Bile acid transformations by *Alcaligenes recti*

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Metabolism of cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, and deoxycholic acid by the grown cells of the bacterium Alcaligenes recti *suspended in water was studied. Each isolated metabolite was characterized by the application of various spectroscopic methods. Cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, and deoxycholic acid yielded methylated derivatives 3α-methoxy-7α, 12α-dihydroxy-5β-cholanoic acid, 3α-methoxy-7α-hydroxy-5β-cholanoic acid, 3α-methoxy-7β-hydroxy-5β-cholanoic acid, and 3α-methoxy-12α-hydroxy-5β-cholanoic acid, respectively. In addition, cholic acid furnished 7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid; chenodeoxycholic acid gave 7α-hydroxy-3-oxo-5β-cholanoic acid and 7α-hydroxy-3-oxochol-4-en-24-oic acid while ursodeoxycholic acid yielded 7β-hydroxy-3-oxochol-4-en-24-oic acid and 3-oxochola-4,6-dien-24-oic acid. The formation of various metabolites showed that two competitive enzymic reactions, i.e., selective methylation of the 3α-hydroxy group and dehydrogenation in the A/B rings, were operative. The methylation process was found to be enzymic involving an S-adenosyl-L-methionine (AdoMet)-dependent methyl transferase, and this reaction appeared to be inhibitory to the process of degradation of the ring system. In the other reaction sequence, degradation of the ring system was initiated by dehydrogenation of the 3α-hydroxy group. A 7β-dehydratase activity producing the Δ^6 double bond was also noticeable in the metabolism of ursodeoxycholic acid.* (*Steroids* **58**:79–86, 1993)

Keywords: sterols; bile acids; metabolites; microbial transformation; *Alcaligenes recti*; bacterial transformation

Introduction

Microbial transformations are used as a general means to prepare derivatives that are difficult to synthesize by chemical methods, and commercially important steroids are now often prepared by a combination of chemical and microbial processes. In our study for obtaining physiologically important steroid derivatives by microbial transformation,[1–3] selective methylation of 3α-hydroxy groups of cholic acid **(1)**, chenodeoxycholic acid **(5)**, ursodeoxycholic acid **(9)**, and deoxycholic acid **(13)** has been found to be the major enzymic transformation reaction when the bile acids were incubated in distilled water with cells of the bacterium *Alcaligenes recti* isolated from soil by enrichment culture technique[4] using cholic acid as the sole carbon source. Catabolism of bile acids by microorganisms has been the subject of much interest in recent years.[5–8]

There are two basic degradative pathways for bacterial bile acid metabolism. In the first pathway the main point of attack for the microorganisms is on the bile acid nucleus with rupture of the B, A, C, and then D rings, with either no or partial removal of the side chain before the ring system is completely degraded. In the second pathway, the side chain is the main point of attack, and rupture of the ring system is not observed until the side chain is completely removed.[9]

In the present study of metabolism of four bile acids by the strain of *A recti*, two competitive enzymic reactions, methylation of 3α-hydroxy group and dehydrogenation of the A ring, appeared to be predominant. The selective methylation of 3α-hydroxy groups of bile acids by bile acid-*O*-methyl transferase generated by an intestinal microorganism,[10] *A recti*, is an inhibitory sequel to the dehydrogenation of 3α-hydroxy group to 3-keto derivatives, which is the first step in the ring degradation sequence. A detailed study on regulation of this enzymic methylation may lead to regulation of bile acid metabolism.

This article reports the aerobic metabolism of bile acids, spectroscopic characterization of the various metabolites formed, and the significance and mechanism of their formation.

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Experimental

Materials

Inorganic salts were purchased from Sarabhai Chemicals, Bombay, India. Peptone, beef extract, yeast extract, and agar were purchased from Difco Laboratories, Detroit, MI, USA. Silica gel for column chromatography and thin-layer chromatography (TLC), and organic solvents were supplied by E. Merck, Bombay, India. The steroid substrates cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, S-adenosyl L-methionine, and S-adenosyl homocysteine were purchased from Sigma Chemical Co., St. Louis, MO, USA. Reagents and solvents were of analytical grade.

General methods

Melting points (mp) were determined in open capillary tubes in an H_2SO_4 bath and are uncorrected. IR spectra in KBr were recorded with a Perkin-Elmer 177 spectrophotometer. Optical rotations were measured in MeOH with a Perkin-Elmer automatic polarimeter. Mass spectra (ms) were obtained in Hitachi RMU-6L (Hitachi, Japan) and MS-50 (Kratos, UK) mass spectrometers at an ionization potential of 70 eV. Fast atom bombardment mass spectra were obtained on a VG ZAB-SE mass spectrometer equipped with a fast atom bombardment (FAB) source operating at an accelerating voltage of 8 keV. FAB mass spectra were also obtained using a Finningan MAT 312 mass spectrometer (Finningan, USA) operating at an accelerating voltage of 2–3 keV. Samples were dissolved in DMSO-d_6 (2–10 $\mu g/\mu l$) and deposited on an FAB probe tip. A thin layer of either glycerol or thioglycerol was applied to the probe tip containing the samples and mixed thoroughly with a Pasteur pipette before insertion into the source. The primary atom (xenon) was produced using a Saddlefield ion source operating at a tube current of 1–1.5 mA at an energy of 8 keV. For the salt-addition technique, samples were dissolved in DMSO-d_6 (2–5 $\mu g/\mu l$) to which salt (KCl, NaCl, or NH_4Cl) was added such that the sample to salt ratio was roughly 1 : 3. ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were obtained with a JEOL FT-100 spectrometer at 100 and 25.05 MHz, respectively, with tetramethylsilane as an internal standard. High-performance liquid chromatography (HPLC) was performed in a Spectra Physics model SP 8000 B apparatus controlled by a microprocessor that allows selection of constant pressure or constant flow for quantification, with an autoinjector, a dual-channel plotter/printer, and model SP 8440 UV/Vis variable-wavelength detector.

Growth and fermentation methods

The strain of *A recti* (Registry no. IICB 331) was isolated from soil by enrichment culture technique[4] with cholic acid as the sole carbon source. It is being maintained on nutrient agar slants at the Culture Collection Unit of Steroid and Terpenoid Division of this Institute.

A young culture of the bacterial strain was grown for 24 hours in either of the two fermentation media: (a) 0.5% peptone, 0.25% yeast extract, 0.25% beef extract, 0.25% NaCl, pH 7.0; or (b) 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4$, 1% glucose, pH 7.0. The cell growth was monitored in a hemocytometer, and, in the optimum condition, cells/ml of the medium was found to be 12.45×10^6. The cells were harvested by centrifuging, washed with 0.5% NaCl, and suspended in distilled water. A 1.2-g portion of each substrate (**1**, **5**, **10**, and **14**) in powder form was evenly distributed in 20 flasks of 500-ml capacity, each containing 100 ml of distilled water, and was incubated with the cell suspension for 120 hours at 37 C in aerobic condition with shaking.

Extraction and isolation of metabolites

Fermentation was terminated after 120 hours of incubation by direct extraction of the metabolites with ethyl acetate. The organic layer was washed with distilled water, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. A little of the residue obtained in each case was dissolved in 1 ml of MeOH and analyzed by TLC on silica gel plates with $CHCl_3$/MeOH/CH_3COOH in different proportions as the developing solvent system. The individual metabolites were separated and purified by column chromatography over silica gel and preparative TLC followed by crystallization. Various spectroscopic techniques, e.g., ultraviolet (UV), IR, ^{1}H-NMR, ^{13}C-NMR, and MS were used for characterization of the metabolites. The purity of each metabolite and its derivatives also was checked by HPLC.

Transformation of cholic acid (1)

Fermentation of 1.2 g of cholic acid yielded the compounds 3α-methoxy-7α,12α-dihydroxy-5β-cholanoic acid (**2**) and 7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid (**4**).

3α-Methoxy-7α,12α-dihydroxy-5β-cholanoic acid (2)

This compound, which is reported for the first time, was crystallized from MeOH as prisms, mp 220–222 C and $[\alpha]_D^{27}$ + 36° (c = 0.03, MeOH); IR, ν_{max} (KBr) 3400 (hydroxy), 2940 (acid hydroxy), 1730 (acid carbonyl), 1185, 1156, 1050, 1030, 975 cm^{-1}; MS m/z 422 (M^+, 2%), 404 (M^+-H_2O, 5%), 386 (M^+-2H_2O, 87%), 368 (M^+-3H_2O, 37%), 357 ($M^+-H_2O-CO_2H$, 20%), 271 (M^+-H_2O–MeOH–side chain, 42%), 253 (M^+-2H_2O–MeOH–side chain, 100%), ^{1}H-NMR (C_5D_5N) δ 0.68 (s, 3H, 18-C$\underline{H_3}$), 0.88 (s, 3H, 19-C$\underline{H_3}$), 3.36 (s, 3H, 3α-OC$\underline{H_3}$). Analysis calculated for $C_{25}H_{42}O_5$: C, 71.05; H, 10.02. Found: C, 71.12; H, 10.06.

Preparation of acetate (3) of 3α-Methoxy-7α,12α-dihydroxy-5β-cholanoic acid (2)

The compound (**2**) (60 mg) was acetylated with acetic anhydride (7 ml) and pyridine (1 ml) at 80 C for 1 hour. The reaction mixture was dried under reduced pressure and the acetate (**3**) was purified by chromatography to yield an amorphous solid. $[\alpha]_D$ + 38.5° (c = 0.25, MeOH); MS m/z 506 (M^+, 1%), 428 ($M^+-CH_3CO_2H-H_2O$, 70%), 368 (M^+-2 $CH_3COOH-H_2O$, 75%), 253 (M^+-2 CH_3CO_2H–MeOH–side chain, 100%), ^{1}H-NMR (C_5D_5N) δ 0.68 (s, 3H, 18-C$\underline{H_3}$), 0.84 (s, 3H, 19-C$\underline{H_3}$), 2.00, 2.04 (s each, 6H, 2x–OCOC$\underline{H_3}$), 3.36 (s, 3H, 3α–OC$\underline{H_3}$). Analysis calculated for $C_{29}H_{46}O_7$: C, 68.74; H, 9.15. Found: C, 68.68; H, 9.12.

7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid (4)

The compound was crystallized from MeOH to give needles. mp 193–195 C; UV, λ_{max} (EtOH), 242 nm; FTIR, ν_{max} 3420 (hydroxy), 1715 (acid carbonyl), 1662 (3-ketone), 1610, 1552, 1535, 1260 cm^{-1}; MS m/z 404 (M^+), 376 (M^+–CO), 358 (M^+-CO-H_2O), 340 ($M^+-CO-2H_2O$), 261 (M^+-42 – side chain), 124; ^{1}H-NMR (C_5D_5N) δ 1.12 (s, 3H, 18-C$\underline{H_3}$), 1.20 (s, 3H, 19-C$\underline{H_3}$), 4.14 (brs, 2H, 7β-$\underline{H}$ and 12β-$\underline{H}$) and 6.00 (s, 1H, 4-$\underline{H}$).

Transformation of chenodeoxycholic acid (5)

Incubation of 1.2 g of chenodeoxycholic acid as described above furnished the compounds 3α-methoxy-7α-hydroxy-5β-chola-

noic acid **(6)**, 7α-hydroxy-3-oxo-5β-cholanoic acid **(7)** and 7α-hydroxy-3-oxochol-4-en-24-oic acid **(8)**.

3α-Methoxy-7α-hydroxy-5β-cholanoic acid (6)

This was obtained as an amorphous solid (single peak in HPLC) $[\alpha]_D^{27}$ = +21.7° (c = 0.23, MeOH); FTIR, ν_{max} 3400 (hydroxy), 1730 (acid carbonyl), 1162, 1070, 980 cm^{-1}; MS m/z 406 (M^+, 5%), 388 (M^+-H_2O, 52%), 355 (M^+–MeOH–H_2O–H, 39%), 316 (M^+–side chain + H, 33%), 273 (M^+–side chain–MeOH, 100%) and 255 (M^+-H_2O–MeOH–side chain, 44%); ^{1}H-NMR (C_5D_5N) 0.73 (s, 3H, 18-C$\underline{H}_3$), 0.96 (s, 3H, 19-C$\underline{H}_3$), 3.34 (s, 3H, 3α-OC$\underline{H}_3$) and 4.20 (brs, 1H, 7β-H). The compound as such has not yet been reported; a report of its methyl ester is found in literature.[11] Analysis calculated for $C_{25}H_{42}O_4$: C, 73.85; H, 10.4. Found: C, 73.78; H, 10.47.

7α-Hydroxy-3-oxo-5β-cholanoic acid (7)

This was crystallized from MeOH as needles, mp 200–202 C; $[\alpha]_D^{28}$ = −30° (c = 0.01, MeOH); FTIR, ν_{max} 3500 (hydroxy), 2925 (acid hydroxy), 1715 (acid carbonyl), 1690 (3-ketone), 1400, 1255, and 1030 cm^{-1}. FAB MS (KCl added) m/z 819 (2M + K, 35%), 429 (M + K, 100%); ^{1}H-NMR (DMSO-d_6) δ 0.60 (s, 3H, 18-C$\underline{H}_3$), 1.10 (s, 3H, 19-C$\underline{H}_3$); TLC R_f 0.71 [isooctane/EtOAc/AcOH (50 : 50 : 10 v/v/v)].

Transformation of ursodeoxycholic acid (9)

Fermentation of 1.2 g of ursodeoxycholic acid as described above yielded three metabolites: 3α-methoxy-7β-hydroxy-5β-cholanoic acid **(10)**, 7β-hydroxy-3-oxochol-4-en-24-oic acid **(11)**, and 3-oxochola-4,6-dien-24-oic acid **(12)**.

3α-Methoxy-7β-hydroxy-5β-cholanoic acid (10)

This compound was obtained as an amorphous solid after purification. $[\alpha]_D^{25}$ = +44° (c = 0.31, MeOH); FTIR, ν_{max} 3350 (hydroxyl), 1725 (acid carbonyl), 1155, 1060, 970 cm^{-1}; MS m/z 406 (M^+), 388 (M^+-H_2O), 273 (M^+–side chain–MeOH), 255 (M^+-H_2O–MeOH–side chain), 213; ^{1}H-NMR (C_5D_5N) δ 0.68 (s, 3H, 18-C$\underline{H}_3$), 0.96 (s, 3H, 19-C$\underline{H}_3$), 3.37 (s, 3H, 3α-OC$\underline{H}_3$). Analysis calculated for $C_{25}H_{42}O_4$: C, 73.85; H, 10.41. Found: C, 73.71; H, 10.48.

7β-hydroxy-3-oxochol-4-en-24-oic acid (11)

This compound was crystallized from ethyl acetate as microneedles, mp 190–191 C (lit[12]: mp 198 C) $[\alpha]_D^{25}$ = +8° (c = 0.01, MeOH); UV, λ_{max} 242 nm (ε 16,300); FTIR, ν_{max} 3500 (hydroxy), 1735 (acid carbonyl), 1650 (3-ketone), 1450, 1200, 1100, and 980 cm^{-1}; MS m/z 388 (M^+, 40%), 371 (M^+–OH, 34%), 370 (M^+-H_2O, 100%), 124 (15%); ^{1}H-NMR (C_5D_5N) δ 0.72 (s, 3H, 18-C$\underline{H}_3$), 1.08 (s, 3H, 19-C$\underline{H}_3$), 3.60 (m, 1H, 7-$\underline{H}$), 5.92 (s, 1H, 4-$\underline{H}$).

3-oxochola-4,6-dien-24-oic acid (12)

This compound was crystallized from methanol as needles, mp 192–194 C; $[\alpha]_D^{25}$ = 0° (c = 0.01, MeOH); UV, λ_{max} 284 nm; FTIR ν_{max} 3100 (acid hydroxy), 1600, 1625, 1725 (4,6-dien-3-one), 1225, 1335 cm^{-1}; MS m/z 370 (M^+, 100%), 355 (M^+-CH_3, 2%), 269 (M^+–side chain, 36%), 136 (24%); ^{1}H-NMR (C_5D_5N) δ 0.64 (s, 3H, 18-C$\underline{H}_3$), 1.00 (s, 3H, 19-C$\underline{H}_3$), 5.88 (s, 1H, 4-H), 6.08 (s, 2H, 6-H and 7-H).

Transformation of deoxycholic acid (13)

Fermentation of 1.2 g of deoxycholic acid as described above afforded only 3α-methoxy-12α-hydroxy-5β-cholanoic acid **(14)**.

3α-Methoxy-12α-hydroxy-5β-cholanoic acid (14)

This compound was also obtained as an amorphous solid. $[\alpha]_D^{25}$ = +21.7° (c = 0.23, MeOH); FTIR, ν_{max} 3600 (hydroxy), 2950 (acid hydroxy), 1720 (acid carbonyl, 1410, 1275, 1050 cm^{-1}; MS m/z 406 (M^+, 2%), 388 (M^+-H_2O, 33%), 355 (M^+–MeOH–H_2O–H, 15%), 273 (M^+–side chain–MeOH, 100%), 255 (M^+–side chain–MeOH–H_2O, 32%); ^{1}H-NMR (C_5D_5N) δ 0.68 (s, 3H, 18-C$\underline{H}_3$), 0.96 (s, 3H, 19-C$\underline{H}_3$), 3.36 (s, 3H, 3α-OC$\underline{H}_3$). Analysis calculated for $C_{25}H_{42}O_4$: C, 73.85; H, 10.41. Found: C, 73.73; H, 10.35.

Acetate of 3α-methoxy-12α-hydroxy-5β-cholanoic acid (15)

This compound was obtained as an amorphous solid. ^{1}H-NMR ($CDCl_3$) δ 0.64 (s, 3H, 18-C$\underline{H}_3$), 0.92 (s, 3H, 19-C$\underline{H}_3$), 2.10 (s, 3H, -OCOC$\underline{H}_3$), 3.34 (s, 3H, 3α-OC$\underline{H}_3$), 4.62 (t, 1H, 12β-$\underline{H}$). Analysis calculated for $C_{27}H_{44}O_5$: C, 72.28; H, 9.89. Found: C, 72.20; H, 9.80.

Results and discussion

Compound **2** was obtained as the major metabolite of cholic acid **(1)**. Its molecular formula ($C_{25}H_{42}O_5$) suggested an addition of CH_2 to the molecular formula of cholic acid. Moreover, its ^{1}H-NMR spectrum displayed a three-proton singlet at δ 3.36, ascribed to a methoxy group. The results demonstrated that one of the three hydroxyl groups of cholic acid has been methylated to yield metabolite **(2)**. The ^{1}H- and ^{13}C-NMR data of the metabolite of **2** were found to be inadequate for unambiguous determination of the location of the methoxy group in **2** because of the absence of discernible methylation-induced shifts. However, the ^{13}C-NMR spectrum of the acetate **(3)** showed that the two acetoxy functions were located at C-7 and C-12. Although C-7 and C-12 were shifted downfield by 5.5 and 2.2 ppm (α-shift), C-6, C-8, C-11, and C-13 were shifted upfield by 3.4, 1.7, 1.3, and 1.4 ppm (β-shift), respectively, in comparison with those of metabolite **2**. Taking into consideration the α- and β-shift due to acetylation, the location of the methoxy function could be determined to be at C-3 in **2**. Thus, the compound was characterized as 3α-methoxy-7α,12α-dihydroxy-5β-cholanoic acid **(2)**, which to our knowledge has not yet been reported in the literature. The metabolite **(4)** was characterized from its mass spectrum showing the molecular ion at m/z 404 and a fragment ion at m/z 124, which is characteristic of the Δ^4-keto system in a steroid skeleton.[13] The presence of a Δ^4-3-keto system in **4** was also supported by its UV spectrum.

Chenodeoxycholic acid **(5)** yielded a major metabolite **(6)** with molecular formula $C_{25}H_{42}O_4$. The mass spectrum of this compound suggested an addition of CH_2 to the molecular formula of **5**. The ^{1}H-NMR spectrum displayed a three-proton singlet assignable to a methoxy group. The location of the methoxy group was established to be at C-3 from a study of its mass spectrum and ^{1}H- and ^{13}C-NMR data. Thus, the metabolite was characterized as 3α-methoxy-7α-hydroxy-5β-cholanoic acid **(6)**.[11]

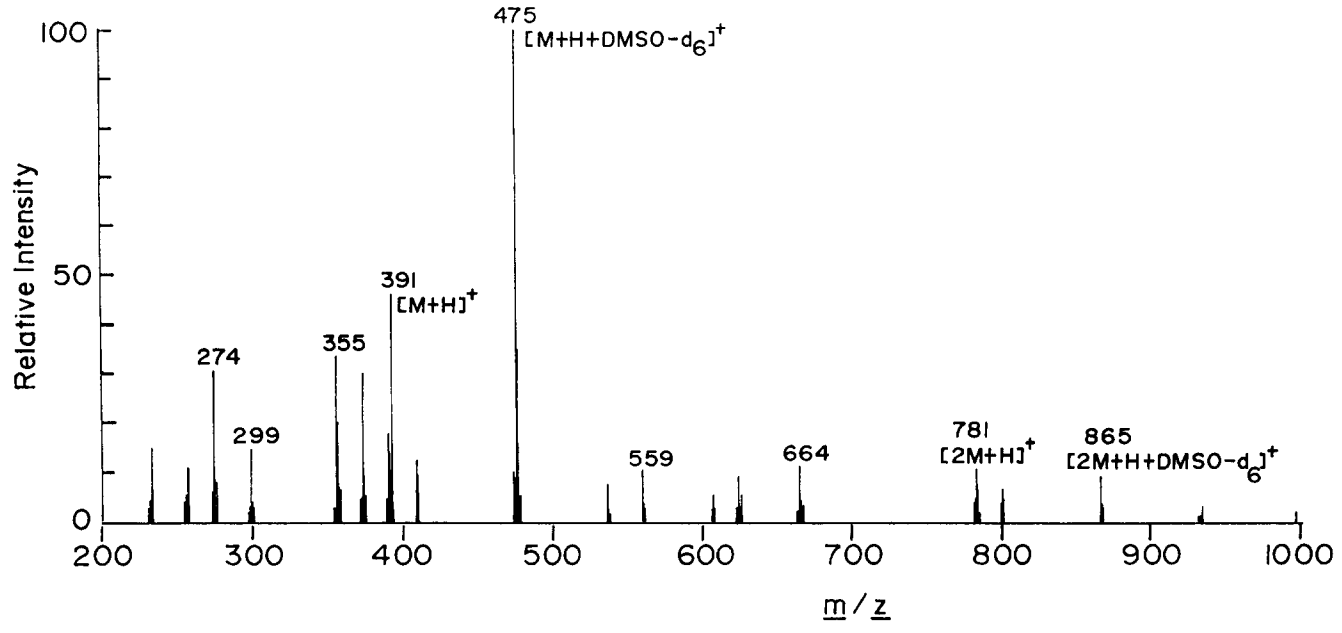

Figure 1 Positive-ion FAB mass spectrum of 7α-hydroxy-3-oxo-5β-cholanoic acid (**7**) at an accelerating voltage of 2–3 KeV. Samples were dissolved in DMSO-d_6 and deposited on an FAB probe tip. A thin layer of either glycerol or thioglycerol was applied to the probe tip containing samples and inserted into the source.

The electron impact mass spectra of the metabolite of **7** did not display a discernible molecular ion. However, the FAB MS, particularly when KCl was added in the positive mode,[14] the $[M + K]^+$ ion was obtained at m/z 429 as the base peak. A peak at m/z 819 attributable to $[2 M + K]^+$ was also obtained. The FAB MS of the metabolite of **7** in the positive mode with and without adding KCl are shown in Figures 1 and 2. Thus, the molecular weight of **7** could be determined to be 390. Moreover, the ^{1}H- and ^{13}C-NMR data suggested it to be a 7α-hydroxy-3-oxo-5β-cholanoic acid. Although its spectroscopic data have not yet been reported in the literature, its identification was confirmed by its TLC behavior[15] in different solvent systems.

A minor metabolite of chenodeoxycholic acid (**5**) was characterized as 7α-hydroxy-3-oxochol-4-en-24-

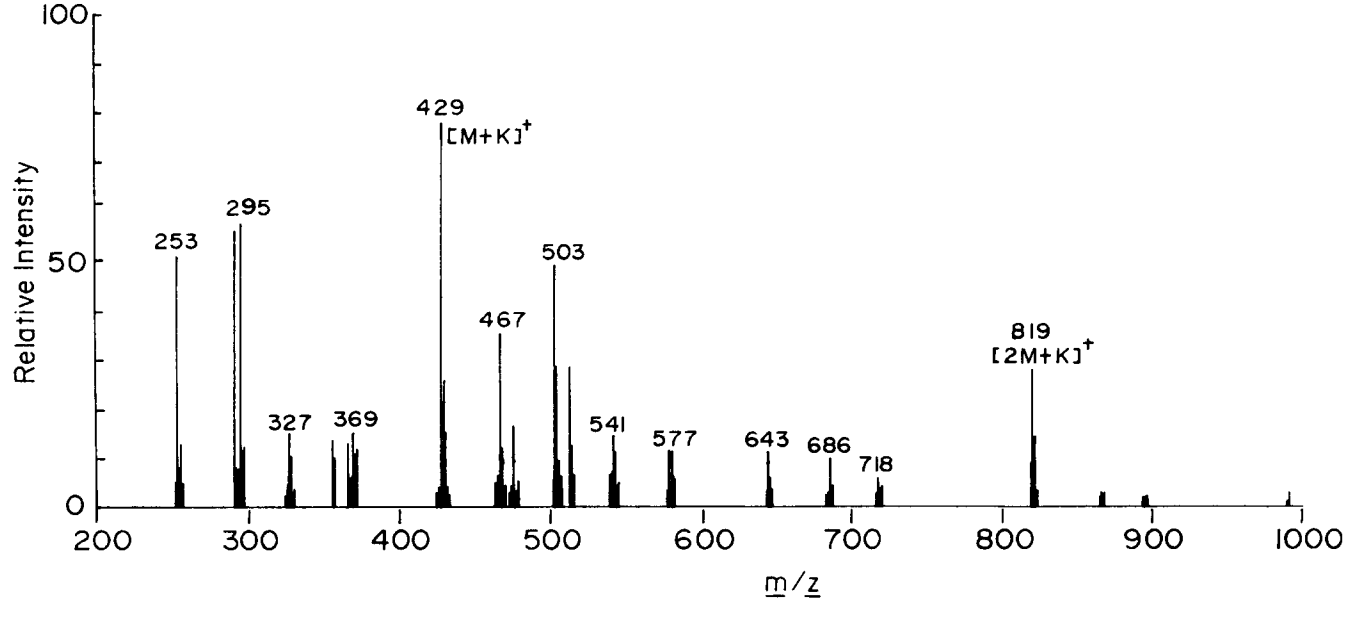

Figure 2 KCl-added positive-ion FAB mass spectrum of 7α-hydroxy-3-oxo-5β-cholanoic acid (**7**). Samples were dissolved in DMSO-d_6 to which KCl was added such that the sample-to-salt ratio was approximately 1 : 3.

Table 1 Different substrates and their transformed products with yields

Metabolites	Identification	Yield (%)
2	3α-Methoxy-7α,12α-dihydroxy-5β-cholanoic acid	45
4	7α,12α-Dihydroxy-3-oxochol-4-en-24-oic acid	5
6	3α-Methoxy-7α-hydroxy-5β-cholanoic acid	40
7	7α-Hydroxy-3-oxo-5β-cholanoic acid	8
8	7α-Hydroxy-3-oxochol-4-en-24-oic acid	0.08
10	3α-Methoxy-7β-hydroxy-5β-cholanoic acid	32
11	7β-Hydroxy-3-oxochol-4-en-24-oic acid	12
12	3-Oxochola-4,6-dien-24-oic acid	36
14	3α-Methoxy-12α-hydroxy-5β-cholanoic acid	31

A young culture of the bacterial strain was grown for 24 h in one of the two fermentation media as detailed in the Experimental section. Substrates **1, 5, 9,** and **13** were then incubated in distilled water for 120 h. The yields of metabolites **2, 4, 6, 7, 8, 10, 11, 12,** and **14** were determined by HPLC.

oic acid **(8)** from its UV spectrum (λ_{max} at 242 nm) and mass spectra (M^+ at 388 and a peak at m/z 124), as well as by comparison of its TLC behavior with that of an authentic sample.[16]

Ursodeoxycholic acid **(9)** afforded three metabolites. The metabolite **(10)** was obtained as a major one and was found to be a mono-methyl derivative of ursodeoxycholic acid **(9)** by its mass and ^{1}H-NMR data. It was finally characterized as 3α-methoxy-7β-hydroxy-5β-cholanoic acid taking into consideration its mass, as well as ^{1}H- and ^{13}C-NMR data. This metabolite **(10)** also appears to be a novel compound.

The minor metabolite **(11)** showed in its mass spectrum the molecular ion at m/z 388 required for $C_{24}H_{36}O_4$. The spectrum displayed the peak at m/z 124 indicating the presence of a Δ^4-3-keto system. The UV and IR data were also commensurate with the presence of this system. Moreover, the ^{1}H- and ^{13}C-NMR data strongly suggested the structure of the metabolite to be 7β-hydroxy-3-oxochol-4-en-24-oic acid. Its mp was comparable with that reported in the literature.[12]

The second major metabolite **(12)** of the substrate **(9)** was found to have the molecular formula $C_{24}H_{34}O_3$ from its MS (M^+ 370) and elemental analysis. A fragment ion at m/z 136 indicated a 4,6-dienone A/B ring structure[13] in **12**. Confirmation of this structural feature was provided by UV, IR, ^{1}H- and ^{13}C-NMR data. Thus, the structure of this metabolite was deduced as 3-oxo-

Table 2 ^{13}C NMR chemical shifts of bile acid metabolites[a]

Carbon	Compound						
	2	**3**	**6**	**7**	**10**	**12**	**14**
1	34.6[b]	34.5	35.8[b]	38.1[b]	35.6	34.2	35.3[b]
2	30.1	30.6[b]	31.1	38.0[b]	31.1[b]	34.4[b]	31.0[c]
3	71.7	70.6	71.7	210.9	71.7	198.1	72.5
4	35.2	34.7	40.0[c]	41.6	40.0	124.1	36.4[b]
5	41.4	40.8	42.7	44.8	42.6	163.2	43.6
6	34.5[b]	31.1[b]	35.5[b]	34.2	35.6	128.0	28.4[c]
7	68.3	73.8	67.5	68.5	67.6	141.2	27.4
8	39.4	37.7	40.1	36.9	40.1	37.9	36.7
9	26.2	28.7	35.8[b]	33.3	33.3	51.0	32.2
10	34.6	34.2	35.5	34.2	35.8	36.2	34.8
11	28.0[c]	26.7[c]	21.0	22.2	21.0	20.9	29.9
12	73.0	75.2	40.8[c]	39.8	40.9	39.8	74.0
13	46.3	44.9	42.5	44.5	42.7	43.6	46.4
14	41.4	43.2	50.7	48.2	50.8	53.6	48.1
15	23.1	22.7	24.0	23.8	24.0	23.9	24.8
16	27.4[c]	27.0[c]	28.4	27.3	28.4	28.3	28.6[c]
17	46.9	47.3	56.2	53.8	56.2	56.1	47.5
18	12.3	12.1	12.0	11.3	12.7	12.0	13.1
19	22.3	21.3	23.2	20.6	23.3	18.6	23.7
20	35.2	34.5	36.2[b]	36.9	36.3	35.6	35.3[b]
21	17.2	17.4	18.5	17.6	18.5	16.3	17.5
22	30.8[d]	30.6[d]	31.4[d]	30.1	31.4[b]	31.7[c]	31.8[d]
23	31.0[d]	31.1[d]	33.3[d]	29.2	31.7[b]	31.8[c]	31.8[d]
24	174.7	174.2	174.2	174.2	174.3	176.2	177.0
$-OCH_3$	51.3	51.3	51.2		51.3		51.9
		171.2					
$-OCOCH_3$		170.1					
		21.3					

[a] Spectra of **2, 3, 6, 10, 12,** and **14** were taken in C_5D_5N and the spectrum of **7** was taken in DMSO-d_6; values are in δ ppm with respect to tetramethylsilane as internal standard.
[b–d] Resonances with the same superscript letters may be reversed.

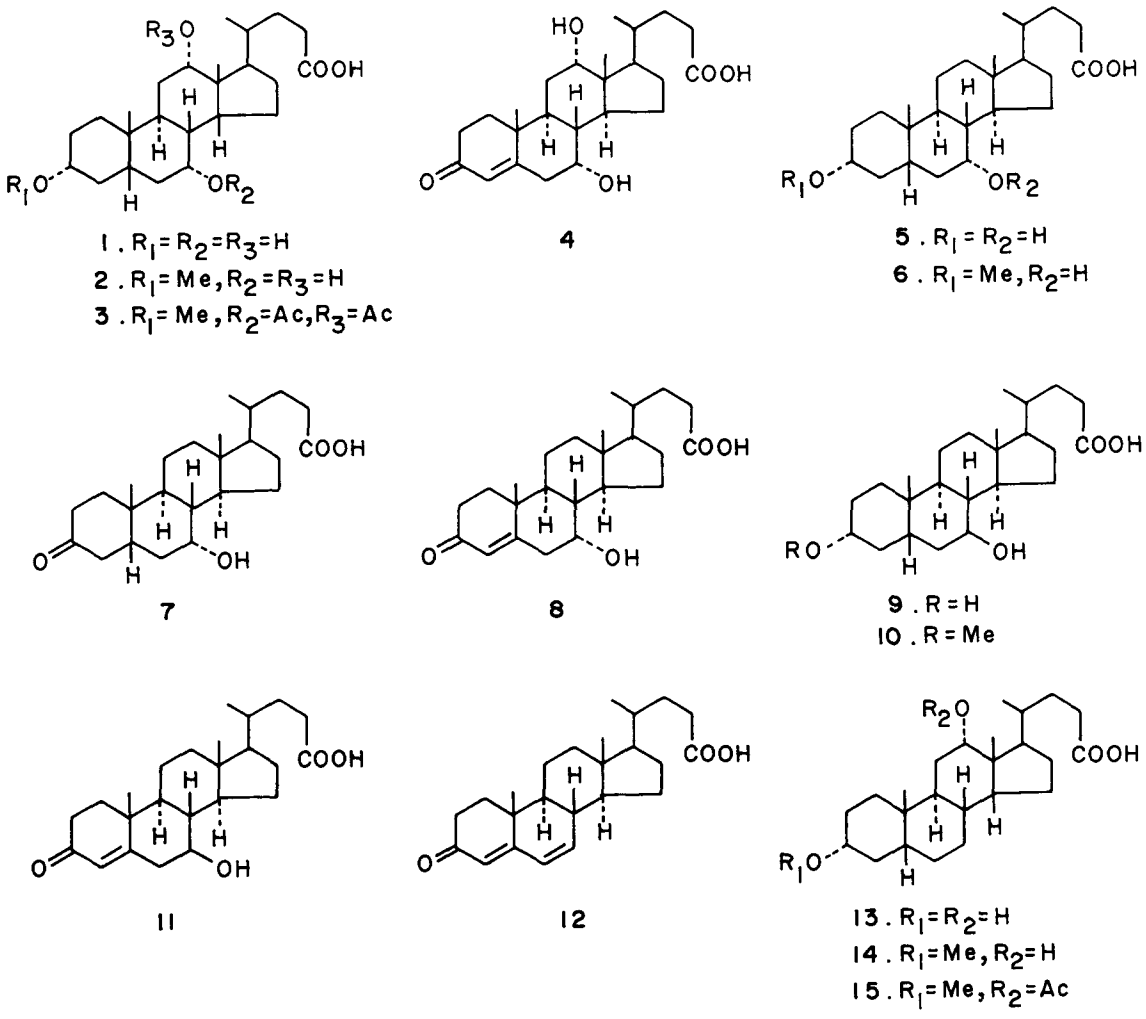

Figure 3 Structures of the substrates and metabolites, as well as their derivatives.

chola-4,6-dien-24-oic acid **(12)**, which has recently been reported as the methyl ester.[17]

When deoxycholic acid **(13)** was used as substrate, the only metabolite **(14)** that could be isolated and in moderate yield showed in its MS the molecular ion at m/z 406 and the fragment ions at m/z 388 and 273, ascribable to (M^+-H_2O) and (M^+–side chain–CH_3OH), respectively. These data indicated that one of the two hydroxy groups of deoxycholic acid **(13)** was methylated to yield the compound **(14)**. The ^1H- and ^{13}C-NMR data of **14** as well as ^1H-NMR data of its acetate **(15)** disclosed the identity of the metabolite to be 3α-methoxy-12α-hydroxy-5β-cholanoic acid **(14)**; to our knowledge this is the first report of the compound. Although no other metabolite of deoxycholic acid was isolated in pure state, UV and IR examination of a minor fraction obtained by preparative TLC showed the formation of 3-keto and Δ^4-3-keto derivatives. The substrates and their transformed products along with the yields are shown in Table 1. The ^{13}C-NMR chemical shifts of various metabolites, which are shown in Table 2, were assigned by the application of known chemical shift rules,[18] off-resonance studies, attached proton test (APT), insensitive nuclei enhancement by polarization transfer (INEPT), distortionless enhancement by polarization transfer (DEPT), and by comparison of ^{13}C data of steroids containing similar carbon atoms.[19] The structures of the substrates, the metabolites, and their derivatives are shown in Figure 3.

The isolation and characterization of various metabolites of four bile acids formed by the strain *A recti* showed that two competitive enzymic reactions (Figure 4), e.g., methylation of the 3α-hydroxy group and dehydrogenation in the A/B rings, were operative in producing the metabolites. In the latter reaction, sequence degradation of the ring system is initated by dehydrogenation of the 3α-hydroxy group. A 7β-dehydratase activity producing the Δ^6 double bond of **12** was also noticeable in the metabolism of ursodeoxycholic acid **(9)**. A comparatively higher yield of the metabolite **(12)** indicated that the 7β-dehydratase produces inhibition in the ring degradation sequence.

The possibility of the involvement of methanol in

the formation of the methylated metabolites is excluded because methanol was not used at any stage of the fermentation experiments or during isolation. Thus, the selective methylation, which is understandable from the point of view of differential activity of hydroxyl groups of bile acids,[20,21] was presumed to be enzymic, involving a methyl transferase designated as bile acid-*O*-methyl transferase (BOMT). This BOMT was found to be S-adenosyl-*L*-methionine (AdoMet) dependent, because addition of adenosyl-*L*-homocysteine (AdoHcy) a product inhibitor of AdoMet-dependent methyl transferase,[22,23] significantly decreased the formation of these metabolites (Table 3). In recent years, AdoHcy hydrolase has emerged as a specific target for the design of potential chemotherapeutic agents.[24,25] This approach has been prompted by recognition of the important role that this enzyme plays in regulating biological methylation reactions, i.e., modulating the intracellular AdoHcy/AdoMet ratio. Inhibition of AdoHcy hydrolase in intact cellular systems results in the accumulation of AdoHcy, which inhibits AdoMet-dependent methylation reactions. The selective methylation of 3α-hydroxy groups of bile acids by this BOMT generated by *A recti* which is an intestinal microorganism, is expected to be associated with the regulation of bile acid metabolism, an important physiological phenomenon. This methylation process obviously inhibits the dehydrogenation of the 3α-hydroxy group forming 3-keto derivatives, which is an obligatory prelude to the events leading to degradation of the ring system. A detailed study on the regulation of this enzymic methylation may lead to the development of potential new drug entities. Moreover, this process of methylation has potential in preparative bile acid chemistry.

Table 3 Effect of AdoHcy on methylation of 3α-hydroxy groups of bile acids[a]

Substrate (100 mg)	AdoHcy added (mg)	Product isolated (mg)
Cholic acid	None	**2**, 45
	19.2	**2**, 23.4
Chenodeoxycholic acid	None	**6**, 40
	19.2	**6**, 20.7
Ursodeoxycholic acid	None	**10**, 32
	19.2	**10**, 18.2
Deoxycholic acid	None	**15**, 31
	19.2	**15**, 17.0

[a] Cholic acid (**1**), chenodeoxycholic acid (**5**), ursodeoxycholic acid (**9**), and deoxycholic acid (**13**); their corresponding 3-methylated derivatives are **2**, **6**, **10**, and **15**.

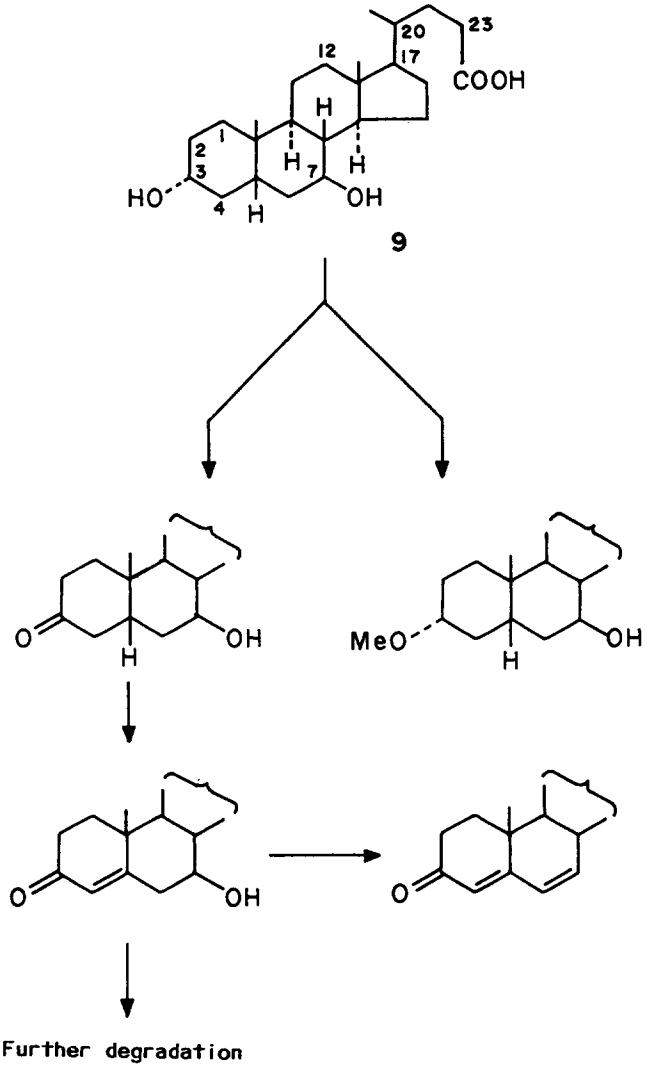

Figure 4 Postulated pathway for the metabolism of bile acids (represented by that of ursodeoxycholic acid) by *Alcaligenes recti*.

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