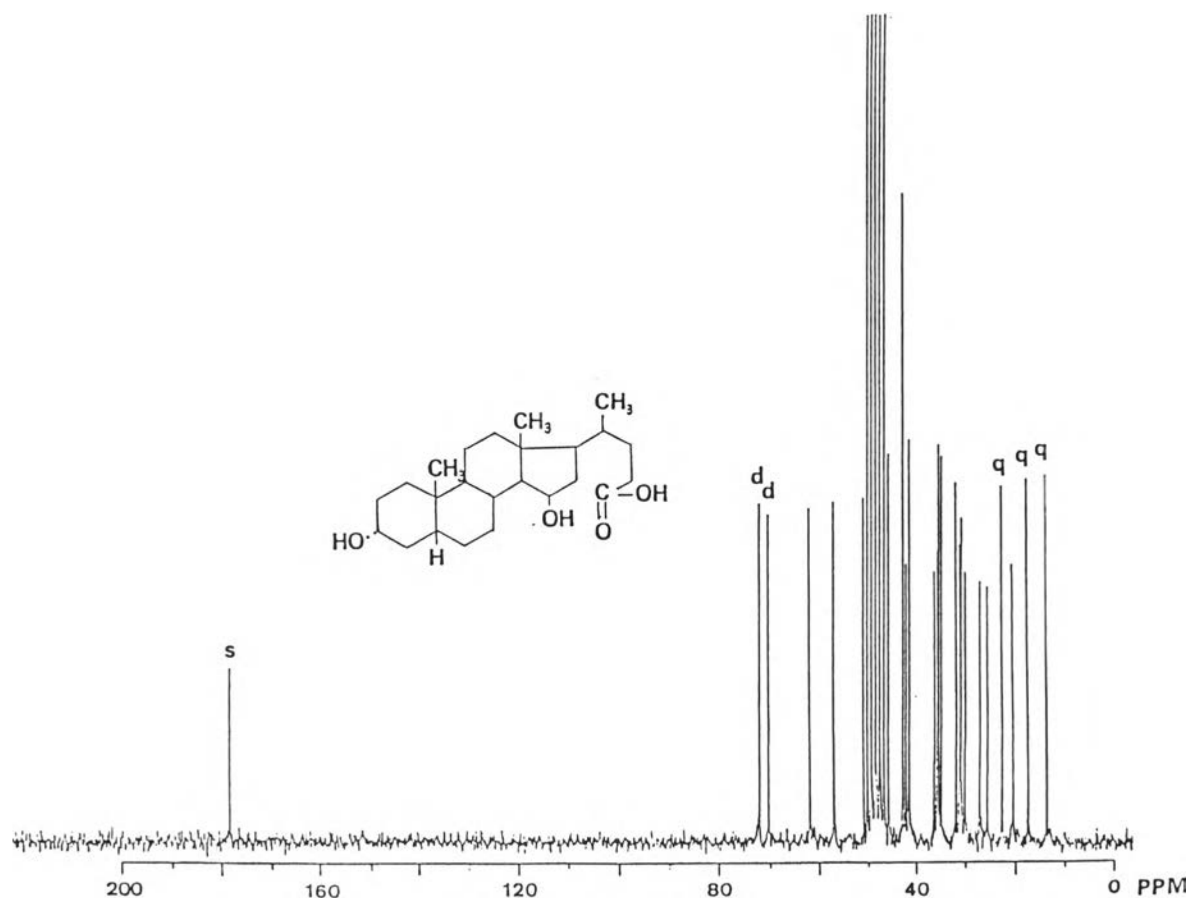


Fig. 4. ^{13}C-NMR spectrum of the product.
Solvent: CO_3OD.

300 MHz ^{1}H-NMR spectrum (C_6D_6: CD_3OD, 10:1, v/v) showed the following resonances: 3.98 (1H, m, J = 5.56 Hz, CHOH), 3.52 (1H, m, J_{ax-ax} = 9.42 Hz, 3α-H), 1.00 (3H, s, 19-CH_3), 0.92 (3H, s, 18-CH_3) and 0.88 ppm (3H, d, J = 8.30 Hz, 21-CH_3). The downfield shift of 18-CH_3 resonance to 0.92 ppm in the product from 0.65 and 0.73 ppm in lithocholic acid methyl ester and 3α, 12α-dihydroxy-5β-cholanic acid respectively (86) indicates that the second hydroxy group is located at the 11β, 12β, 15β, or 16β position. And the J value of the 3.98 ppm signal suggested the location of the hydroxy group on a 5-membered ring. Thus, the 15β or 16β position was most probable. To identify the position of the hydroxy group further, two-dimensional J-correlated ^{1}H-NMR spectroscopy (87) of the product was performed (Fig. 6),

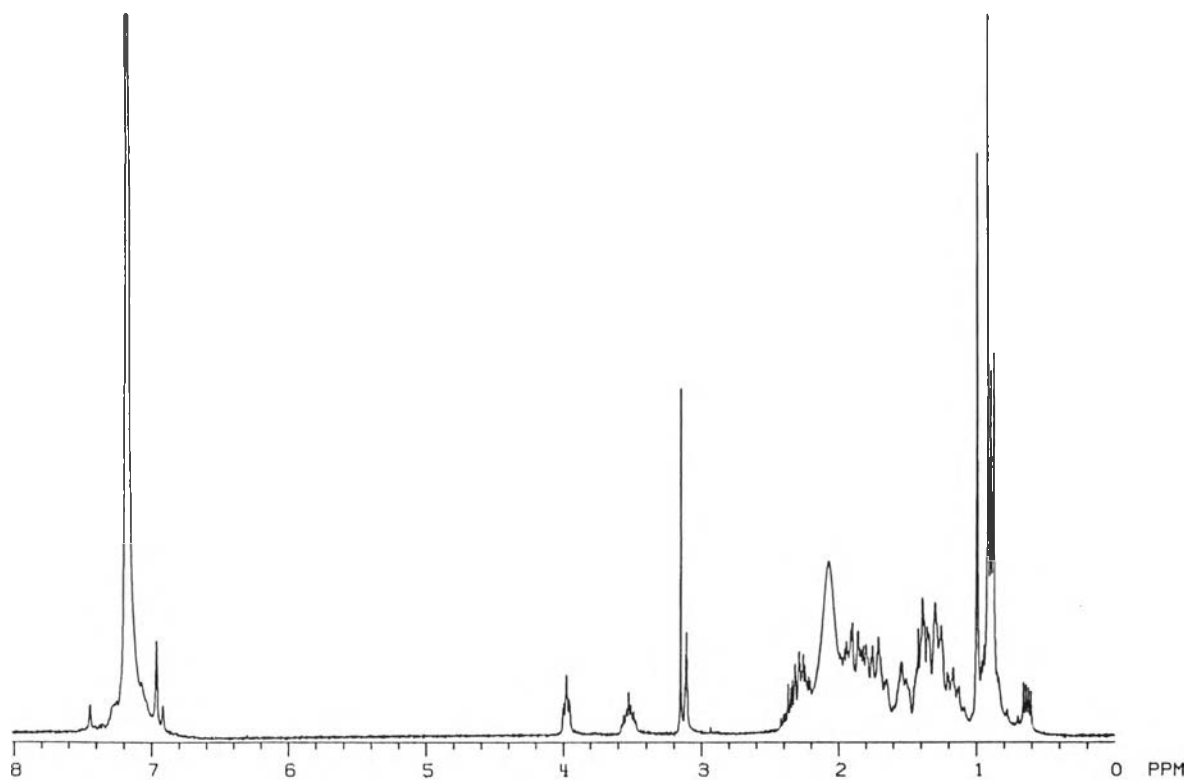


Fig. 5. ^{1}H-NMR spectrum of the product.

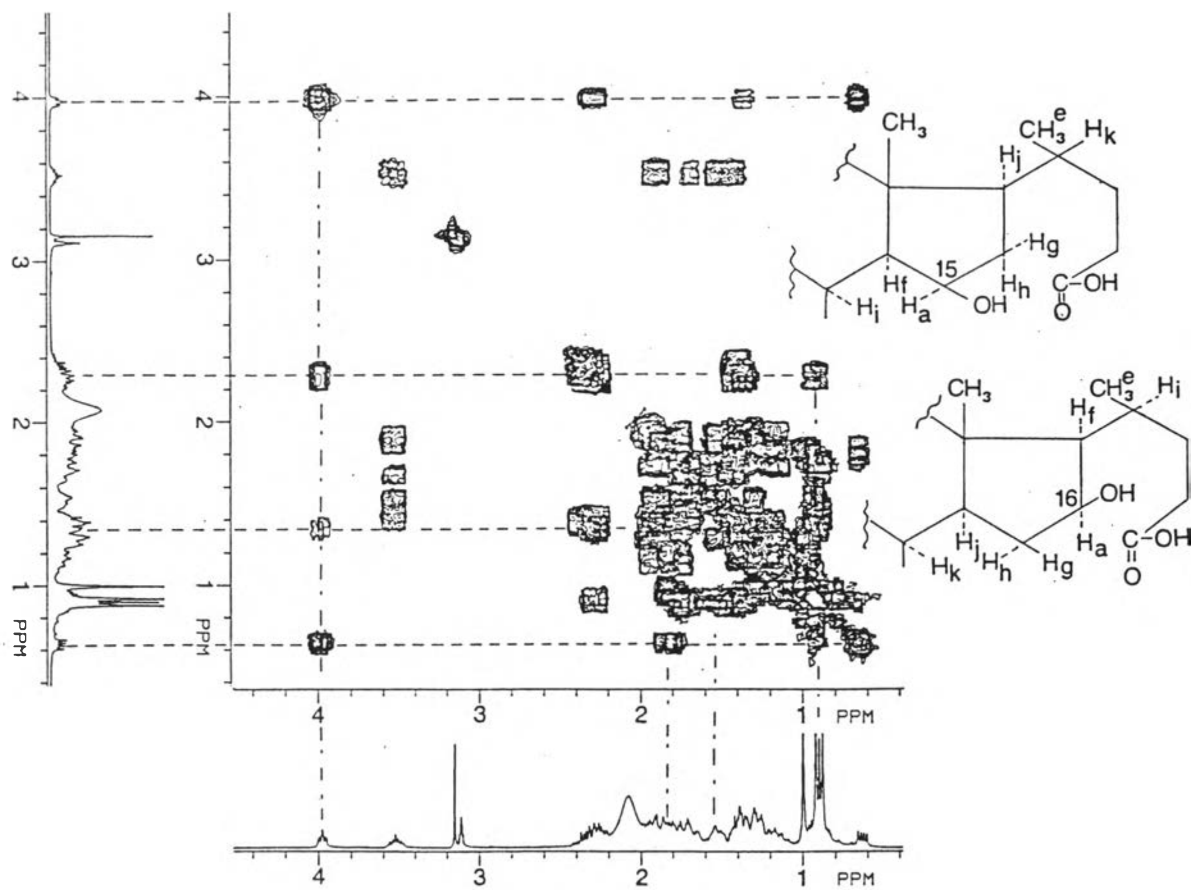
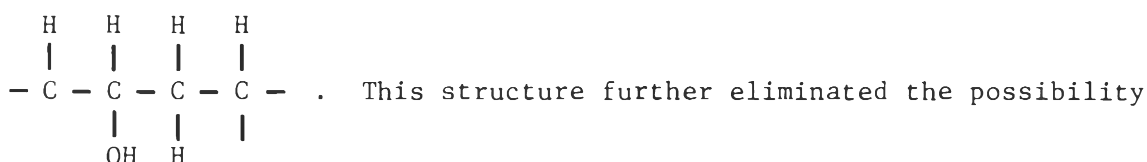


Fig. 6. Two-dimensional J-correlated ^{1}H-NMR spectrum of the product.

in which 3.98 ppm proton showed the coupling with 2.28, 1.36 and 0.65 ppm protons. The strong coupling between 2.28 and 1.36 ppm protons (J = 10.9 Hz) indicated that these protons were both geminal protons of a methylene carbon. The 2.28 ppm proton exhibited one more coupling, with a 0.9 ppm proton (resonance j). These couplings established the partial structure of



. This structure further eliminated the possibility that the hydroxy group is located at the 11β or 12β position. The 0.65 ppm methine signal also showed coupling with the 1.82 ppm signal (resonance i, Fig. 6). This 1.82 ppm proton should be located at C-20 if the second hydroxy group is located at 16β, or at C-8 if the hydroxy group is located

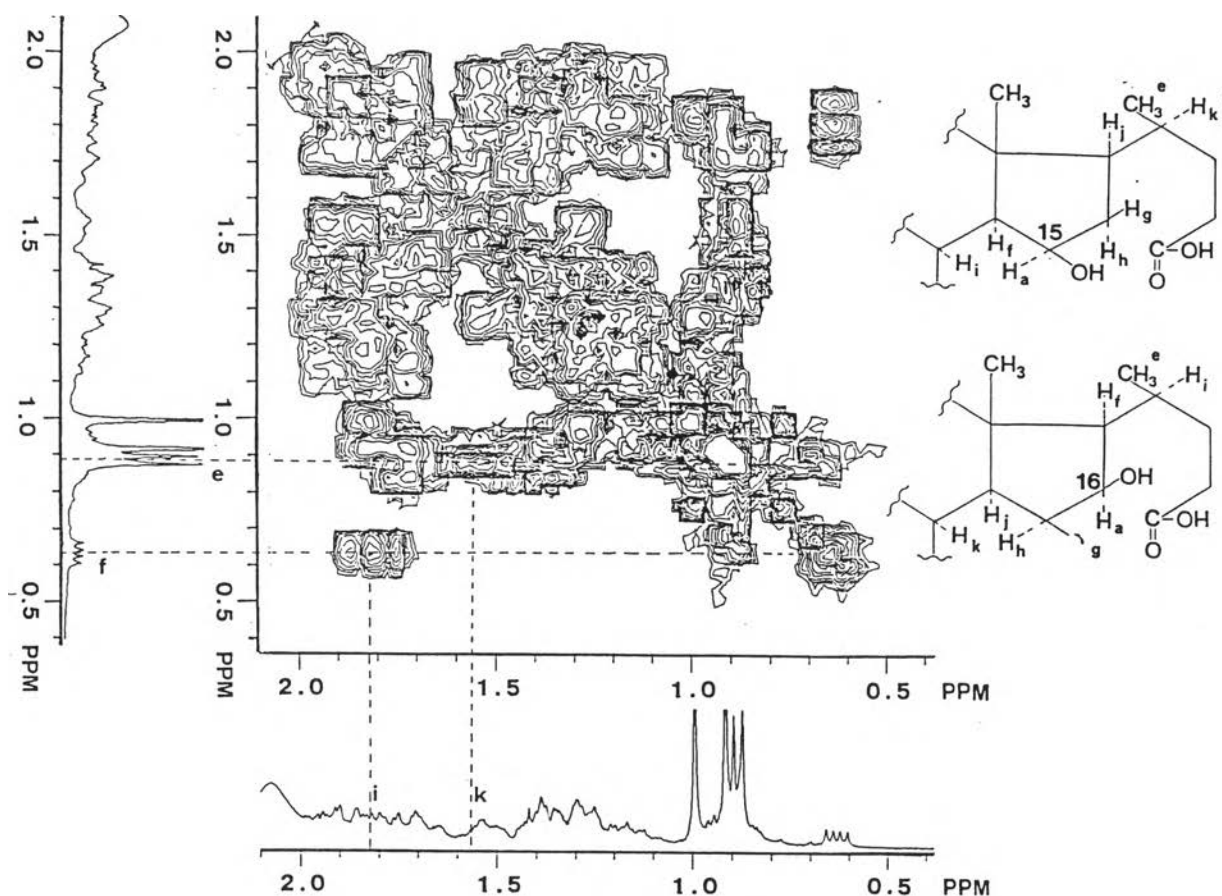


Fig. 7. Two-dimensional J-correlated ^{1}H-NMR spectrum of the product.

at 15β. Because the resonance arising from the C-20 methine proton was at 1.56 ppm (resonance k, Fig. 7), as evidenced by the coupling with methyl protons at C-21 (0.88 ppm, resonance e), the 1.82 ppm signal was assigned as 8-H. From this assignment the 0.65 ppm signal was determined as the C-9 methine signal, thus indicating the location of the second hydroxy group at 15β. The coupling between 1.56 ppm proton and 0.88 ppm proton was further confirmed by the difference spectrum between coupled and 0.88 ppm decoupled spectra. In conclusion, strain ST22 converts lithocholic acid into 3α,15β-dihydroxy-5β-cholanic acid, which is the first known bile acid derivative containing a 15β-hydroxy moiety.

Summary

A product of the microbiological transformation of lithocholic acid by *Cunninghamella blakesleeana* ST22 was identified as 3α,15β-dihydroxy-5β-cholanic acid. Its structure was determined on the basis of IR, ^{13}C-NMR and two-dimensional ^{1}H-NMR spectra.

Part 3 Biological Activity of 3α,15β-Dihydroxy 5β-Cholanic Acid

Introduction

Among natural bile acids, ursodeoxycholic acid (43) and chenodeoxycholic acid (37) were found to be effective for the treatment of cholesterol-gallstone disease by oral administration.

Because both compounds are dihydroxy derivatives of 5β-cholanic acid, the possibility that a new bile acid (88) with two hydroxyl groups may have similar therapeutic effects. This part deals with the cholesterol solubilizing activity of the product compared with those of ursodeoxycholic acid and chenodeoxycholic acid.

Materials and Methods

Chemicals Taurine- and glycine-conjugated forms of 3α,15β-DHC were synthesized respectively by the methods of Lack *et al.* (89) and Hofmann (46). Purity was confirmed by silica gel thin layer chromatography (*n*-butanol: acetic acid:water, 10:1:1, by vol.) and by high performance liquid chromatography (methanol:water, 7:3, pH 3 with conc. H_3PO_4). The taurine- and glycine-conjugated forms of other bile acids were the product of Gasukuro Kogyo, Tokyo. Sodium salts of bile acids were prepared by the method of Igimi *et al.* (9). The purity of each bile salt was checked by elemental analysis, infrared spectrometry and flame photometry. Other reagents were all of commerically available reagent grade and were used

without further purification.

Analysis High performance liquid chromatographic analyses were performed with a Trirotar II (Japan Spectroscopic Co. Ltd.) equipped with Zorbax-ODS column (Dupont Instruments), flow rate of 1 ml/min and detection at 210 nm.

Assay of the *in vitro* cholesterol solubilizing activity of the product Portions of 25 mg of cholesterol were added to test tubes containing 0.4 ml of a bile salt solution (150 mM with or without 12.5 mM lechthin in 0.15 M sodium phosphate buffer pH 8.0), and the tubes were agitated at 80 rpm at 20°C for 48 h. The solutions were then filtered through 0.45-μm filters, and amounts of solubilized cholesterol in the filtrate were determined by the enzymatic method of Fromm *et al.* (91).

Toxicity test of 3α,15β-DHC in mice

a) Sample preparation for injection: 3α,15β-DHC was dissolved in conc. NaOH and diluted with phosphate buffer composed of 0.3% KH_2PO_4 and 0.3% K_2HPO_4 to a final concentration of 28 mg/ml after pH had been adjusted to pH 7.0 with 5 N HCl. The solution was sterilized at 120°C for 10 min before injection into mice.

b) Injection: Five groups of five male mice each, with body weights of 30.0 to 30.5 g, were injected intraperitoneally with 0.1, 0.25, 0.5, 0.75 and 1.0 ml of the 3α,15β-DHC solution prepared as described above. The control groups received 0.5 and 1.0 ml of the buffer. Treated mice were observed for a period of 14 days. LD_{50} value was calculated from the curve of the relationship between log of percent of dead mice and the amount of bile acid injected.

Results and Discussion

1. Hydrophilicity of 3α,15β-dihydroxy 5β-cholanic acid

The solubilization of cholesterol gallstones by ursodeoxycholic acid *in vivo* is known to proceed mainly by a mechaism involving formation of liquid crystals with lecithin and cholesterol. Salvioli *et al.* (92) reported that the ability to form liquid crystals was closely correlated with the hydrophilicity of the bile acid involved: i.e., the more hydrophilic the bile acid, the more easily the liquid crystal of bile acid, lecithin and cholesterol was formed, thus the higher the cholesterol gallstone-solubilizing activity of the bile acid. Therefore, in order to estimate the ability of 3α,15β-DHC to solubilize cholesterol gallstones, its hydrophilicity was measured by reversed-phase HPLC and compared with that of known bile acids (Table 1). For the free acids, hydrophilicity increased in the order of LCA < deoxycholic acid < chenodeoxycholic acid < cholic acid < ursodeoxycholic acid, which agreed well with the data of Armstrong and Carey (78).

The Hydrophilicity of 3α,15β-DHC was found to be the highest. *In vivo*, almost all of bile acids exist in either taurine- or glycine-conjugated form, and these conjugated forms are the actual participants in gallstone solubilization, and therefore the hydrophilicity of conjugated bile acids was estimated. Similar orders of hydrophilicity were also observed for the taurine- and glycine-conjugated forms; and 3α,15β-DHC was more hydrophilic than UDCA.

2. The *in vitro* cholesterol solubilizing activity of 3α,15β-dihydroxy 5β-cholanic acid

The cholesterol-solubilizing activity of the sodium salt of 3α,15β-DHC, UDCA and CDCA in solution with or without 12.5 mM lecithin is shown in

Table 1. Hydrophilicity of several unconjugated and conjugated bile acids analyzed by reverse-phase HPLC.

	HPLC retention factor (k')		
	Unconjugated[a)]	Glycine-conjugated[b)]	Taurine-conjugated[c)]
3α,15β-Dihydroxy-5β-cholanic acid	0.52	0.34	0.58
Ursodeoxycholic acid (3α,7β-Dihydroxy-)	0.58	0.35	0.65
Cholanic acid (3α,7α,12α-Trihydroxy-)	0.86	0.71	1.30
Chenodeoxycholic acid (3α,7α-Dihydroxy-)	1.97	1.47	3.97
Deoxycholic acid (3α,12α-Dihydroxy-)	2.11	1.72	4.74
Lithocholic acid (3α-Hydroxy-)	4.28	-	12.23

Reverse-phase HPLC was performed as described in Materials and Methods using the following mobile phases: a) methanol:water, 75:25, pH 5 with 5 mM H_3PO_4/KH_2PO_4; b) methanol:water, 7:3, pH 3 with conc. H_3PO_4; c) methanol:water, 5:5, pH 3 with conc. H_3PO_4. Three μl of each bile acid solution (1 mg/ml) was injected. Retention factor (k'), which is inversely proportional to hydrophilicity, was calculated according to Armstrong and Carey (1982) (78):

$$k' = \frac{t_r - t_o}{t_o}$$

where t_o = retention time of the solvent front and t_r = retention time of the bile acid.

Table 2. Effect of bile acid on the amount of solubilized cholesterol in solution with or without 12.5 mM lecithin.

Bile salt (50 mM)	Amount of solubilized cholesterol (g/ℓ)	
	without lecithin	with lecithin
CDCA	0.48	0.60
UDCA	0.10	0.83
3α,15β-DHC	0.06	0.60

Table 2. It is from the table that lecithin enhanced the solubilizing activity of the bile acids. And increase in solubilized cholesterol in the presence of lecithin indicates the participation of liquid crystals. The

increase in the solubilizing activity of 3α,15β-DHC was greater than that of the other two bile acids, which indicates its higher capacity for liquid crystal formation.

3. Toxicity test of 3α,15β-DHC in mice

The chronic toxicity of 3α,15β-DHC was determined by intraperitoneal injection into male mice. As shown in Table 3, 80% of mice in group 3 died and 0% mice in group 4 died. The amount of 3α,15β-DHC administered was 14 mg in group 3 and 7 mg in group 4. From the results of this experiment, the calculated LD_{50} value is 400 mg/kg of body weight, compared with that of UDCA of 1000 mg/kg. It might thus be worth considering the use of 3α,15β-DHC for treatment of cholesterol-gallstone disease, since toxicity is gnerally decreased greatly by oral administration.

Table 3. The toxicity test of 3α,15β-DHC in mice.

Group [3α,15β-DHC injected (mg/head)]	No.	Weight (g)	No. dead	No. alive
1 [28]	5	30.1 - 30.5	5	-
2 [21]	5	30.0 - 30.5	5	-
3 [14]	5	30.1 - 30.4	4	1
4 [7]	5	30.1 - 30.3	-	5
5 [2.8]	5	30.1 - 30.3	-	5
Control (1)	3	30.0 - 30.3	-	3
Control (2)	3	30.2 - 30.5	-	3

Mice in control group (1) and (2) were injected with 1.0 ml and 0.5 ml of the same buffer, respectively.

Summary

The new bile acid, 3α,15β-dihydroxy-5β-cholanic acid, was found to possess similar properties to ursodeoxycholic acid in cholesterol solubiliza-

tion, which was estimated by hydrophilicity measurement and *in vitro* cholesterol solubilization. A toxicity test of 3α,15β-DHC in mice indicated that the LD_{50} value by intrapertoneal injection of 3α,15β-dihydroxy-5β-cholanic acid was 400 mg/kg of body weight, which was lower than the value of ursodeoxycholic acid of 1000 mg/kg of body weight.