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# Biotransformation of Bile Acids by Pathogenic Actinomycetes *Nocardia otitidiscaviarum* and *Amycolatopsis* sp. Strains

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**Abstract** Three sterol-type compounds (compounds **4**, **5** and **6**) were isolated from culture broth of pathogenic *Nocardia otitidiscaviarum* IFM 0988 and *Amycolatopsis* sp. IFM 0703 strains which were isolated from Japanese patients. The structures of the compounds were determined by NMR and mass spectrometric analyses. The structural studies indicated that compound **4** is a biotransformation product from cholic acid derivative in a nutrient culture medium constituent by a reductase-type enzyme, and the remaining two compounds **5** and **6** are also biotransformation ones by oxidase-type enzymes.

**Keywords** *Nocardia otitidiscaviarum*, *Amycolatopsis* sp., biotransformation, cholic acid

Pathogenic actinomycete strains such as *Nocardia brasiliensis*, *N. asteroides* and *N. otitidiscaviarum* invade the human host by specific mechanisms of infections and metabolize the host's cellular constituents [1, 2]. However, little information is available regarding the metabolism of special compounds by such pathogens [1, 2]. *Nocardia* species produce various bioactive secondary metabolites, some of which have various antifungal, antibacterial, and immunosuppressive activities [3, 4]. Our ongoing search for metabolites of *Nocardia* and related actinomycete species has revealed that most media used in our experiments, such as nutrient broth (Difco Laboratories, Detroit, USA), contain cholic acid and its derivatives: cholic acid (**1**), taurocholic acid (**2**), and glycocholic acid (**3**) (Fig. 1). Furthermore, our screening studies of new metabolites of the cultured *Nocardia* and *Amycolatopsis* strains using a nutrient medium suggested the presence of additional cholic acid related compounds, implying microbial conversion of cholic acid by pathogenic *Nocardia*. We isolated such compounds and designated them as compound **4** from *N. otitidiscaviarum* IFM 0988 strain, and compounds **5** and **6** from *Amycolatopsis* sp. IFM 0703 strain (Fig. 1). Subsequently, we elucidated their structures using physicochemical methods such as NMR and MS. Our preliminary structural studies suggested that compound **4** was produced by enzymes such as reductases of *N. otitidiscaviarum* IFM 0988; compounds **5** and **6** are also produced by an oxidase type enzyme of *Amycolatopsis* sp. IFM 0703. Such a hypothesis was also supported by the fact that sterol-type compounds have never been reported as procaryotic metabolites. This paper describes the isolation and structural elucidation of compounds **4**, **5** and **6** as new biotransformation products from pathogenic *N. otitidiscaviarum* IFM 0988 and *Amycolatopsis* sp. IFM 0703, respectively.

## Fermentation and Isolation

The seed broth was prepared by inoculating mycelial elements of *N. otitidiscaviarum* IFM 0988 grown on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, USA) in 10 ml of brain heart infusion broth (BHI, Difco Laboratories) with 2% glucose in a 50 ml Erlenmeyer

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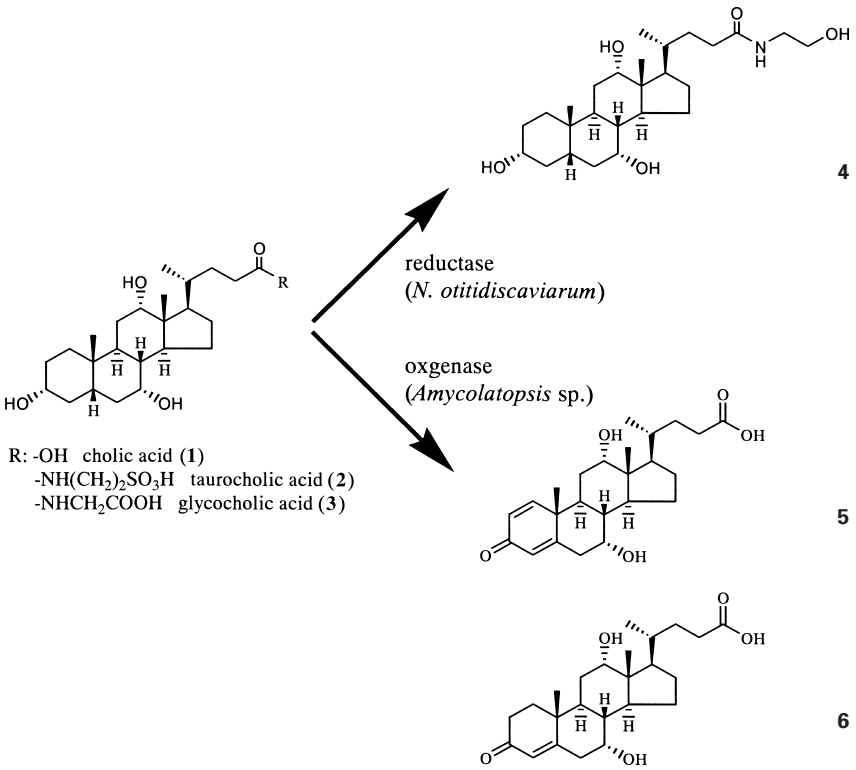


**Fig. 1** Possible biotransformation routes of bile acids [cholic acid (**1**), taurocholic acid (**2**), and glycocholic acid (**3**)] by *Nocardia otitidiscaviarum* IFM 0988 and *Amycolatopsis* sp. IFM 0703.

shake flask. The culture was incubated on a rotary shaker at 250 rpm for 4 days. We transferred 10% of inoculum to a 500 ml Erlenmeyer flask containing 150 ml of the production medium (two times concentrated nutrient broth medium, Difco Laboratories) with 2% glycerol and 0.05% antifoam. We adjusted the medium to pH 7.4; the culture was incubated on a rotary shaker at 250 rpm for 6 days. After incubation, an equal volume of MeOH was added to the culture broth, which was then incubated for another 3 hours to kill the microorganisms. Cholic acid spots were visualized on silica gel TLC plates by spraying a 5% ethanolic solution of molybdophosphoric acid, followed by heating at 120°C for 5 minutes. Thereafter, the broth was filtered and evaporated under vacuum to one-third of its original volume. The filtrate was applied to a Diaion HP-20 column (3×30 cm; Mitsubishi Chemical Corp.) and washed with distilled water (100 ml). The fractions were eluted with 100 ml of $CH_3OH$ and then evaporated to dryness. The cholic acid fractions were extracted with 50 ml of a 1 : 1 volume mixture of BuOH and water. Then the solvent layer was evaporated to dryness. The dried fractions were chromatographed on a silica gel column (3×30 cm) and eluted with acetone/$CH_3OH$ (7 : 1). Subsequently, the fractions were rechromatographed on silica gel using an elution solvent mixture of AcOEt/*i*-PrOH/$H_2O$ (4 : 2 : 1, upper phase). Compound **4** was obtained through further purification by preparative TLC using the same solvent system. From 2-liter cultures, 2 mg of compound **4** was obtained.

The fraction containing sterol-type compounds from *Amycolatopsis* sp. IFM 0703 strain was applied to Diaion HP-20 column (Mitsubishi Chemical Corp.) and washed with distilled water and 50% aq. $CH_3OH$. Elution with $CH_3OH$ yielded a fraction containing cholic-acid related compounds. This fraction was rechromatographed on a silica gel column (2.5×60 cm) with $CHCl_3$/$CH_3OH$/$H_2O$ (65 : 15 : 5, lower phase). The fraction that included compound **5** was further purified by gel filtration chromatography (Toyopearl HW-40; TosoHaas) to yield compound **5** (25.9 mg). The fraction including compound **6** was further rechromatographed on a silica gel column (1.5×40 cm) and eluted with AcOEt/*i*-PrOH/$H_2O$ (4 : 2 : 1, upper phase). It was then purified by gel filtration chromatography to yield compound **6** (2.3 mg).

**Detection of Bile Acids by LC/MS**

Our preliminary studies suggested the presence of sterol-type compounds in the culture broths of *N. otitidiscaviarum*

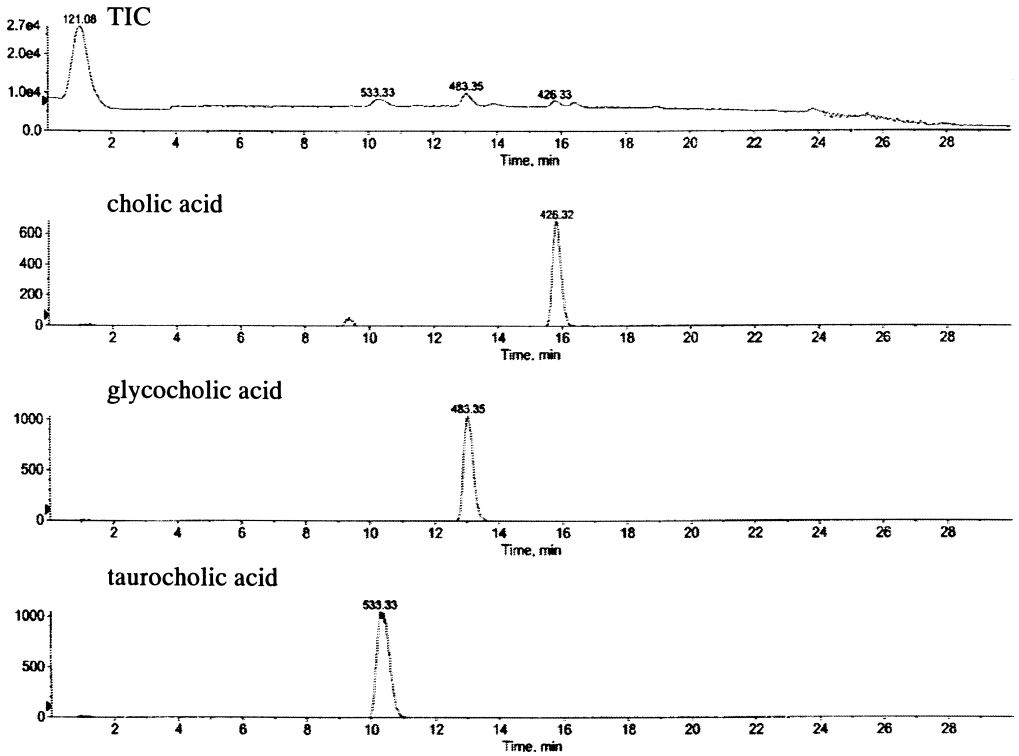


**Fig. 2** Detection by LC/MS of bile acids in the nutrient medium (Difco Laboratories). LC conditions for EIS-TOF LC/MS: column: TSK gel super ODS (100×2.0 mm i.d.), mobile phase; $CH_3OH/H_2O$ containing 0.1% formic acid, gradient rate; $CH_3OH$ 40 →100% (30 minutes), linear, flow rate: 0.2 ml/minute, splitting ratio; 1/40.

IFM 0988 and *Amycolatopsis* sp. IFM 0703. However, such sterol-type compounds have never been reported as procaryotic metabolites in bacteria such as *Nocardia* and *Amycolatopsis* species. This information indicated that sterol-type compounds may exist in the nutrient broth without inoculation of the bacterial culture, prompting us to seek and thereby detect sterol-type compounds in the non-inoculated nutrient broth. LC conditions for ESI-TOF (electrospray-time of flight) LC/MS (API QSTAR Pulsar-I; MDS Sciex) were the following: column; TSK gel super ODS (100×2.0 mm, i.d.), mobile phase; $CH_3OH/H_2O$ containing 0.1% formic acid, gradient rate $CH_3OH$ 40%→100% (30 minutes), linear, flow rate; 0.2 ml/minute, splitting ratio, 1/40. Using this LC/MS detection method, three sterol-type compounds (**1**, **2** and **3**) were detected (cholic acid (**1**): 14.4 minutes, *m/z* 426 $[M+NH_4]^+$, glycocholic acid (**2**): 13.3 minutes, *m/z* 483 $[M+NH_4]^+$, and taurocholic acid (**3**): 10.1 minutes, *m/z* 533 $[M+NH_4]^+$) (Fig. 2). Their presence was also confirmed by comparison with authentic respective reference samples. These data showed clearly that **1**, **2** and **3** are included in the non-cultured nutrient medium such as nutrient broth (Difco Laboratories).

**Structure Elucidation**

Compound **4** was isolated from the culture broth of *N. otitidiscaviarum* IFM 0988 and compounds **5** and **6** were isolated from the culture broth of *Amycolatopsis* sp. IFM 0703, when grown on media containing cholic acid (**1**), taurocholic acid (**2**), and glycocholic acid (**3**) as trace constituents. Structures of compounds **4**, **5** and **6** were elucidated by MS and 1D/2D NMR spectroscopy. The IR spectrum of compound **4** (film, satellite FTIR; Mattson Instruments) showed absorbance at 2940 (C–H), and 3397 $cm^{-1}$ (OH). The HRESI-MS (95XL; Finnigan MAT GmbH) of compound **4** displayed the sodiated ion at *m/z* 474.32510 ($[M+Na]^+$, calcd. 474.3195) suggesting $C_{26}H_{45}NO_5$ as its chemical formula. Structures of compounds **5** and **6** were elucidated using FAB mass spectrometry (JMS-700 MS-station; JEOL) and 1D/2D NMR spectroscopy. Positive ion FAB mass spectra showed the protonated molecule $[M+H]^+$ at *m/z* 403 for compound **5** and at *m/z* 405 for compound **6**. The molecular formulae of **5** and **6** were established to be $C_{24}H_{34}O_5$ and $C_{24}H_{36}O_5$ on the basis of HR-FAB mass measurements (*m/z* 403.2496 $[M+H]^+$ $\Delta+1.2$ mmu) and (*m/z* 405.2621 $[M+H]^+$ $\Delta-2.0$ mmu), respectively. Thus, their chemical compositions suggested the presence of eight double bonds

**Table 1** Assignment of $^{1}H$ and $^{13}C$ NMR spectra of **4**, **5** and **6** (in $CDCl_3$, 500 MHz, chemical shifts ($\delta$) in ppm, coupling constants in Hz, s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, br: broad, multiplicity of carbons in parentheses)

| | Compound **4** | | | Compound **5** | | | Compound **6** | | |
|---|---|---|---|---|---|---|---|---|---|
| Position | $^{13}C$ (ppm) | $^{1}H$ (ppm) | (mult; *J*, Hz) | $^{13}C$ (ppm) | $^{1}H$ (ppm) | (mult; *J*, Hz) | $^{13}C$ (ppm) | $^{1}H$ (ppm) | (mult; *J*, Hz) |
| 1 | 34.9 | 1.79 | (dd; 4.1; 7.1) | 159.2 | 7.25 | (d; 9.6) | — | — | |
| 2 | 30.4 | 1.81 | | 126.9 | 6.20 | (d; 9.0) | — | — | |
| 3 | 66.2 | 3.55 | (dd; 7.1, 7.1,4.60 (OH) br) | 188.3 | | | 201.9 | | |
| 4 | 34.9 | 1.80 | | 127.6 | 6.08 | (s) | 126.7 | 5.73 | (s) |
| 5 | 41.5 | 2.15 | | 170.1 | | | 172.9 | | |
| 6 | 33.4 | 1.10 | | 42.2 | 2.47 | (dd; 13.6, 2.9) | 42.2 | 2.39 | (dd; 15.0, 3.0) |
| | | | | | 2.76 | (dd; 13.7, 1.7) | | 2.65 | (dd; 11.7, 2.4) |
| 7 | 71.0 | 3.65 | (7.1, 1.0, 4.60 (OH) br) | 70.4 | 4.01 | | 69.2 | 3.92 | |
| 8 | 40.4 | 1.20 | | 41.2 | 1.75 | | 41.4 | 1.67 | |
| 9 | 30.9 | 1.23 | | 39.7 | 1.97 | | 43.2 | 1.97 | |
| 10 | 44.3 | — | | 44.9 | | | 39.4 | | |
| 11 | 35.3 | 1.30, 1.42 | | 31.3 | 1.85, 1.98 | | 24.1 | 1.76, 1.89 | |
| 12 | 71.4 | 3.80 | (4.6 (OH) br) | 73.3 | 4.00 | | 173.4 | 3.99 | |
| 13 | 46.1 | — | | 47.7 | | | 147.5 | | |
| 14 | 46.8 | 1.80 | | 43.2 | 1.92 | | 140.1 | 1.92 | |
| 15 | 26.8 | 1.15 | | 24.2 | 1.22, 1.77 | | — | — | |
| 16 | 27.8 | 1.11 | | 28.5 | 1.35, 1.91 | | — | — | |
| 17 | 46.1 | 1.80 | | 48.0 | 1.84 | | 48.9 | 1.85 | |
| 18 | 12.3 | 0.59 | (s) | 13.0 | 0.78 | (s) | 13.0 | 0.76 | (s) |
| 19 | 22.6 | 0.80 | (s) | 18.7 | 1.25 | (s) | 17.3 | 1.21 | (s) |
| 20 | 35.1 | 1.38 | | 36.8 | 1.43 | | 37.3 | 1.41 | |
| 21 | 18.2 | 0.93 | (d, 7.5 ) | 17.5 | 1.01 | (d; 6.2) | 17.8 | 1.01 | (d; 6.6) |
| 22 | 31.7 | 1.31 | | 32.6 | 1.33, 1.79 | | 36.3 | 1.78, 2.02 | |
| 23 | 32.5 | 1.98, 2.05 | | 32.5 | 2.20, 2.34 | | 34.7 | 2.29, 2.47 | |
| 24 | 172.7 | — | | 178.2 | | | 178.2 | | |
| 1′ | 49.8 | 3.22 | (d,t, 7.5, 6.50) | | | | | | |
| 2′ | 60.0 | 3.32 | (t, 6.5, 4.77 (OH) t,br) | | | | | | |

or rings in **5** and seven in **6**.

The chemical structures of compounds **4**, **5** and **6** (Fig. 1) were elucidated based on NMR spectra ($^{1}H$, $^{13}C$, DEPT, HSQC, HMBC, NOESY). Table 1 lists the assignments of proton and carbon signals.

The $^{13}C$ NMR spectrum of compound **4** showed 26 carbon signals. Among them were one carbonyl and four oxygen-bonded carbons. One of the latter showed triplet multiplicity. Moreover, the DEPT spectrum attested to the presence of two quaternary carbons in the molecule. The $^{13}C$ NMR spectra of compounds **5** and **6** showed 24 carbon signals, among which were two carbonyl and two oxygen-bonded carbons. Presence of a carboxyl group in compound **5** was confirmed by converting it to the corresponding methyl ester with trimethylsilyldiazomethane. The proton NMR spectra showed many overlapping signals in a characteristic sterol skeleton. Details of the C-17 side chain and the tetracyclic ring system were unveiled by the $^{1}H,^{1}H$ coupling pattern, as suggested by the $^{1}H,^{1}H$-COSY spectra. For full structural assignment the H,C long-range heteronuclear coupled NMR spectra (HMBC) and NOESY spectra were essential. The $^{2}J_{H,C}$ and $^{3}J_{H,C}$ couplings of the methyl protons (C-18, C-19, C-21) and protons of the oxymethine groups with neighboring carbons were particularly helpful. Relative stereochemistry at the bridge positions of the ring system was assigned based on the NOESY spectra.

Compounds **4** and **5** therefore appear as new natural products that belong to the family of cholestane-type sterols and compound **6** is already known as a biotransformational product [5]. These sterols owe their occurrence in the culture broth of *N. otitidiscaviarum* IFM 0988 and *Amycolatopsis* sp. IFM 0703 to the biotransformation of sterol-type nutrient constituents such

as cholic (**1**), taurocholic (**2**), and glycocholic acids (**3**). We consider these compounds to be produced from **1**, **2** and **3** by enzymes in the *Nocardia* strains used. A reductase-type enzyme produced compound **4**; compounds **5** and **6** were produced by oxidase-type enzymes.

We have reported the microbial inactivation of fusidic acid by *N. brasiliensis*. Our data showed that this inactivation is a species-specific phenomenon in this bacterium [6]. We also reported unique inactivation mechanisms of pathogenic *Nocardia* against clinically useful antibiotics such as rifampicin and erythromycin [7, 8]. The present biotransformation of sterol-type compounds is commonly observed in pathogenic *Nocardia* species (data not shown), but our preliminary study indicates that other *Nocardia* species have a different transformation mechanism against bile acids (**1**, **2** and **3**). The genus *Amycolatopsis* is originally separated from genus *Nocardia*. Therefore, *Amycolatopsis* is related closely to the genus *Nocardia* in its physiological and biochemical characteristics as well as phylogenetic position. Moreover, its pathogenicity potentials have been confirmed [9]. Therefore, further detailed studies regarding microbial transformation of sterol-type compounds by pathogenic *Nocardia* and *Amycolatopsis* strains are of interest because both genus strains can cause opportunistic infections in immunocompromised patients.

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