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NOTE

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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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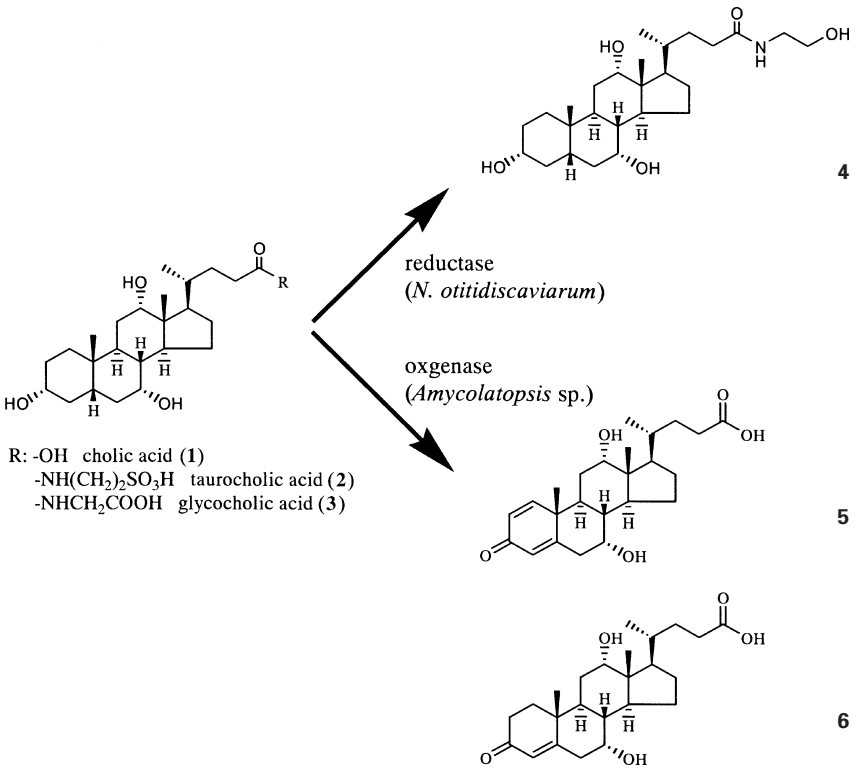

Fig. 1 Possible biotransformation routes of bile acids [cholic acid (**1**), taurocholic acid (**2**), and glycocholic acid (**3**)] by *Nocardia otitidiscaviarum* IFM 0988 and *Amycolatopsis* sp. IFM 0703.

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*

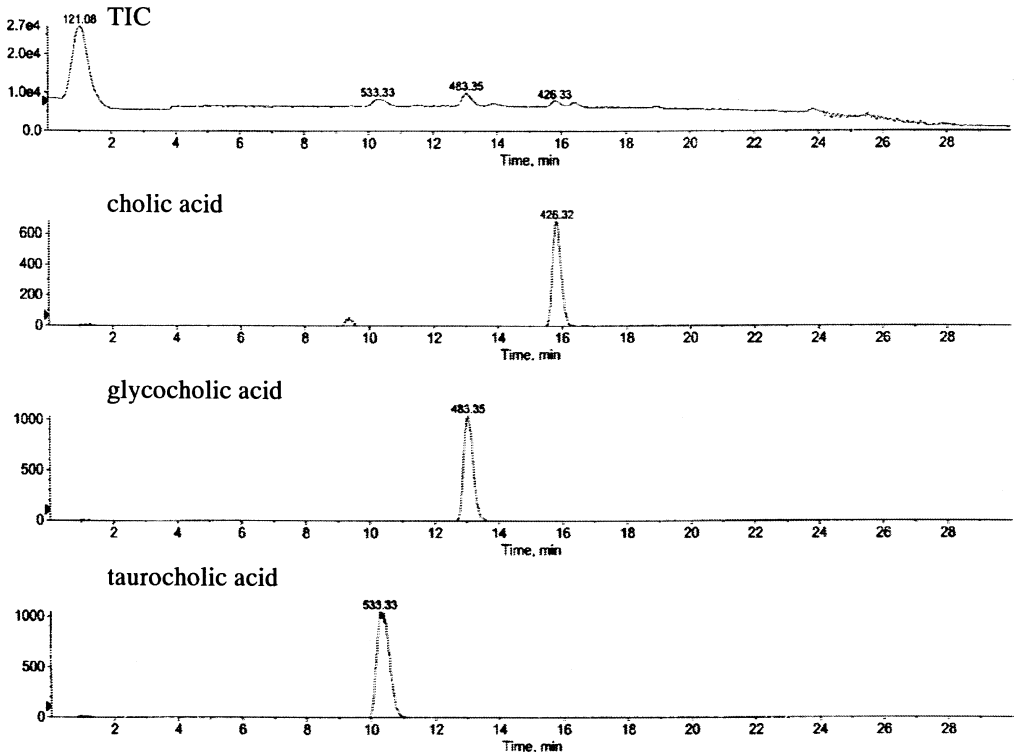

Fig. 2 Detection by LC/MS of bile acids in the nutrient medium (Difco Laboratories). LC conditions for EIS-TOF LC/MS: column: TSK gel super ODS (100×2.0 mm i.d.), mobile phase; CH_3OH/H_2O containing 0.1% formic acid, gradient rate; CH_3OH 40 →100% (30 minutes), linear, flow rate: 0.2 ml/minute, splitting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