

Microbial Conversion of EM574 and EM523, Gastrointestinal Motor Stimulating Agents

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EM574 exerts gastrointestinal motor stimulating (GMS) activity even after being converted to its metabolites P1 and P2 in dogs. These metabolites were isolated from dog liver using a series of chromatographic procedures. Their structures were determined to be the 15- and 14-hydroxyl derivatives of EM574, respectively, by spectral analysis.

Large scale preparation by microbial transformation was investigated for further evaluation of the metabolites, because the amounts obtained by oxidation with dog liver homogenate were limited. Three strains of actinomycetes, *Amycolatopsis tolypophorus* IFO 13151, *Dactylosporangium variesporum* IFO 14104 and *Nocardia capreola* IFO 12847, were found to have the aiming oxidative potency. HPLC analysis of the crude extracts from these three cultures showed that the bioactive metabolites, EM574 P1 and P2 were produced. They were isolated from the culture broth with the other bioactive products EM574 P3 and P4. These bioactive products were prepared by large scale cultivation. EM574 P3 and P4 showed GMS activity comparable to that of EM574 P1 and P2.

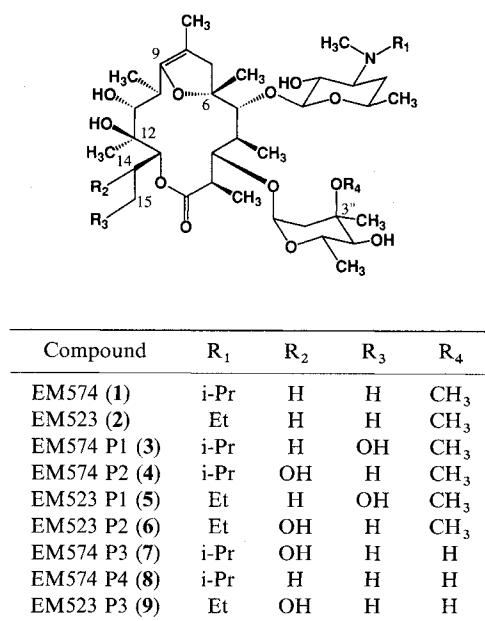
The structures of EM574 P3 and P4 were elucidated by spectral analysis and found to be the 3'-*O*-demethyl derivatives of EM574 P2 and EM574, respectively. Moreover, the absolute configuration at the C14 position of P2 was determined to be R by spectral analysis of the 6-membered cyclic carbonate of EM574 P2.

Erythromycin (EM) has been recognized to have prokinetic activities in addition to antibacterial activities. Its action resembles that of a gastrointestinal hormone, motilin.^{1~4)} Recent findings have provided evidence that EM is a motilin receptor agonist.^{5~7)} Accordingly, these evidences shed light on EM derivatives as promising new prokinetic drugs. Investigations on EM derivatives developed two motilides, EM574 (**1**) and EM523 (**2**), which showed stronger gastrointestinal motor stimulating (GMS) activity than EM A without antibacterial activities.^{8,9)} However, the plasma levels of the agents were very low upon oral or intraduodenal administration in dogs and all of proposed metabolites did not have enough activity to explain the strong activity. These observations suggested that unknown "bioactive metabo-

lites" might be responsible for the GMS activity.

Two bioactive metabolites, P1 and P2, were isolated from dog liver by chromatographic procedures as detected by contractile activity using an isolated rabbit duodenum. Their structures were determined to be the 15- and 14-hydroxyl derivatives of the parent compounds, respectively, by spectral analysis. Preparation of the metabolites was accomplished by oxidation with dog liver homogenates, but only limited quantities were obtained.¹⁰⁾ For pharmaceutical and pharmacokinetic studies and further chemical characterization, much larger amounts of the metabolites were necessary. Chemical modification to prepare the 15- and 14-hydroxyl derivatives seemed to be next to impossible without using partial synthetic methods. Thus, we

Fig. 1. Structures of EM574, EM523 and their metabolites.



examined microbial conversion to produce these metabolites more efficiently. A part of this paper has been orally reported.¹¹⁾

Results

Preparation of the Metabolites P1 and P2 by Microbial Conversion

Eleven strains of actinomycetes stored at Institute for Fermentation, Osaka were selected and tested for their potency to consume **1**. They were cultured at 28°C for 24 hours. Lactobionate of **1** (1L, final conc. 100 µg/ml) was then added followed by incubation for another 24 hours. The supernatant was extracted with ethyl acetate to give a crude extract. The remaining **1** in the extract was estimated by HPLC analysis (ODS, 37% acetonitrile-0.02 M phosphate buffer; pH 4.0=solvent A) as listed in Table 1. The results indicated that **1** was consumed by three strains of actinomycetes, *Amycolatopsis tolypophorus* IFO 13151, *Dactylosporangium variesporum* IFO 14104 and *Nocardia capreola* IFO 12847. The presence of EM574 P1 (**3**) or P2 (**4**) was determined by HPLC (ODS, 32% acetonitrile-0.02 M phosphate buffer; pH 4.0=solvent B) of the crude extracts derived from these three strains. *A. tolypophorus* and *D. variesporum* were found to produce **4**, and *N. capreola* was found to produce both **3** and **4**.

In the case of *N. capreola*, the optimal incubation period was found to be 4~6 hours after the addition of

Table 1. Metabolism of EM574 by actinomycetes.

Strain	IFO No.	pH	Growth	Residual rate (%)
<i>Actinoplanes azureus</i>	13993	6.8	±	119.6
<i>Actinoplanes caeruleus</i>	13939	7.3	++	97.0
<i>Amycolatopsis mediterranei</i>	13415	8.5	+++	61.8
<i>Amycolatopsis tolypophorus</i>	13151	8.1	+	0.0
<i>Dactylosporangium variesporum</i>	14104	8.6	+++	2.9
<i>Nocardia capreola</i>	12847	8.1	+++	0.0
<i>Saccharothrix australiensis</i>	14444	8.8	+++	53.9
<i>Streptomyces griseobrunneus</i>	12775	8.4	+++	48.0
<i>Streptomyces lactamdurans</i>	13305	8.6	+++	78.4
<i>Streptomyces nigrescens</i>	12894	5.8	+++	90.2
<i>Streptomyces violaceoruber</i>	12826	7.0	++	48.0

1. The seed culture was transferred to the media and incubated at 28°C for 24 hours. The culture broth thus obtained was mixed with 1L followed by incubation for 6 hours. The broth was purified according to the procedure¹⁰⁾ for the isolation of **3** and **4** from the liver homogenate to give a crude powder. This was subjected to silica gel chromatography to afford crude powders of **3** and **4**, respectively. These were purified by preparative HPLC (ODS, methanol or acetonitrile in phosphate buffer; pH 4.0) to give **3** and **4**, respectively. As a result, **3** and **4** (total yield 9.5%) were isolated from the culture broth of *N. capreola*.

In the case of *D. variesporum*, the optimal incubation period was found to be 24 hours after the addition of **1**. The cultivation and isolation were carried out in the same procedure as in the case of *N. capreola*. From the broth, **4** (yield 3.0%) was isolated. In the case of *A. tolypophorus*, the optimal incubation period was found to be 20 hours after the addition of **1**. From the broth of *A. tolypophorus*, **4** (yield 20%) was isolated.

The Unique Metabolites in Microbial Conversion

Prokaryotes, such as bacteria and actinomycetes, possibly have a metabolic system different from that of eukaryotes, such as fungi and mammals. For example, in the oxidative metabolism by cytochrome P450, the former demands NAD,¹²⁾ the latter requires NADP. Therefore, actinomycetes might convert **1** to unique metabolites of their own. The metabolic products of the three strains were subjected to preparative HPLC (ODS, solvent B). All the fractions were subjected to the contraction assay.¹⁰⁾

The products of *A. tolypophorus* gave no bioactive fraction but that of **4**. On the other hand, the extracts from *D. variesporum* contained bioactive fractions at

Table 2. Microbial conversion of EM574/523 by actinomycetes.

Broth (liter)	Substrate (g)	P1 (mg) (%)	P2 (mg) (%)	P3 (mg) (%)	P4 (mg) (%)
<i>Nocardia capreola</i>					
3	0.5 (1)	4 (0.8)	43 (8.4)	—	—
120	12.0 (1)	105 (0.9)	338 (2.8)	76 (0.6)	—
120	12.0 (1)	149 (1.2)	1.4 g (11.4)	95 (0.8)	—
1200	120.0 (1)	1.0 g (0.8)	13.2 g (10.8)	3.3 g (2.7)	—
120	12.0 (2)	15 (0.1)	812 (6.8)	200 (1.7)	—
120	12.0 (2)	14 (0.1)	4.1 g (34.0)	194 (1.6)	—
<i>Dactylosporangium variesporum</i>					
10	1.0 (1)	—	42 (4.1)	46 (4.6)	49 (5.0)
120	12.0 (1)	—	104 (0.8)	129 (1.1)	133 (1.1)
120	12.0 (2)	—	—	383 (3.2)	—
<i>Amycolatopsis tolypophorus</i>					
3	0.5 (1)	—	102 (20.0)	—	—

—: Not detected.

Table 3. ¹³C NMR data for EM574, EM523 and their metabolites.

C-No.	1	2	3	4	5	6	7	8	9
1	178.4	178.4	178.6	177.5	178.8	177.5	177.2	178.2	177.2
2	44.8	44.8	44.2	44.5	44.2	44.6	44.7	45.0	44.6
3	76.5	76.3	75.9	75.9	75.0	76.3	76.4	76.5	76.3
4	43.4	43.5	43.7	43.6	43.7	43.5	43.5	43.3	43.6
5	80.2	80.1	79.8	79.9	79.8	80.1	80.1	80.4	79.9
6	85.6	85.6	85.6	85.8	85.7	85.8	86.1	86.0	86.1
7	42.7	42.6	42.5	42.6	42.5	42.6	42.8	42.8	42.7
8	101.6	101.6	101.7	101.9	101.7	102.0	102.3	101.9	102.2
9	151.7	151.7	151.8	151.3	151.8	151.2	151.1	151.5	151.2
10	30.5	30.5	30.6	30.5	30.6	30.4	30.5	30.5	30.6
11	70.2	70.0	70.1	69.5	70.2	69.9	69.8	70.2	69.7
12	75.4	75.4	74.9	77.6	74.9	75.4	ND	75.4	77.2
13	78.3	78.3	74.6	75.9	74.6	72.1	75.9	78.4	76.0
14	21.1	21.1	31.3	66.4	31.3	66.4	66.3	21.0	66.4
15	10.9	10.9	59.2	20.0	59.2	20.0	20.0	10.9	19.9
2-Me	13.5	13.4	12.7	13.1	12.8	13.3	13.2	13.6	13.0
4-Me	8.7	8.7	8.8	8.6	8.8	8.6	8.4	8.5	8.4
6-Me	26.3	26.3	26.5	26.2	26.5	26.2	25.9	26.0	25.9
8-Me	12.0	12.0	11.9	12.0	11.9	12.0	12.0	11.9	12.0
10-Me	15.0	15.1	14.9	15.3	14.8	15.2	15.5	15.2	15.6
12-Me	16.2	16.1	16.4	16.7	16.4	16.6	16.6	16.1	16.8
1'	103.1	103.1	103.0	103.2	103.0	103.1	104.0	104.1	104.0
2'	70.3	70.4	70.1	70.2	70.3	70.3	70.1	70.2	70.3
3'	63.1	65.2	63.1	63.1	65.1	65.2	63.1	63.1	65.0
4'	33.2	29.6	33.1	33.1	29.6	29.7	33.2	33.2	29.5
5'	68.9	69.0	68.9	69.0	69.0	69.0	69.4	69.4	69.6
6'	21.4	21.4	21.4	21.4	21.4	21.3	21.4	21.4	21.4
N-Me	30.9	36.3	30.9	30.9	36.3	36.3	30.9	30.6	36.2
N-R	52.7	47.6	52.8	52.8	47.7	47.7	53.0	52.8	47.5
	21.1	13.9	21.1	21.1	13.8	14.4	20.6	20.7	13.9
	20.6	—	20.5	20.5	—	—	20.4	20.5	—
1''	94.6	94.6	94.5	94.5	94.5	94.6	96.3	96.6	96.2
2''	34.7	34.6	34.6	34.6	34.6	34.6	40.2	40.3	40.2
3''	73.1	73.0	73.1	73.1	73.1	73.0	ND	69.8	69.8
4''	78.2	78.1	78.0	78.0	78.0	78.1	77.2	77.2	76.3
5''	65.7	65.7	65.9	65.8	65.9	65.8	66.8	66.8	66.9
6''	18.2	18.2	18.1	18.1	18.1	18.2	18.1	18.2	18.1
7''	21.6	21.6	21.6	21.6	21.6	21.6	22.7	25.6	25.6
O-Me	49.5	49.6	49.6	49.6	49.6	49.6	—	—	—

ND: Not detected.

δ ppm, CDCl₃, 75 MHz

retention times (Rt) 5~6 and 13~14 minutes along with that of **4** (Rt 7~8 minutes). The conversion mixture of *N. capreola* contained a bioactive fraction at Rt 5~6 minutes accompanied by those of **3** and **4**. On analytical HPLC (ODS, solvent B) with detection by UV absorption at 214 nm, the unknown bioactive fractions corresponded to the peaks at Rt 5.3 and 14.0 minutes. They were designated EM574 P3 (**7**) and P4 (**8**), and purified by silica gel and preparative HPLC (ODS) from the crude extracts of the two strains. From the culture broth of *D. variesporum* (mentioned above) **7** and **8** were obtained as white powders along with **4** (total yield 9.0%). In that of *N. capreola* (mentioned above) **7** was detected, along with **3** and **4**. Substrate **2** was converted by *N. capreola* and analyzed in the same manner as described above. EM523 P1 (**5**), EM523 P2 (**6**) and EM523 P3 (**9**) were detected in the crude extract by HPLC analysis.

Preparation of the Metabolites in Large Scale Cultivation

The production process described above was carried out on a large scale with a 200-liter fermenter. In this case, the substrate was supplied as an 80% ethanol solution. The crude products were purified by combinations of silica gel and HP-20 chromatography and preparative HPLC (ODS). As a result, from the culture broth of *D. variesporum*, **4**, **7** and **8** (total yield ca. 3%) were obtained. From that of *N. capreola*, **3**, **4** and **7** were obtained in a ca. 4% yield totally. From that of *N. capreola* fed on **2**, **5**, **6** and **9** were obtained in a ca. 8.5% yield totally. However, in the culture mixture of *D. variesporum* fed on **2**, **6** and **9** were produced but not EM523 P4. Yields of the products in these cultures are summarized in Table 2.

The Structures of P3 and P4

In the NMR spectra, **7** and **8** showed no 3''-O-methyl signal. Along with this, the chemical shifts of C1'', C2'' and C3'' changed by 2~6 ppm in the ¹³C NMR spectra. Apart from these changes, the other signals of **7** and **8** were nearly the same as those of **4** and **1**, respectively. The UV and IR spectra of **7** and **8** were very similar to those of **1**. The molecular ion peaks (MH⁺) of **7** and **8** were detected at 730 and 746, respectively, in the SI-MS, indicating that they decreased from those of **4** and **1** by 14 mass units (corresponding to one CH₂). Therefore, **7** and **8** were concluded to be the 3''-O-demethyl derivatives of **4** and **1**, respectively. The structure of **9** was determined to be the 3''-O-demethyl derivative of **6** in the same way. The structures are shown in Fig. 1, and the ¹³C NMR spectral data for all the bioactive metabolites

and the substrates are listed in Table 3.

Absolute Configuration at the C14 Position of P2

The structures of P1, P2, P3 and P4 were determined except for the absolute configuration at the C14 position of P2 and P3. Even with the intensive NMR spectroscopic analysis of **4**, the configuration at the position remained uncertain. Being overlapped by other signals, the 14-H signal could not be distinguished from the others in the NOE experiment. Therefore, **4** was converted to corresponding 6-membered cyclic carbonate.

Erythromycin A has three (C11, C2' and C4'') secondary and two (C6 and C12) tertiary hydroxyl (OH) groups. In the reaction with acid anhydride, 2'-OH reacts first, 4''-OH second and then 11-OH follows.¹³⁾ The tertiary OH groups are expected to react very slowly. Compound **4** has a secondary OH at C14 instead of 6-OH. In the reaction of **4** with an excess of 1,1'-carbonyldiimidazole (CDI), 2'- and 4''-OH are expected to be easily carbamated. There are two possibilities that a cyclic carbonate is formed over C11~C12 (5-membered ring) or C12~C14 (6-membered ring), and the remaining OH (14-OH or 11-OH) is to be carbamated. Compound **4** was heated at 80°C in toluene with 5 equivalents of CDI for 18 hours affording the two main products **4a** and **4b**. The physico-chemical (especially NMR) data showed that they have two imidazinocarbonyl groups and one cyclic carbonate group. Both **4a** and **4b** have 4''-carbamate and 2'-OH groups (2'-H, δ 3.17 and 3.16 ppm). The 2'-carbamate seems so unstable as to have been hydrolyzed during the work up. When 4''-OH of **4** was converted to 4''-carbamate **4a** and **4b**, the chemical shifts of C4'' and 4''-H showed downfield shifts from δ_C 78.0 and δ_H 3.06 to δ_C 83.4 and δ_H 4.88 (**4a**) and to δ_C 83.3 and δ_H 4.81 ppm (**4b**). In the ¹H NMR spectra of **4a**, the 11-H was observed at δ 5.30 ppm, showing a 1.88 ppm downfield shift from δ 3.42 ppm, while 14-H was at δ 4.46 ppm showing only a 0.36 ppm downfield shift from δ 4.10 ppm. This indicated that 11-OH was converted to 11-carbamate and the cyclic carbonate was formed over C12 and C14. On the other hand, **4b** showed 14-H at δ 5.51 ppm and 11-H at δ 4.23 ppm indicating that 14-OH was substituted by the carbamate and the carbonate was formed over C11 and C12. The structures of **4a** and **4b** are illustrated in Fig. 2. As for the reactivity in the acylation reaction, the 14-hydroxyl group of **4** seems as reactive as the 11-hydroxyl group.

Concerning the determination of the configuration at C14, information from the NMR data of **4a** was very

Fig. 2. Cyclic carbonylation of EM574 P2 (4) with carbonyldiimidazole (CDI).

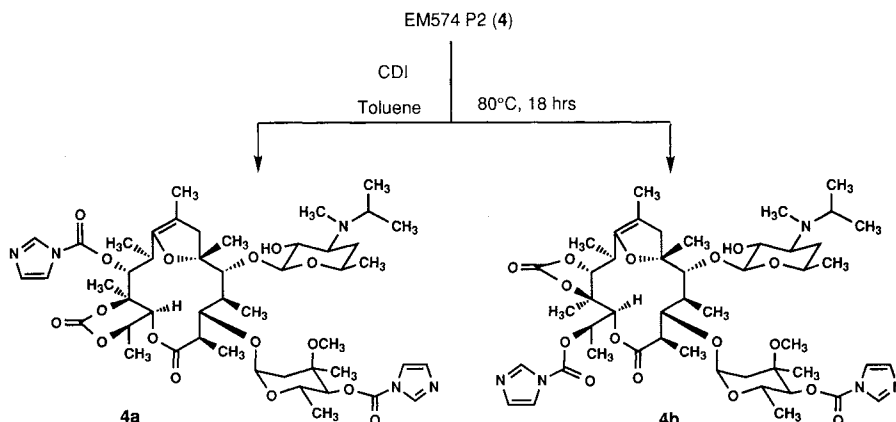


Fig. 3. Determination of the absolute configuration at the C14 position of 4a.

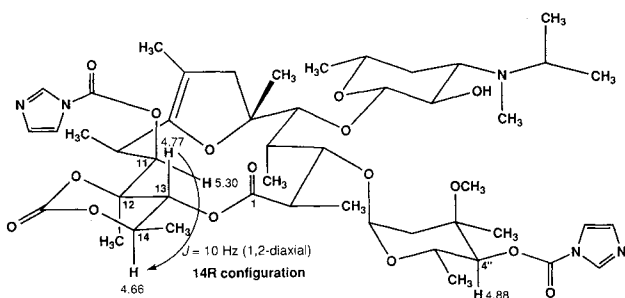


Table 4. Pharmacological activities of EM574, EM523 and their metabolites.

Compound	Contractile activity (EC ₅₀ , nM)	GMS activity (ED ₂₀₀ mg/kg, <i>iv</i>)
EM574 (1)	1.6	0.96
EM574 P1 (3)	2.6	0.38
EM574 P2 (4)	1.3	0.40
EM574 P3 (7)	2.5	0.21
EM574 P4 (8)	1.7	0.28
EM523 (2)	3.6	6.1
EM523 P1 (5)	2.7	4.0
EM523 P2 (6)	3.6	2.1
EM523 P3 (9)	4.8	15.5

helpful. In the ¹H NMR spectrum of 4a, 13-H was observed at δ 4.77 ppm as a doublet ($J=10.1$ Hz) showing a coupling with 14-H (δ 4.66 ppm, double quartet, $J=10.1, 6.3$ Hz). The coupling constant (10.1 Hz) indicated that two protons located in the 1,2-diaxial position on the 6-membered cyclic carbonate ring as shown in Fig. 3. This means the absolute configuration at C14 to be "R".

Biological Activities

The contractile activity¹⁰⁾ in isolated rabbit duodenum (*in vitro*) and GMS activity¹⁰⁾ (*in vivo*) of the obtained products are summarized in Table 3. All the metabolites showed activity comparable to that of the parent compound in the *in vitro* assay. On the other hand, except for 9, they exhibited 2- to 4-fold stronger GMS activity than 1 and 2 *in vivo*.

Discussion

Here we have described the microbial production of 14- and 15-hydroxyl derivatives of EM574 and EM523.

The bioactive metabolites 3 and 4 were detected in the plasma after oral administration of [¹⁴C]-labeled 1 to dogs,¹¹⁾ and therefore, the significance of the metabolites has become of greater interest. The main products, 14-hydroxyl derivatives (4 and 6), were obtained in sufficient amounts for further pharmaceutical investigation, while the yields for the 15-hydroxyl derivatives (3 and 5) were very low. The production utilized the three strains of actinomycetes for giving the metabolites in different yield from one another. It is quite interesting that such oxidative activity is observed in these strains belonging to the rare actinomycetes.

On the other hand, the 3'-*O*-demethyl derivatives, P3 and P4, were isolated as unique microbial products. As the substrate for microbial conversion, 1 and 2 show some different characteristics. EM523 gave only a small amount of P1 and didn't give P4 even on a cultivation scale of 200 liters, while EM574 gave P1~P4 to some extent. EM574 and EM523 differ only in the 3'-*N*-alkyl group. This difference causes a slight change in the C14/15 position, but the alkyl group should affect the oxidation at the 3'-*O*-position. It may be speculated that the alkyl group occupies the space near to the 3'-*O*-methyl group as shown in Fig. 3.

The absolute configuration at the C14 position of EM574 P2 was determined to be "R" by converting the cyclic carbonate **4a**. As the metabolites of erythromycin derivatives in humans, 14-hydroxy-6-*O*-methylerythromycin A¹⁴⁾ has been reported. The stereochemistry at the C14 position of the major metabolite (M-5) was elucidated to be "R", and that of the minor one (M-6) was found to be "S". In the microbial (*Mucor* species) transformation of 6-*O*-methylerythromycin derivatives,¹⁵⁾ only (14*R*)-14-hydroxy-6-*O*-methylerythromycin A and B were isolated. These results partially agree with our results. We couldn't isolate "S"-derivatives having GMS activity in the microbial conversion, while the conversion using dog liver homogenates afforded the 14- and 15-hydroxyl derivatives in nearly the same yields.¹⁰⁾ This might mean that microorganisms, including actinomycetes and *Mucor* oxidize erythromycin-type antibiotics stereo- and regio-selectively at the C14 position.

Experimental

Measurement of Physico-chemical Data

The UV spectra were measured at 23~28°C in methanol. The IR spectra were measured in KBr pellets using a Horiba FT-200 instrument. The SI-MS spectra were measured on a Hitachi M-80A instrument using glycerol as the matrix. The NMR spectra were measured in deuteriochloroform. The δ -values in the NMR spectra were recorded in ppm downfield from tetramethylsilane (TMS) as the external standard using a Bruker AC-300 spectrometer.

Preparation of Seed Cultures of Actinomycetes

Each actinomycete grown on a yeast extract-malt extract agar slant was inoculated into a 200-ml Erlenmeyer flask containing a medium consisting of glucose 1%, tryptone 1% and yeast extracts 0.6% (40 ml, pH 7.0) and shaken on a reciprocal shaker at 28°C for 24 hours. Five-milliliter portions of the resulting culture fluid were dispensed into test tubes and frozen at -80°C as the working stock. The stock was thawed at room temperature, and a 1-ml portion was transferred to a 200-ml Erlenmeyer flask containing 40 ml of the medium and shaken on a reciprocal shaker at 28°C for 24 hours to obtain the seed culture fluid.

Preparation of **3** and **4** by *N. capreola* (flask scale)

One-milliliter portions of the seed culture of *N. capreola* IFO 12847 were transferred to a 200-ml Erlenmeyer flask containing 40 ml of the medium and shaken on a reciprocal shaker at 28°C for 24 hours. Compound 1L (6 mg/ml in water, 1.0 ml) was added to each flask and incubated at 28°C for 6 hours. The obtained culture broth (3.0 liters) was centrifuged at 4°C and 8000 rpm for 10 minutes. The supernatant (2.8 liters) was adjusted to pH 7.0 and subjected to Diaion HP-20 (20~50 mesh, 300 ml, Mitsubishi Chem. Ind., Japan)

column chromatography. The desired fraction was washed with 50% MeOH aq. (1.5 liters) and eluted with 80% MeOH in 5 mM HCl (900 ml). The eluate was neutralized, concentrated to ca. 200 ml, adjusted to pH 8.5 and extracted with EtOAc (100 ml) three times. The EtOAc layers were combined, washed with water (100 ml), dried over anhydrous sodium sulfate and evaporated to give a crude powder (203 mg). This powder (200 mg) was subjected to silica gel column chromatography (E. Merck, silica gel 60, Art. 7734, 5.0 g) eluting with MeOH-CHCl₃. The fractions eluted with MeOH-CHCl₃ (2:98~5:95 and 5:95) were independently collected and concentrated to give crude powders of **4** (111 mg) and **3** (45 mg), respectively. The former was subjected to preparative HPLC (column: YMC-Pack, D-ODS-5, YMC, Japan, mobile phase: 55% MeOH-0.02 M phosphate buffer; pH 4.0). The latter was subjected to prep. HPLC (28% acetonitrile-0.02 M phosphate buffer). The fractions of **4** and **3** were independently collected, adjusted to pH 7.4, concentrated to about 20 ml, adjusted to pH 8.5 and extracted with EtOAc (12 ml) three times. The organic layers were combined, washed with saline, dried over anhydrous sodium sulfate and evaporated to give powders of **4** (43 mg) and **3** (4.3 mg), respectively (total yield, 9.5%).

UV: **4**, λ_{\max} 207 nm (ϵ 7700), IR: **4**, 3460, 2970, 2940, 1740, 1640, 1460, 1380, 1330, 1270, 1170, 1100, 1060, 1010, 940 (cm⁻¹). SI-MS: m/z 760 (MH⁺), 602 (MH⁺-cladinose), Anal Calcd. for C₃₉H₆₉NO₁₃·0.5H₂O: C 60.92, H 9.17, N 1.82. Found: C 61.06, H 9.37, N 1.81.

Preparation of **4** by *A. tolypophorus* (flask scale)

One-milliliter portions of the seed culture of *A. tolypophorus* IFO 13151 were transferred to a 200-ml Erlenmeyer flask containing 40 ml of the medium and shaken on a reciprocal shaker at 28°C for 48 hours. Compound 1L (12 mg/ml in water, 1.0 ml) was added to each flask and incubated at 28°C for 20 hours. The obtained culture broth (3.0 liters) was treated in the same manner as described in the case of *N. capreola* to give a crude powder (415 mg). This powder (400 mg) was subjected to silica gel column chromatography (10 g) eluting with MeOH-CHCl₃. The fractions eluted with MeOH-CHCl₃ (2:98) were collected and concentrated to give a crude powder of **4** (218 mg). This powder was subjected to prep. HPLC (D-ODS-5, 57% MeOH-0.02 M phosphate buffer; pH 4.0). The fractions of **4** were collected, adjusted to pH 7.4, concentrated to about 20 ml, adjusted to pH 8.5 and extracted with EtOAc (12 ml) three times. The organic layers were combined, washed with saline, dried over anhydrous sodium sulfate and evaporated to give a powder of **4** (102 mg, yield 20%).

Preparation of **4**, **7** and **8** by *D. variesporum* (flask scale)

One-milliliter portions of the seed culture of *D. variesporum* IFO 14104 were transferred to a 200-ml

Erlenmeyer flask containing 40 ml of the medium and shaken on a reciprocal shaker at 28°C for 24 hours. Compound **1L** (4 mg/ml in water, 1.0 ml) was added to each flask and incubated at 28°C for 24 hours. The obtained culture broth (9.0 liters) was treated in the same manner as described in the case of *N. capreola* to give a crude powder (896 mg). This powder (895 mg) was subjected to silica gel (20 g) column chromatography eluting with MeOH-CHCl₃ to give a mixture of **4**, **7** and **8** (468 mg). This mixture was subjected to prep. HPLC (D-ODS-5, solvent B) to give powders of **4** (43 mg), **8** (49 mg) and crude **7**, respectively. The crude powder of **7** was purified by prep. HPLC (25% acetonitrile-0.02 M phosphate buffer, pH 4.0) to give a powder of **7** (46 mg, total yield, 9.5%).

UV: **7**, λ_{\max} 206 nm (ϵ 8500), IR: 3460, 2970, 2940, 1740, 1640, 1460, 1380, 1330, 1270, 1170, 1110, 1060, 1010, 940 (cm⁻¹). SI-MS: m/z 746 (MH⁺), 602 (MH⁺ - cladinose), Anal Calcd. for C₃₈H₆₇NO₁₃ · 0.5H₂O: C 60.46, H 9.08, N 1.86. Found: C 60.48, H 9.21, N 1.84. **8**; UV: λ_{\max} 206 nm (ϵ 7400), IR: 3490, 2970, 2940, 1730, 1640, 1460, 1380, 1330, 1270, 1200, 1160, 1110, 1060, 1020 cm⁻¹, SI-MS: m/z 730 (MH⁺), 586 (MH⁺ - cladinose), Anal Calcd. for C₃₈H₆₇NO₁₂ · 0.5H₂O: C 61.77, H 9.27, N 1.90. Found: C 61.86, H 9.43, N 1.90.

Preparation of **6** and **9** by *N. capreola* (flask scale)

One-milliliter portions of the seed culture of *N. capreola* IFO 12847 were transferred to a 200-ml Erlenmeyer flask containing 40 ml of the medium and shaken on a reciprocal shaker at 28°C for 24 hours. Compound **2** (8 mg/ml in 80% EtOH, 0.5 ml) was added to the flask and incubated at 28°C for 4 hours. The obtained culture broth (40 ml) was treated in the same manner as described in the case of *N. capreola* to give a crude powder (4.0 mg). This powder was analyzed by HPLC (ODS; A-312, 25% acetonitrile-0.02 M phosphate buffer; pH 4.0) and determined to contain **5** (46 mg) and **6** (2.0 mg). In another experiment, **9** was detected (7 µg/ml) in the culture broth obtained in the same manner.

Preparation of Seed Cultures for Large Scale Cultivation

The frozen stock was thawed at room temperature, and a 5-ml portion was transferred to a 2-liter Sakaguchi flask containing 500 ml of the medium. The mixture was shaken on a reciprocal shaker at 28°C for 24 hours to obtain the seed culture fluid.

Preparation of **4**, **7** and **8** by *D. variesporum* (fermenter scale)

The above-mentioned seed culture fluid of *D. variesporum* (1.5 liters) was transferred to a 200-liter stainless fermenter containing 120 liters of the above-mentioned medium. The mixture was cultivated at 28°C with aeration of 120 liter/minute and agitation of 180 rpm

for 24 hours. A solution of **1** (16 mg/ml in 80% EtOH, 750 ml) was added to the tank and then incubated for 24 hours. The obtained culture broth (112 liters) was filtered with a filter aid Radiolite (3 kg, Showa Kagaku Kougyou, Japan). The filtrate (108 liters) was adjusted to pH 7.0 and subjected to HP-20 (10 liters) column chromatography. The desired fraction was eluted with 80% MeOH in 5 mM HCl (30 liters). The eluate was neutralized, concentrated to ca. 7 liters, adjusted to pH 8.5 and extracted with EtOAc (5 liters) twice. The EtOAc layers were combined, washed with water (5 liters), dried over anhydrous sodium sulfate and evaporated to give a crude powder (4.3 g). This powder was subjected to silica gel column chromatography (silica gel 60, 100 g) eluting with MeOH-CHCl₃. The fractions eluted with MeOH-CHCl₃ (2:98 and 2:98 ~ 5:95) were independently collected and concentrated to give powder-I (972 mg) and powder-II (392 mg), respectively. Powder-I was purified by Sephadex LH-20 (500 ml, Pharmacia, Sweden) column chromatography eluting with MeOH to give powder-III (506 mg). Powder-II and -III were each subjected to prep. HPLC (ODS, YMC-Pack S-363 I-15) eluting with 30% acetonitrile-0.02 M phosphate buffer (pH 4.0). The fractions containing **4** and a mixture of **7** and **8** were independently collected, adjusted to pH 7.4, concentrated, adjusted to pH 8.5 and extracted with EtOAc three times. The organic layers were combined, washed with saline, dried over anhydrous sodium sulfate and evaporated to give powders of **4** (104 mg) and a mixture of **7** and **8**. The mixture was subjected to prep. HPLC (D-ODS-5) eluting with 25% acetonitrile-0.02 M phosphate buffer (pH 4.0). The fractions containing **7** and **8** were independently treated in the same manner to give powders of **7** (129 mg) and **8** (133 mg), respectively (total yield, ca. 3%).

Preparation of **3**, **4** and **7** by *N. capreola* (fermenter scale)

The seed culture fluid of *N. capreola* was transferred and cultured in the same manner as described in the case of *D. variesporum* for 24 hours. A solution of **1** (16 mg/ml in 80% EtOH, 750 ml) was added to the fermenter and then incubated for 6 hours. The obtained culture broth (120 liters) was treated in a manner similar to that described above to give a crude powder (6.1 g). This powder was subjected to silica gel column chromatography (150 g) eluting with MeOH-CHCl₃. The fractions eluted with MeOH-CHCl₃ (2:98 ~ 5:95 and 5:95) were independently collected and concentrated to give powder-I (2.55 g) and powder-II (1.59 g), respectively. Powder-I and -II were each purified by HP-20 (100 ~ 200 mesh, 100 and 50 ml) column chromatography eluting with 80% MeOH in 5 mM HCl. The eluates were concentrated and extracted with EtOAc at pH 8.5, and the organic layers were dried and evaporated to give powder-Ia (1.58 g) and -IIa (722 mg), respectively. Powder-Ia and -IIa were each subjected to prep. HPLC (ODS, YMC-Pack SH-363-15, S-15) eluting with 30%

and 26% acetonitrile-0.02 M phosphate buffer (pH 4.0) to give crude powders of **3** (300 mg), **4** (534 mg) and **7** (323 mg). These were each subjected to prep. HPLC (D-ODS-5 for **3**, and SH-363-15 for **4**, **7**) eluting with solvent C, 55% and 54% MeOH-0.02 M phosphate buffer (pH 4.0) to give powders of **3** (105 mg), **4** (338 mg) and **7** (76 mg), respectively (total yield, ca. 4%).

3; UV: λ_{\max} 206 nm (ϵ 8500), IR: 3440, 2970, 2940, 1730, 1640, 1450, 1380, 1200, 1170, 1050, 1010 cm^{-1} , SI-MS: m/z 760 (MH^+), 602 (MH^+ - cladinose), Anal Calcd. for $\text{C}_{39}\text{H}_{69}\text{NO}_{13} \cdot 0.5\text{H}_2\text{O}$: C 60.92, H 9.17, N 1.82. Found: C 60.63, H 9.15, N 1.78.

From another cultivation mixture (120 liters) incubated for 4 hours followed by an isolation procedure as described above, powders of **3** (149 mg), **4** (1.40 g) and **7** (95 mg, total yield 13.4%) were obtained.

From another cultivation mixture (1200 liters) incubated for 4 hours followed by an isolation procedure as described above, powders of **3** (1.0 g), **4** (13.2 g) and **7** (3.3 g, total yield 14.3%) were obtained.

Preparation of **5**, **6** and **9** by *N. capreola* (fermenter scale)

The seed culture fluid of *N. capreola* was transferred and cultured in the same manner as described in the case of *D. variesporum* for 24 hours. A solution of **2** (16 mg/ml in 80% EtOH, 750 ml) was added to the fermenter and then incubated for 6 hours. The obtained culture broth (116 liters) was treated in a manner similar to that described above to give a crude powder (9.0 g). This powder was subjected to silica gel column chromatography (200 g) eluting with MeOH- CHCl_3 . The fractions eluted with MeOH- CHCl_3 (2:98 and 5:98) were independently collected and concentrated to give powder-I (2.9 g) and powder-II (1.68 g), respectively. Powder-II was subjected to prep. HPLC (SH-363-15) eluting with 26% acetonitrile-0.02 M phosphate buffer (pH 4.0) to give crude powders of **5** (80 mg) and **9** (783 mg). They were each subjected to prep. HPLC (D-ODS-5 for **5** and SH-363 for **9**) eluting with 25% acetonitrile and 52% MeOH-0.02 M phosphate buffer (pH 4.0) to give powders of **5** (15 mg) and **9** (200 mg), respectively. Powder-I was triturated from MeOH- Et_2O to give a powder of **6** (812 mg, total yield, 8.5%).

5; UV: λ_{\max} 209 nm (ϵ 8900), IR: 3450, 2970, 2940, 1720, 1640, 1450, 1380, 1200, 1170, 1050, 1010 cm^{-1} , SI-MS: m/z 746 (MH^+), 588 (MH^+ - cladinose), Anal Calcd. for $\text{C}_{38}\text{H}_{67}\text{NO}_{13} \cdot 0.5\text{H}_2\text{O}$: C 60.37, H 9.20, N 1.80. Found: C 60.46, H 9.08, N 1.86. **6**; UV: λ_{\max} 207 nm (ϵ 7600), IR: 3440, 2980, 2930, 1740, 1650, 1450, 1380, 1340, 1280, 1170, 1050, 1010, 940 cm^{-1} , SI-MS: m/z 746 (MH^+), 588 (MH^+ - cladinose), Anal Calcd. for $\text{C}_{38}\text{H}_{67}\text{NO}_{13} \cdot \text{H}_2\text{O}$: C 59.74, H 9.10, N 1.83. Found: C 59.83, H 9.14, N 1.73. **9**; UV: λ_{\max} 207 nm (ϵ 7700), IR: 3460, 2980, 2940, 1740, 1650, 1460, 1380, 1330, 1270, 1170, 1110, 1050, 1020, 940 cm^{-1} , SI-MS: m/z 732 (MH^+), 588 (MH^+ - cladinose), Anal Calcd. for $\text{C}_{37}\text{H}_{65}\text{NO}_{13}$: C 60.72, H 8.95, N 1.91. Found: C 60.32,

H 9.01, N 1.97.

From another cultivation mixture (120 liters) incubated for 4 hours followed by an isolation procedure as described above, powders of **5** (14 mg), **6** (4.16 g) and **9** (194 mg, total yield 34.0%) were obtained.

Preparation of **9** by *D. variesporum* (fermenter scale)

From another cultivation mixture (120 liters) incubated for 24 hours and followed by isolation procedure as described above was obtained a powder of **9** (383 mg, yield 3.2%).

Preparation of Cyclic Carbonates **4a** and **4b**

To a stirred solution of **4** (112 mg, 0.148 mmol) in toluene (5.6 ml) was added 1,1'-carbonyldiimidazole (122 mg, 5.0 eq.). The mixture was heated at 80°C for 18 hours. The reaction mixture was diluted with EtOAc (20 ml), washed with water (10 ml) twice and then brine (10 ml), dried over anhydrous sodium sulfate and evaporated to give a powder (160 mg). This was subjected to silica gel chromatography eluting with a mixture of acetone and toluene. The fractions eluted with acetone-toluene (3:7 ~ 4:6) were concentrated and subjected to prep. HPLC (ODS, YMC pack, D-ODS-5) eluting with 45% acetonitrile in phosphate buffer (pH 3.0). The fractions of **4a** were collected, concentrated to ca. 10 ml and extracted at pH 6.5 with EtOAc (10 ml) twice. The organic layers were combined, washed with water (10 ml) twice and then brine (10 ml), dried over anhydrous sodium sulfate and evaporated to give a powder of **4a** (45 mg, yield 31%). The fractions of pure **4b** were treated in the same manner to give a powder of **4b** (31 mg, yield 22%).

4a; SI-MS: m/z 974 (MH^+), 722 (MH^+ - cladinose unit), Anal Calcd. for $\text{C}_{48}\text{H}_{71}\text{N}_5\text{O}_{16} \cdot \text{H}_2\text{O}$: C 58.11, H 7.42, N 7.06. Found: C 58.06, H 7.29, N 6.62. ^{13}C NMR: δ 176.1 (Q), 148.9 (Q), 148.7 (Q), 148.4 (Q), 148.0 (Q), 137.6 (CH), 136.9 (CH), 131.0 (CH), 130.8 (CH), 117.4 (CH), 117.1 (CH), 103.8 (Q), 102.9 (CH), 95.3 (CH), 86.6 (Q), 84.5 (Q), 83.4 (CH), 80.4 (CH), 77.3 (CH), 76.2 (CH), 73.0 (Q), 71.2 (CH), 70.2 (CH), 70.1 (CH), 68.5 (CH), 62.8 (CH), 62.7 (CH), 52.9 (CH), 49.7 (CH₃), 44.4 (CH), 43.8 (CH), 42.4 (CH₂), 35.0 (CH₂), 33.1 (CH₂), 31.1 (CH₃), 30.9 (CH), 25.9 (CH₃), 21.5 (CH₃), 21.4 (CH₃), 21.2 (CH₃), 20.5 (CH₃), 18.9 (CH₃), 17.7 (CH₃), 16.9 (CH₃), 15.2 (CH₃), 13.0 (CH₃), 11.7 (CH₃), 8.8 (CH₃). **4b**; SI-MS: m/z 974 (MH^+), 722 (MH^+ - cladinose unit), Anal Calcd. for $\text{C}_{48}\text{H}_{71}\text{N}_5\text{O}_{16} \cdot \text{H}_2\text{O}$: C 58.11, H 7.42, N 7.06. Found: C 58.12, H 7.23, N 6.82. ^{13}C NMR: δ 176.1 (Q), 152.4 (Q), 148.7 (Q), 147.9 (Q), 147.2 (Q), 137.3 (CH), 136.9 (CH), 130.9 (CH), 130.8 (CH), 117.3 (CH), 117.1 (CH), 105.3 (Q), 103.1 (CH), 95.2 (CH), 87.7 (Q), 84.3 (Q), 83.3 (CH), 82.8 (CH), 79.9 (CH), 76.6 (CH), 74.0 (CH), 72.9 (Q), 72.4 (CH), 70.1 (CH), 68.6 (CH), 62.9 (CH), 62.7 (CH), 52.9 (CH), 49.8 (CH₃), 43.6 (CH), 43.5 (CH), 42.0 (CH₂), 35.0 (CH₂), 33.1 (CH₂), 31.7 (CH), 31.1 (CH₃), 25.7 (CH₃), 21.5 (CH₃), 21.3 (CH₃), 21.2 (CH₃), 20.4 (CH₃), 17.8 (CH₃), 16.7 (CH₃),

15.7 (CH₃), 14.7 (CH₃), 12.6 (CH₃), 12.1 (CH₃), 8.6 (CH₃).

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