Tetrahedron Vol. 40, No 15, pp 2843 to 2846, 1984
Printed in the U.S.A.

0040-4020/84 $3.00 + .00
Pergamon Press Ltd.

15β-Hydroxylation of Lithocholic Acid by *Cunninghamella* sp.

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(*Received in Japan* 1 *March* 1984)

Abstract - The microbiological transformation of lithocholic acid (3α-hydroxy-5β-cholanic acid) into 3α, 15β-dihydroxy-5β-cholanic acid by *Cunninghamella* sp. is investigated. The structure has been determined on the basis of two-dimensional ^{1}H-NMR.

Although microbial transformation of a variety of steroidal compounds has been well studied[1], little knowledge is available on the hydroxylation of bile acids. While most studies on microbial transformation have been done with respect to the cholesterol metabolism[2,3], few reports have been published on obtaining useful bile acid derivatives.

Since Danzinger et al.[4] and Makino et al.[5] discovered that 7-hydroxy derivatives of 5β-cholanic acid, i.e. ursodeoxycholic acid (3α, 7β-dihydroxy-5β-cholanic acid) and chenodeoxycholic acid (3α, 7α-dihydroxy-5β-cholanic acid) are effective in dissolving cholesterol gallstone and they have been used successfully for patients of gallstone disease, more attention has been raised on the hydroxylation of bile acids.

In the course of our studies on the biotransformation of bile acids by means of fungi[6], a fungus *Cunninghamella* sp. ST-22 was isolated from acid soil at Bangkok, Thailand in 1982, and was characterized by its ability to convert lithocholic acid into more hydrophilic compounds. In this report we described the structural elucidation of the main product as 3α, 15β-dihydroxy-5β-cholanic acid (Figure 1).

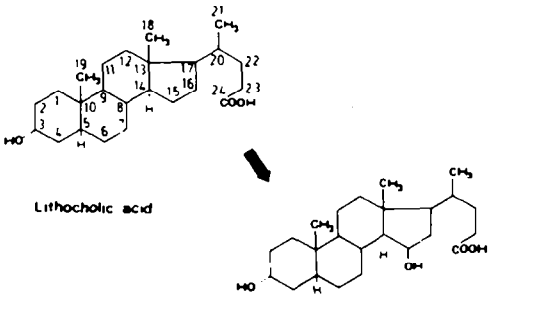


Figure 1. Conversion of lithocholic acid into 3α, 15β-dihydroxy-5β-choloanic acid

Results and Discussion

The microbiological transformation was carried out in submerged cultures of *Cunninghamella* sp. ST-22 containing 1 g of lithocholic acid, 40 g dextrin, 12 g asparagine, 3 g KCl, 2 g KH_2PO_4, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract and 0.1 g each of $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $MnSO_4 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$ per ℓ of the culture. After 6 days of cultivation at 30°C on a reciprocal shaker, the product was isolated by extraction with ethyl acetate. The ethyl acetate extract was chromatographed twice on a silica gel column using first ethyl acetate-2,2,4-trimethylpentane-acetic acid (10:10:2, v/v/v) and then chloroform-acetone-acetic acid (50:10:0.6, v/v/v) as eluent. The material was crystallized twice from ethyl acetate/hexane to afford white crystals (475 mg, 31.0 % yield). The homogeneity was checked by high performance liquid chromatography and the crystals dissolved in methanol showed a single peak. The compound, 1, m.p. 185.5-186.5°C and $[\alpha]_D^{25}$+3.4 (c 1, EtOH), was shown to have molecular formula $C_{24}H_{40}O_4$ 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[7] [$(M + Na)^+$ at m/z 415 and $(M + H + glycerol)^+$ at m/z 485]. Considering that the starting material was lithocholic acid ($C_{24}H_{40}O_3$), 1 was assumed to be a dihydroxy derivative of 5β-cholanic acid. The IR spectrum of 1 which exhibited absorption at ν_{max} (nujol) 3280 (bonded OH) and 1700 cm^{-1} (C=O dimer) supported the above estimation. Further confirmation of the structure came from the ^{13}C-NMR of 1 (Table 1) which showed 24 signals. By INEPT ^{13}C-NMR[8], 1 was found to contain 3 methyl carbons, 10 methylene carbons, 8 methine carbons and 3 quaternary carbons. Two methine carbons

Table 1. ^{13}C-NMR data [a] for 1 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-$\underline{C}H_2$	36.6	(t)
11-$\underline{C}H_2$	21.8	(t)	23-$\underline{C}H_2$	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.

showed $\underline{C}HOH$ resonances (70.6 and 72.7 ppm) and one quaternary carbon showed $\underline{C}OOH$ resonance (178.0 ppm). From the data mentioned above, 1 is concluded as dihydroxy derivative of 5β-cholanic acid.

Although there already known several dihydroxy derivatives[9], comparison of several characters of 1, 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[9-11] indicated that 1 is the new compound. Therefore, we investigated the ^{1}H-NMR spectrum to assign

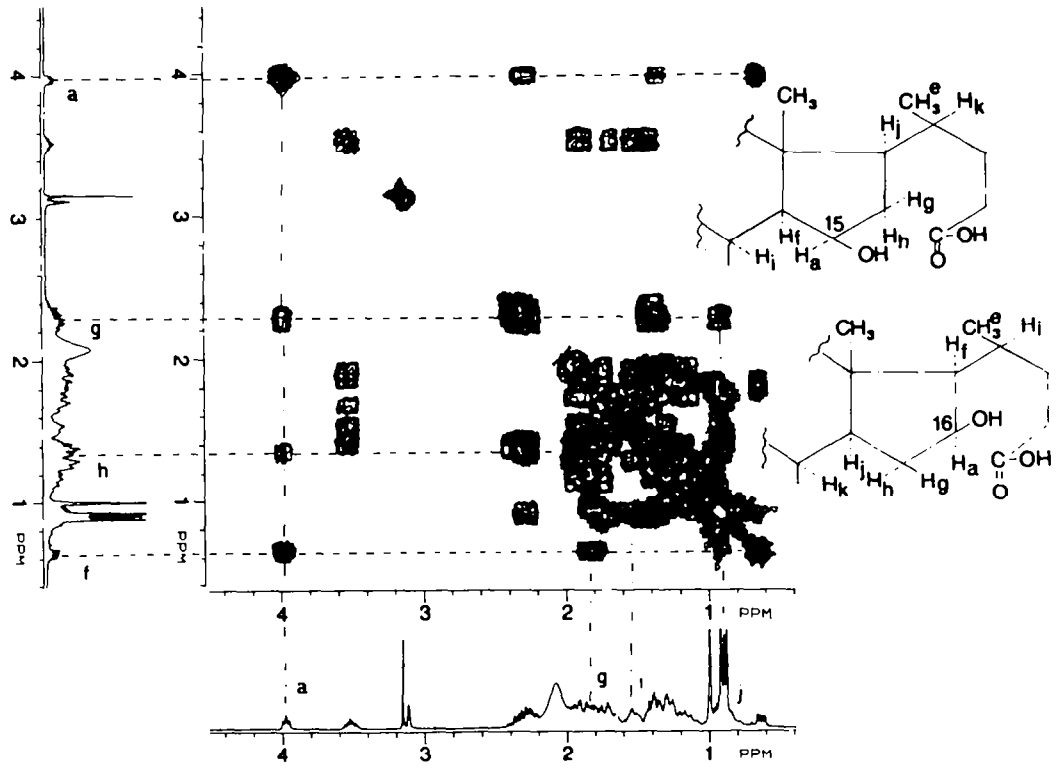


Figure 2. Two-dimensional J-correlated ^{1}H-NMR spectrum of 1

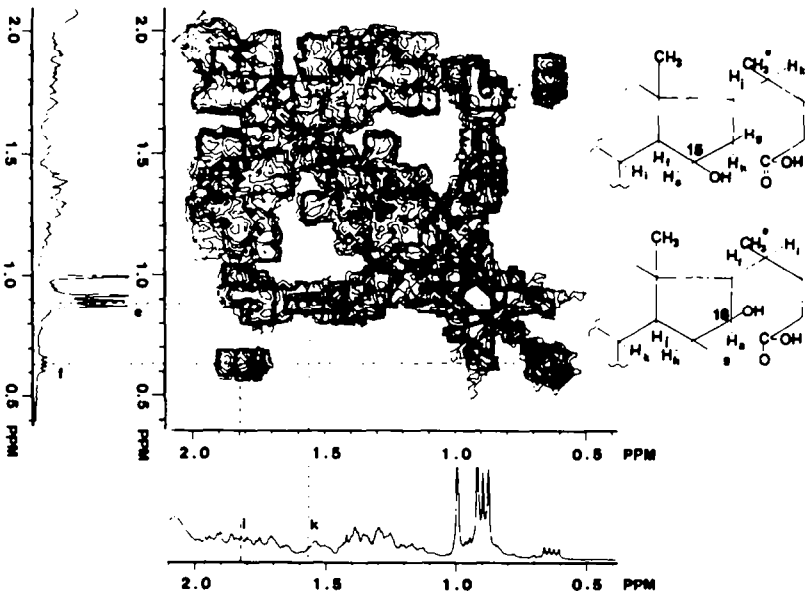
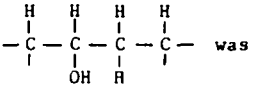


Figure 3. Two-dimensional J-correlated ^{1}H-NMR spectrum of 1

the position of the hydroxy group other than 3α in this compound. In 300 MHz ^{1}H-NMR spectrum (C_6D_6:CD_3OD, 10:1, v/v) following resonances were observed: 3.98 (1H, m, J=5.56 Hz, CHOH), 3.52 (1H, m, J_{ax-ax}=9.42 Hz, 3β-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). From the down field shift of 18-CH_3 resonance (0.92 ppm in 1 from 0.65 and 0.73 ppm in lithocholic acid methyl ester and 3α, 12α-dihydroxy-5β-cholanic acid, respectively)[12], the hydroxy group other than 3α in 1 seemed to locate either at 11β, 12β, 15β, 16β position. Furthermore, the J value of 3.98 ppm signal suggested the location of the hydroxy group on 5 membered ring. Thus, for the position of the hydroxy group, 15β or 16β was most probable. To confirm the position of the hydroxy group further, we performed the two-dimensional J-correlated ^{1}H-NMR spectroscopy[13] of 1 (Figure 2), 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 each proton was the geminal proton of methylene carbon. The 2.28 ppm proton exhibited the one more coupling with 0.9 ppm proton (resonance j). Based on these couplings, the

partial structure of  was

demonstrated. This structure further eliminated the possibility that the hydroxy group may exist at 11β or 12β position. The 0.65 ppm methine signal further showed the coupling with 1.82 ppm signal (resonance i, Fig. 2). This 1.82 ppm proton should locate at C-20 when the hydroxy group locates at 16β, or at C-8 when the hydroxy group locates at 15β. Because the resonance arising from the C-20 methine proton was at 1.56 ppm (resonance k, Figure 3) 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 C-9 methine signal, thus indicating the location of the 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 *Cunninghamella* sp. ST-22 converts lithocholic acid into 3α, 15β-dihydroxy-5β-cholanic acid.

In our knowledge this compound is the first bile acid derivative containing 15β-hydroxy moiety. The pharmacological study in detail is in progress.

EXPERIMENTAL

Instrumentation

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 analyses were performed by the

analytical center of Applied Chemistry (Faculty of Engineering, Osaka University). ^{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 analyses of the product were carried out with a Trirotar-II (Japan Spectroscopic Co. Ltd.) equipped with Zorbax-ODS column (DuPont Instruments). Solvent used was methanol-water (73:27, v/v, pH 3 with H_3PO_4) with detection at 210 nm. Gas chromatography was performed with a Hitachi 163 equipped with FID. A glass column 3 mm × 1 m 2 % Silicone DC-QF-1 on Uniport HP 80/100 mesh was used, col. temp. and inj. temp. 220°C and 240°C, respectively. Samples for GC were derivatized with hexafluolo isopropanol and trifluoroacetic anhydride according to the method of Imai and Tamura[15].

Cultivation of Cunninghamella *sp. ST-22 and isolation of the compound* 1.

The cultivation was performed either with 2-ℓ Sakaguchi flasks on a reciprocal shaker (120 rpm, 30°C) or with a 10-ℓ jar fermentor (type MD-500; L.E. Marubishi). For jar fermentation, the pH was kept 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.

For silica gel chromatography of compound 1, Wakogel C-300 (Wako Pure Chemical Industries, Ltd.) was used. All solvents used were purchased from commercial sources and were used without further purification. The typical purification procedure of 1 was as follows: Nine ℓ of culture broth was adjusted to pH 3 with 3 N HCl, followed by the extraction with 18-ℓ of ethyl acetate with vigorous mixing for 2-3 hr at room temperature (1.53 g of 1). After drying ethyl acetate layer with anhydrous Na_2SO_4, the extract was concentrated by rotary evaporator (50°C). The oily material was dissolved in the minimum amount of ethyl acetate-2,2,4-trimethylpentane-acetic acid (10:10:2, v/v/v) and was chromatographed on a silica gel column (3.0 × 96 cm) using the same mixture as eluent. Fractions of 10 ml were collected and were analysed by TLC (ethyl acetate-2,2,4-trimethylpentane-acetic acid, 10:10:2, v/v/v) and gas chromatography. Fractions containing the compound 1 (711 mg) was mixed, concentrated with a rotary evaporator, and was rechromatographed on a silica gel (chloroform-acetone-acetic acid, 50:10:0.6, v/v/v) column (3.0 × 96 cm). Fractions containing compound 1 were mixed (527 mg), evaporated to dryness by rotary evaporator (50°C), and crystallized twice from ethyl acetate/hexane (475 mg, 31.0 % yield).

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Acknowledgements - Authors wish to express great gratitude to Dr. Hideo Naoki (Suntory Institute for Bioorganic Research) for performing several spectral measurements, to Dr. Yasuhiro Yamada for his critical evaluation of the manuscript and helpful discussions. Thanks are also extended to Mrs. Mami Sugimoto for her excellent assistance during purification.