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ORIGINAL PAPER

Dehydropropylpantothenamide isolated by a co-culture of *Nocardia tenerifensis* IFM 10554^T in the presence of animal cells

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Abstract
A new amide, named dehydropropylpantothenamide (**1**), was obtained by a co-culture of *Nocardia tenerifensis* IFM 10554^T in the presence of the mouse macrophage-like cell line J774.1 in modified Czapek-Dox (mCD) medium. Compound **1** was synthesized from D-pantothenic acid calcium salt in 6 steps. The absolute configuration of natural compound **1** was determined by comparisons of the optical rotation and CD spectra of synthetic **1**. In the present study, a new method for producing secondary metabolites was demonstrated using a "co-culture" in which the genus *Nocardia* was cultured in the presence of an animal cell line.

Keywords Actinomycetes · *Nocardia* · Co-culture · Amide

Introduction

Actinomycetes produce many secondary metabolites with diverse structures and activities [1], and many of these metabolites produce antibiotics including streptomycin [2], erythromycin [3], and kanamycin [4]. Many biosynthesis genes on the genomes of actinomycetes are involved in the production of secondary metabolites; however, a large proportion is considered to be cryptic genes that are not activated to produce secondary metabolites [5, 6]. In order to obtain new secondary metabolites, it is important to activate these cryptic gene clusters. Recent studies have attempted to produce secondary metabolites by stimulating gene expression using several methods such as culture conditions [7], drug stimulation [8], the forced expression of genes [9], and combined cultures [10–12].

Actinomycetes of the genus *Nocardia* are Gram-positive bacteria found in the lungs, skin, brain, and other organs and cause infections in immunocompromised patients. When bacteria of the genus *Nocardia* infect the human body, they are attacked by immune cells such as macrophages. However, the activation of biosynthesis genes by mimicking this phenomenon, namely, an infection state for the isolation of new secondary metabolites, has not yet been investigated. Therefore, we herein examined a new method for producing secondary metabolites using a "co-culture" in which the genus *Nocardia* is cultured in the presence of an animal cell line.

We describe the isolation and structural elucidation of a new compound (**1**) isolated in modified Czapek-Dox (mCD) [13] medium by a co-culture of *Nocardia tenerifensis* IFM 10554^T in the presence of J774.1. Five known steroids were also isolated from *N. tenerifensis* IFM 10554^T, and the results obtained revealed that two out of the five compounds were obtained only under co-culture conditions in DMEM with 10% FBS medium.

Results and discussion

Strains for the co-culture study were initially selected from 76 strains belonging to the genus *Nocardia* obtained from the Medical Mycology Research Center, Chiba University.

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Biosynthetic gene clusters were examined in a gene analysis of the genus *Nocardia* with antiSMASH [14]. A phylogenetic tree was prepared with two analytical software packages, Clustal X [15] and MEGA [16], using the nocobactin-related biosynthetic gene [17] group, a pathogenic factor, as an index. Strains were classified into five clades. Based on these clades and the number of biosynthetic gene clusters, six strains (*N. altamirensis* IFM 10819^T, *N. mexicana* IFM 10801^T, *N. tenerifensis* IFM 10554^T, *N. terpenica* IFM 0706^T, *N. mexicana* IFM 10801^T, and *N. vulneris* NBRC 108936^T) were selected.

Bacteria of the genus *Nocardia* receive supplementation with macrophages when they infect the human body [18]. Therefore, a mouse macrophage-like cell line (J774.1) was selected as an animal cell line for co-culturing in order to reconstruct the initial infection state.

Morphological changes in and the cell viability of J774.1 were investigated using a co-culture with the six selected *Nocardia* strains. All six strains caused morphological changes in J774.1 and ultimately led to cell death, suggesting an interaction between the strain and cell line.

Culture conditions for the six selected strains were examined in the presence or absence of J774.1 using a combination of various media mCD, nutrient broth (NB) [19], Waksman [20], Yeast-Malt-Glucose (YMG) [21], DMEM, and DMEM with 10% FBS media], temperatures (28 or 37 °C), air compositions (atmosphere or 5% CO_2), containers (50-mL Erlenmeyer flask or 75-cm^2 cell culture flask), and shaking conditions (static or rotary shaking culture).

LC–MS analyses of the extracts of culture broths obtained under the various conditions described above revealed that multiple peaks were selectively noted for the extract of *N. tenerifensis* IFM 10554^T cultured in the presence of J774.1. Thus, this strain was selected for further study. The ratio of the cell numbers of *N. tenerifensis* IFM 10554^T and J774.1 was also examined under various conditions (1:1, 5:1, 10:1, 50:1, and 100:1) in order to demonstrate that selective LCMS peaks were present for the extracts obtained from the culture at a ratio of 10:1 for mCD medium and 5:1 for DMEM with 10% FBS medium.

A large-scale co-culture (7.3 L) of *N. tenerifensis* IFM 10554^T and J774.1 in mCD medium at a cell number ratio of 10:1 was performed at 28 °C in 175-cm^2 cell culture flasks under static conditions for 2 weeks in atmospheric air. After centrifugation of the culture broth, the supernatant and methanol extract of mycelia were combined and subjected to partition between ethyl acetate (EtOAc) and water. The EtOAc fraction was subjected to fractionation with ODS column chromatography (the MeOH–H_2O system), followed by reversed-phase HPLC separation (50% MeOH) to give a new compound (**1**), named dehydropropylpantothenamide (Fig. 1).

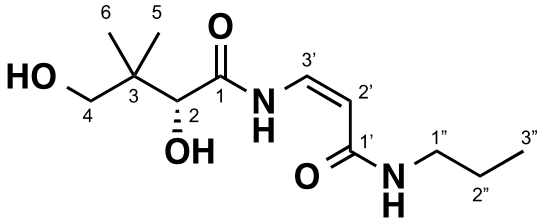


Fig. 1 Structure of **1**

Table 1 ^{1}H-and ^{13}C-NMR chemical shifts for dehydropropylpantothenamide (**1**)

Position	δ_H	δ_C
1		171.8
2	4.18 (d, 4.1)	78.1
3		39.5
4	3.53, 3.60 (d, 11.2)	71.3
5	0.99 (s, 3H)	20.8
6	1.06 (s, 3H)	20.7
1’		167.9
2’	5.01 (d, 9.0)	100.5
3’	7.32 (dd, 9.0, 10.7)	133.7
1”	3.27 (dt, 7.6, 6.2, 2H)	41.0
2”	1.54 (m, 2H)	22.9
3”	0.94 (d, 7.6, 3H)	11.3
2-OH	3.61 (brs)	
4-OH	2.60 (brs)	
1-NH	11.9 (d, 10.7)	
1’-NH	5.42 (brs)	

Measured in CDCl3. 600 MHz (^{1}H), 150 MHz (^{13}C) δ in ppm. J in Hz

Compound (**1**) was revealed to have the molecular formula $C_{12}H_{22}N_2O_4$ by high resolution ESIMS (obsd. *m/z* 539.3080 [2 M + Na]$^+$, calcd for $C_{24}H_{44}N_4O_8Na$, 539. 3057). The ^{1}H-NMR spectrum of **1** measured in $CDCl_3$ showed one *Z*-olefin [δ_H 7.32 (1H) and 5.01 (1H); J = 9.0 Hz], one oxymethine [δ_H 4.18 (1H)], one oxymethylene [δ_H 3.53 (1H), 3.60 (1H)], two other methylene [δ_H 3.27 (2H) and 1.54 (2H)], three methyl [δ_H 1.06 (3H), 0.99 (3H) and 0.94 (3H, J = 7.6 Hz)], two NH [δ_H 11.9 (1H) and 5.42 (1H)], and two OH signals [δ_H 3.61 (1H) and 2.60 (1H)] (Table 1). The ^{13}C-NMR spectrum of **1** measured in $CDCl_3$ showed twelve peaks. The cross-peaks observed in the 2D TOCSY data of **1** suggested the presence of two partial structures, A and B (Fig. 2). The HMBC spectrum of **1** showed correlations from the methylene protons (H_2-1″, δ_H 3.27) of partial structure B to the carbonyl carbon (C-1′, δ_C 167.9), which also correlated with the *Z*-olefinic protons [H-2′ (δ_H 5.01) and H-3′ (δ_H 7.32)] of partial structure A, suggesting that partial structure A was connected to partial structure

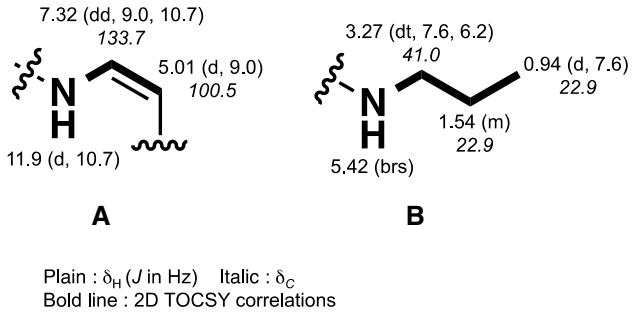


Fig. 2 Partial structures, **a** and **b**, for **1**

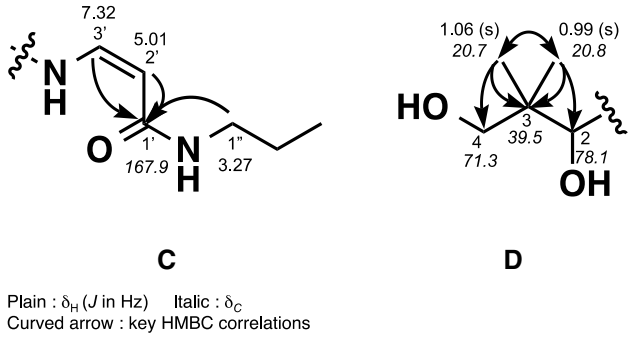
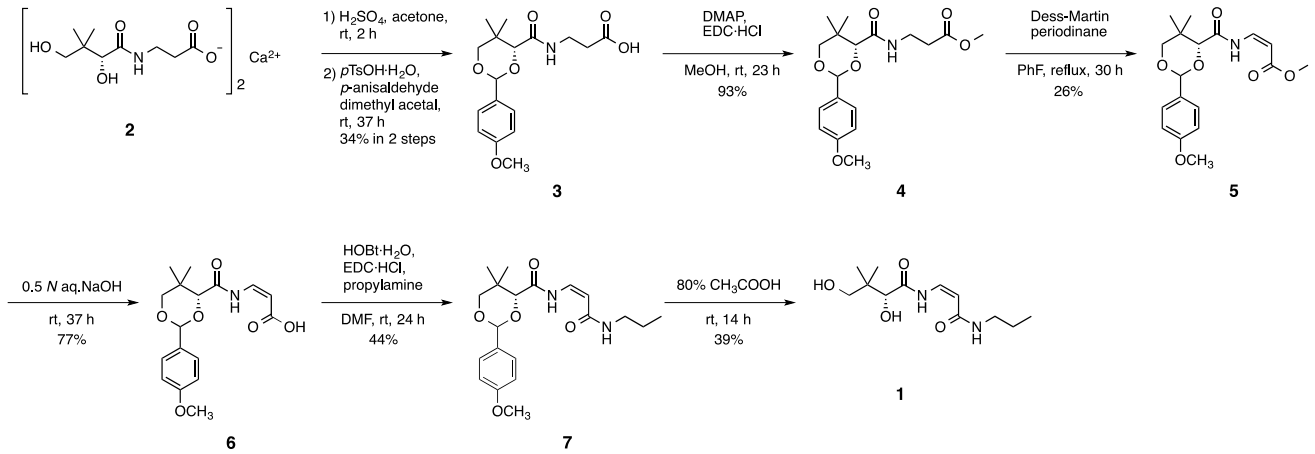


Fig. 3 Partial structures, **c** and **d**, and HMBC correlations for **1**

B through the carbonyl carbon C-1′ to give partial structure C (Fig. 3). The HMBC correlation observed from two methyl protons [H_3-5 (δ_H 0.99) and H_3-6 (δ_H 1.06)] to sp^3 quaternary carbon [C-3 (δ_C 39.5)] and two sp^3 oxygenated carbons [C-2 (δ_C 78.1) and C-4 (δ_C 71.3)] suggested the presence of partial structure D (Fig. 3). The remaining carbonyl group (δ_C 171.8) had to be located at the C-1 position, connecting partial structures C and D, through the process of elimination to construct the complete structure **1**, which was further supported by comparisons of spectral data with those of CJ15, 801 [22] having a related structure. In order to elucidate the absolute stereochemistry of **1**, its synthesis was examined as described in Scheme 1. Two hydroxy groups of D-pantothenic acid calcium salt (**2**) were protected with *p*-anisaldehyde dimethyl acetal to give **3**. After the conversion of **3** to its methyl ester **4**, the treatment of **4** with Dess-Martin periodinane [23] afforded *Z*-olefin (**5**), and the hydrolysis of **5** gave acid **6**. Compound **6** was subjected to amidation with propylamine to give **7**, and deprotection of the acetal group of **7** afforded **1**. The optical rotation and CD spectrum of natural compound **1** corresponded well with those of synthetic **1** (Fig. 4). Therefore, the configuration at position 2 was elucidated as the *R* configuration. An HPLC examination revealed that compound **1** was preferentially produced under co-culture conditions in the presence of J774.1 only, while it was not produced under single culture conditions in the absence of J774.1 (Fig. 5).

A large-scale co-culture (8.0 L) of *N. tenerifensis* IFM 10554^T and J774.1 in DMEM with 10% FBS medium at a cell number ratio of 5:1 was performed in 175-cm^2 cell culture flasks under static conditions at 37 °C for 24 h under 5% CO_2. After centrifugation of the culture broth, the supernatant and methanol extract of mycelia were combined and subjected to partition between EtOAc and water. The EtOAc fraction was treated by reversed-phase HPLC separation (10–100% MeOH gradient) to give five known compounds. They were identified as 1, 4-androstadine-16α,17β-diol-3-one (**8**) [24], 4-androstene-16α,17β-diol-3-one (**9**) [25], 1,4-androstadine-3,17-dione (**10**) [26], 4-androstene-3,17-dione (**11**) [27], and cholesteryl oleate (**12**) [28], based on analyses of NMR and ESIMS data. Comparisons of HPLC data revealed that **8** and **9** were produced only under co-culture conditions in the presence of J774.1 in DMEM with 10% FBS medium (Fig. 6) and were not produced in the absence of J774.1.



Scheme 1 Synthesis of **1**

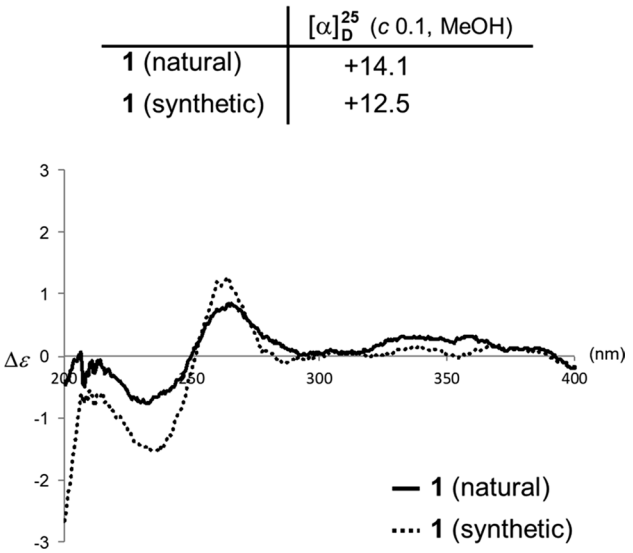


Fig. 4 Comparison of optical rotation and CD spectra of **1** (natural) and **1** (synthetic)

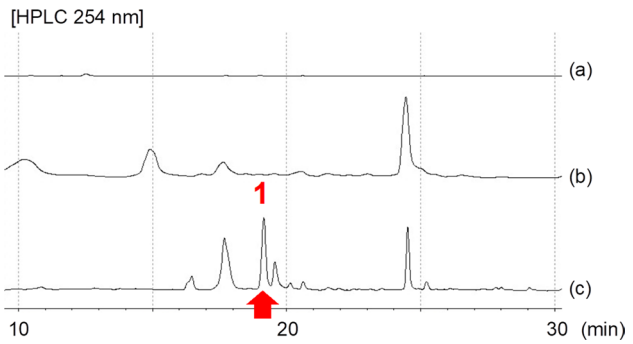


Fig. 5 Comparison of extracts of culture broth in mCD medium. **a** Single culture (J774.1), **b** single culture (*N. tenerifensis*), **c** co-culture (*N. tenerifensis* and J774.1)

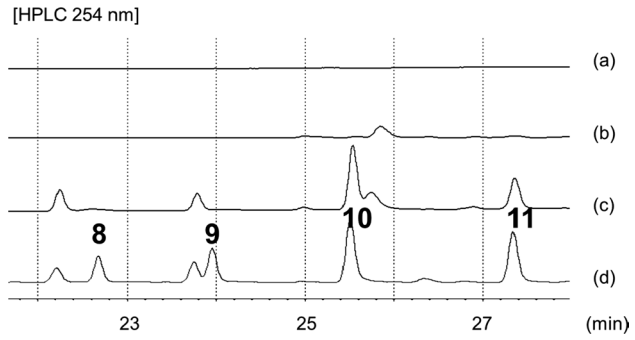


Fig. 6 Comparison of extracts of culture broth in DMEM or DMEM + FBS medium, **a** Single culture (J774.1, DMEM + FBS), **b** single culture (*N. tenerifensis,* DMEM), **c** single culture (*N. tenerifensis*, DMEM + FBS), **d** co-culture (*N. tenerifensis* and J774.1, DMEM + FBS)

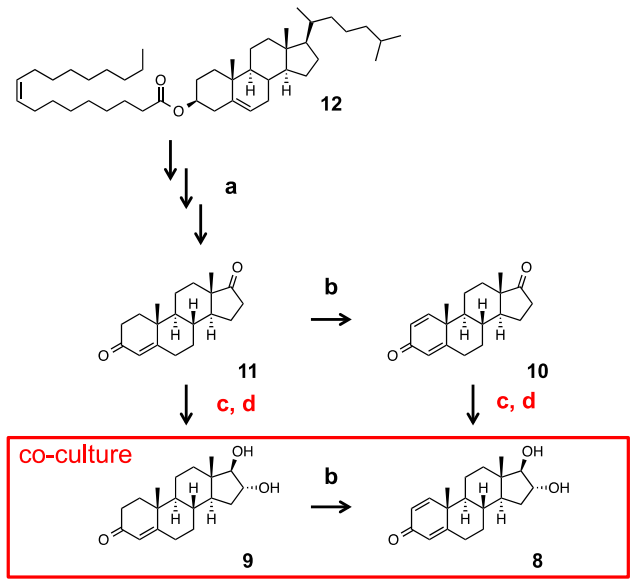


Fig. 7 The presumed biosynthetic pathway. **a** Ketosteroid isomerase (an enzyme involved in reactions from **12** to **11**), **b** 3-Ketosteroid-delta-1-dehydrogenase, **c** 17β-hydroxysteroid dehydrogenase, **d** 16α-hydroxylase

The biosynthetic pathway of this compound group was proposed, as shown in Fig. 7, based on performing a MiGAP analysis [29] and BLAST search [30] on the draft genome of *N. tenerifensis*. Compounds **8**–**11** were proposed to be biosynthesized from **12** by a group of enzymes including ketosteroid isomerase, 3-ketosteroid-delta-1-dehydrogenase, 17β-hydroxysteroid dehydrogenase, and 16α-hydroxylase. The expression levels of the biosynthesis genes in a single culture of *N. tenerifensis* in DMEM medium and those in DMEM with 10% FBS medium were then compared by RNA-seq. Two compounds, **10** and **11** were not produced in DMEM medium, but were produced in DMEM with 10% FBS medium (Fig. 6b and c). By analyzing RNA-seq, the expression of the genes coding two enzymes, ketosteroid isomerase and 3-ketosteroid-delta-1-dehydrogenase, was 4.0–4.6-fold stronger in DMEM with 10% FBS medium than in DMEM medium (Table 2). When compounds **10** and **11** were added to J774.1 and cultured for 24 h, the production of compounds **8** and **9** was not observed (Fig. 8). These results suggested that the conversion from compound **10** to **8** and compound **11** to **9** was not due to J774.1. The expression of genes derived from two enzymes, 17β-hydroxysteroid dehydrogenase (Fig. 7c) and 16α-hydroxylase (Fig. 7d), in the strain (Table 3) may be activated by a co-culture of *N. tenerifensis* IFM 10554^T and J774.1.

Since compounds **1**, **8**, and **9** were preferentially produced by a co-culture, their cytotoxicities against J774.1 were

Table 2 Presumed genes expressed in DMEM with FBS medium

Gene location[a]	Length aa	Function[a]	Similar proteins[b]	%Identity[b]	Fold changes[c]
Contig00701 (142297..142714)	138	Ketosteroid isomerase	Ketosteroid isomerase [*Nocardia brasiliensis*]	93	4.6
			Ketosteroid isomerase [*Nocardia altamirensis*]	92	
Contig04601 (26751..28467)	571	3-Ketosteroid-delta-1-dehydrogenase	3-Ketosteroid-delta-1-dehydrogenase [*Nocardia brasiliensis*]	93	4.0
			3-Ketosteroid-delta-1-dehydrogenase [*Nocardia vulneris*]	93	

[a] Results of the MiGAP analysis

[b] Results of the NCBI BLAST search

[c] Results of the RNA-seq [fold change in RPKM (10- and 11-producing and non-producing conditions)]

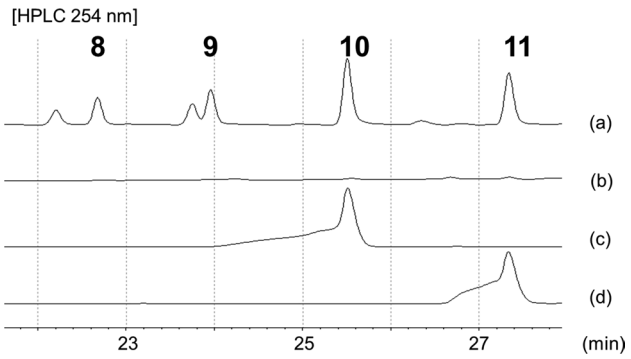


Fig. 8 Comparison of extracts of culture broth in DMEM + FBS medium. **a** Co-culture, **b** single culture [J774.1 cell, addition of DMSO (control)], **c** single culture [J774.1 cell, addition of **10**], **d** single culture [J774.1 cell, addition of **11**]

examined and the results obtained revealed that they did not exhibit cytotoxicity against J774.1.

In the present study, we demonstrated a new method to activate biosynthesis genes by culturing actinomycetes of the genus *Nocardia* in the presence of animal cells and obtained a new compound **1**. By using this method, the further isolation of new secondary metabolites is expected in the future.

Experimental

General experimental procedures

The following instruments were used in the present study: a P-2200 polarimeter (JASCO) for optical rotations; a UV mini 1240 UV–Vis spectrophotometer (Shimadzu) for UV–Vis spectroscopy; an ECZ-600 spectrometer (JEOL) for NMR spectroscopy (solvent chemical shifts were used as the internal standard); and a JMS-T100LP (JEOL) for HR-ESIMS. An HPLC system (Shimadzu) consisting of a SCL-10AVP system controller, LC-20AD pumps, DGU-12A on-line degasser, CTO-10AS VP column oven, SIL-20A autosampler, and SPD-M20A PDA detector was used and chromatographic data were collected and processed using Shimadzu CLASS-VP software (version 6.14 SP1, Shimadzu). A Shimadzu LCMS system (Shimadzu) consisting of LC-20AD pumps, a DGU-12A$_3$ on-line degasser, CTO-20A column oven, SIL-20A autosampler, SPD-M20A PDA detector, FCV-20AH$_2$ valve unit, LCMS 2020 for ESIMS, and N_2 Supplier Model 24F for the N_2 generator was used and chromatographic data were collected and processed using LabSolution software (version 5.42 SP4, Shimadzu). The conditions of the LCMS analysis were as

Table 3 Genes presumed to be expressed by the co-culture

Gene location[a]	Length aa	Function[a]	Similar proteins[b]	%Identity[b]
Contig15901 (1139..1957)	272	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	Oxidoreductase [17β-HSD-like, SDR(c)] [*Streptomyces acidiscabies*]	69
			Oxidoreductase [17β-HSD-like, SDR(c)] [*Streptomyces olivochromogenes*]	68
Contig04001 (28393..29613)	406	Cytochrome P450 monooxygenase	CYP154C5 (16α-hydroxylase) [*Nocardia farcinica*]	80
			CYP154C3 (16α-hydroxylase) [*Streptomyces griseus* subsp. *griseus*]	77

[a] Results of the MiGAP analysis

[b] Results of the NCBI BLAST search

follows: 0–100% MeOH in 0.1% HCOOH, 0–30 min, linear gradient, and 100% MeOH in 0.1% HCOOH, isocratic 30–60 min; flow rate: 0.2 mL/min; UV detection: photodiode array (190–600 nm); MS detection: ESI (positive and negative) (*m*/*z* 100–2000); guard column: Develosil ODS-HG-S (ϕ1.5 × 10 mm, Nomura chemical); column: COSMOSIL 5C_{18}-AR-II (ϕ2.0 × 150 mm, Nacalai Tesque). The following adsorbents were used for purification: Silica gel 60 F_{254} (0.25 mm, Merck) and Silica gel 60 RP-8 F_{254} S (0.25 mm, Merck) for analytical TLC; Silica Gel 60 N (Kanto chemical), Silica Gel PSQ 100B (Fuji Silysia chemical), and Chromatrex ODS (Fuji Silysia chemical) for column chromatography; COSMOSIL 5C_{18}-AR-II (ϕ 10.0 × 250 mm, Nacalai Tesque) and COSMOSIL Cholester column (ϕ 10.0 × 250 mm, Nacalai Tesque) for preparative HPLC. The following instruments were used for cultivation and extraction: GeneQuant pro (GE) for OD_{600} spectroscopy; FMC 1000 (EYELA) and SOFT INCUBATOR SLI-450ND (EYELA) for an incubator; BIOLABO BL-171 (Juji Field) for a CO_2 incubator; M150-IVD (Sakuma), HIMAC CENTRIFUGE (Hitachi), KS-5000 (Kubota), and Avanti centrifuge HP-60XP (Beckman Coulter) for a centrifuge.

Microbial strain

Strains of the genus *Nocardia* were stored in a freeze-dried state at the Medical Mycology Research Center, Chiba University, Japan. These strains were reidentified by sequencing the 16S rRNA gene.

Seed culture of a mouse macrophage-like cell line (J774.1) in a 175-cm^2 cell culture flask

A mouse macrophage-like cell line (J774.1) was incubated at 37 °C in a 5% CO_2 atmosphere in 25 mL of DMEM (Wako) with 10% fetal bovine serum (FBS, Bio West) in 175-cm^2 cell culture flasks (Violamo), which reached approximately 80–90% confluency after 5–7 days. Old culture medium was then removed and 5 mL of DMEM with 10% FBS medium was added to the flask. Cells were scraped and collected from the bottom of the flask with a cell scraper. Cell medium was transferred to a 50-mL tube, which was centrifuged at 2000 rpm at 20°C for 5 min. After the removal of medium, 5 mL of DMEM with 10% FBS medium was added to the tube. The residue was stirred by pipetting and transferred to a hemocytometer. The number of viable cells in the hemocytometer was counted using trypan blue. Cell medium was added to each new cell culture flask with 25 mL of DMEM with 10% FBS medium. Cells were cultured for 24 h.

Seed culture of *Nocardia* sp. for a co-culture in modified Czapek-Dox medium

Each strain of *Nocardia* sp. was cultivated in 5 mL of BHI + liquid medium consisting of Bacto™ brain heart infusion (3.7 g/100 mL, Becton, Dickinson and Company), glucose (1 g/100 mL, Wako), and glycerol (1 mL/100 mL, Nacalai Tesque) in a 10-mL Erlenmeyer flask at 28 °C for 5 days with shaking (160 rpm). The culture broth was added to a 50-mL tube. The supernatant was removed after centrifugation at 3000 rpm at 20°C for 2 min. Two milliliters of modified Czapek-Dox medium consisting of sucrose (3 g/100 mL, Wako), $NaNO_3$ (0.3 g/100 mL, Wako), KH_2PO_4 (0.1 g/100 mL, Nacalai Tesque), KCl (0.05 g/100 mL, Nacalai Tesque), $MgSO_4 \cdot 7H_2O$ (0.05 g/100 mL, Wako), and $FeSO_4 \cdot 7H_2O$ (0.001 g/100 mL, Nacalai Tesque) was added to the tube. The supernatant was removed after centrifugation at 3000 rpm at 20°C for 2 min. A strain suspension was prepared by the addition of 11 mL of mCD medium to the tube.

Co-culture in mCD medium in a cell culture flask

Fifty milliliters of mCD medium were added to J774.1 in a cell culture flask after the removal of DMEM with 10% FBS medium. A suspension of *Nocardia* sp. was added to the flasks until the cell number ratio was reached (J774.1:*Nocardia* sp. = 1:10). Flasks were incubated at 28 °C for 2 weeks.

Seed culture of *Nocardia* sp. for a co-culture in DMEM with 10% FBS medium

Each strain of the *Nocardia* sp. culture was cultivated in 5 mL of BHI + liquid medium in a 10-mL Erlenmeyer flask at 28 °C for 5 days with shaking (160 rpm). The culture broth was transferred to a 50-mL tube. The supernatant was removed after centrifugation at 3000 rpm at 20 °C for 2 min. Two milliliters of DMEM were added to the tube. The supernatant was removed after centrifugation at 3000 rpm at 20°C for 2 min. A strain suspension was prepared by the addition of 11 mL of DMEM to the tube.

A co-culture in DMEM with 10% FBS medium in a cell culture flask

Fifty milliliters of DMEM with 10% FBS medium were added to J774.1 in a cell culture flask after the removal of medium. A suspension of *Nocardia* sp. was added to the flasks until the cell number ratio was reached

(J774.1:*Nocardia* sp. = 1:5). The flasks were incubated at 37 °C for approximately 24 h under 5% CO_2.

Fermentation for mCD medium and isolation

The strain *N. tenerifensis* IFM 10554^T was cultured at 28 °C for 2 weeks on mCD medium in 63 cell culture flasks in the presence of J774.1 (cell number ratio J774.1: *N. tenerifensis* = 1:10). The culture broth (3.2 L) was centrifuged at 3000 rpm for 20 min to give the supernatant and mycelial cake; the mycelial cake was extracted with MeOH (1.6 L × 2). The MeOH extract was combined with the supernatant obtained above, and the combined materials were partitioned with EtOAc (3.2 L × 3) and water. Some of the EtOAc-soluble fraction (132.0 mg) was evaporated and MeOH (13.2 mL) was added to the residue. A MeOH-soluble fraction (89 mg) was subjected to ODS column chromatography (ϕ 15 × 130 mm) eluted with 0–100% MeOH to afford 9 fractions. The fraction (4.9 mg) eluted with 40–60% MeOH was purified by reversed-phase HPLC [COSMOSIL 5C_{18}-AR-II (ϕ 10.0 × 250 mm); eluent: 50% MeOH; flow rate: 5 mL/min; UV detection: photodiode array (190–400 nm)] to give compound **1** (1.5 mg, t_R 5.2 min).

Dehydropropylpantothenamide (1)

Colorless amorphous solid. $[\alpha]_D^{25}$ +14.1 (*c* 0.1, MeOH). HRESIMS *m/z*: 539.3080 $[2\ M + Na]^+$ (calcd for $C_{24}H_{44}N_4O_8Na$, 539.3057). UV λ_{max} (MeOH) nm (log ε): 266 (3.8). IR (ATR) cm^{-1}: 1738, 1366, and 1217. CD λ_{nm} (MeOH) nm ($\Delta\varepsilon$): 232 (− 0.7), and 264 (0.9). ^{1}H and ^{13}C NMR data in Table 1.

N-[[(4R)-2-(4-Methoxyphenyl)-5,5-dimethyl-1,3-dioxan-4-yl]carbonyl]-β-alanine (3)

Conc. H_2SO_4 (110 µL, 2.1 mmol, Wako) was added to a solution of D-pantothenic acid calcium salt (**2**: 1.0 g, 2.1 mmol, TCI) in dry acetone (4 mL, Wako), which was then stirred at room temperature for 2 h. *p*-Toluenesulfonic acid monohydrate (*p*-TsOH·H_2O, 80 mg, 0.42 mmol, Wako). *p*-anisaldehyde dimethyl acetal (357 µL, 2.1 mmol, Avocado Research Chemicals) were added to the reaction mixture, which was stirred at room temperature for 37 h. After the addition of H_2O (20 mL), the reaction mixture was extracted with EtOAc 3 times. The organic layer was evaporated *in vacuo*. The residue was subjected to flash silica gel column chromatography (ϕ 20 × 200 mm) eluted with hexane/acetone (1/1) to afford the single diastereomer **3** (481 mg, 1.42 mmol, 34% yield).

^{1}H-NMR (600 MHz, $CDCl_3$) δ: 7.40 (2H, d, J = 9.0 Hz), 6.90 (2H, d, J = 9.0 Hz), 5.45 (1H, s), 4.10 (1H, s), 3.79 (3H, s), 3.70 (1H, d, J = 11.3 Hz), 3.64 (1H, d, J = 11.3 Hz), 3.55 (1H, m), 3.47 (1H, m), 2.57 (2H, m), 1.09 (3H, s), and 1.07 (3H, s). ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 176.5, 169.5, 160.2, 130.1, 127.4, 113.7, 101.2, 83.6, 78.4, 55.3, 34.1, 33.8, 33.1, 21.7, and 19.0. $[\alpha]_D^{20}$ +37.0 (*c* 0.1, MeOH). IR (ATR) cm^{-1}: 2949, 2362, 1653, 1520, 1250, 1103, and 1032. HRESIMS *m/z*: 697.2991 $[2\ M + Na]^+$ (Calcd for $C_{34}H_{46}N_2O_{12}Na$: 697.2948).

Methyl 3-[(4R)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido] propanoate (4)

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 623.4 mg, 3.25 mmol, TCI) and 4-dimethylaminopyridine (DMAP, 40 mg, 0.33 mmol, TCI) were added to a solution of **3** (220 mg, 0.65 mmol) in dry MeOH (13 mL, Wako), which was then stirred at room temperature for 22 h. After the addition of H_2O (30 mL), the reaction mixture was extracted with CH_2Cl_2 3 times. The organic layer was evaporated *in vacuo* and the residue was separated by reversed-phase HPLC [COSMOSIL Cholester (ϕ 10.0 × 250 mm); eluent: 80% MeOH; flow rate: 4 mL/min; UV detection: photodiode array (190–400 nm)] to give **4** (214 mg, 0.61 mmol, 94% yield, t_R 5.1 min).

^{1}H-NMR (600 MHz, $CDCl_3$) δ: 7.41 (2H, d, J = 9.0 Hz), 6.90 (2H, d, J = 9.0 Hz), 5.45 (1H, s), 4.06 (1H, s), 3.80 (3H, s), 3.70 (1H, d, J = 11.0 Hz), 3.64 (1H, d, J = 11.0 Hz), 3.62 (3H, s) 3.55 (1H, m), 3.47 (1H, m), 2.54 (2H, m), 1.08 (3H, s), and 1.08 (3H, s). ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 172.3, 169.1, 160.2, 130.2, 127.4, 113.7, 101.2, 83.7, 78.4, 55.3, 51.7, 34.3, 33.9, 33.0, 21.7, and 19.0. $[\alpha]_D^{20}$ +30.0 (*c* 0.1, MeOH). IR (ATR) cm^{-1}: 2956, 2364, 2348, 2310, 1733, and 1519. HRESIMS *m/z*: 374.1540 $[M + Na]^+$ (Calcd for $C_{18}H_{25}NO_6Na$: 374.1580).

Methyl (Z)-3-[(4R)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido] acrylate (5)

Dess–Martin periodinane (154 mg, 0.36 mmol, TCI) was added to a solution of **4** (14 mg, 0.04 mmol) in fluorobenzene (PhF, 4 mL, Wako), which was then refluxed for 30 h. The reaction mixture was quenched with saturated aqueous (aq.) $Na_2S_2O_4$ and saturated aq. $NaHCO_3$, and was then stirred until the solution became clear. The mixture was extracted with hexane 3 times. The organic layer was evaporated *in vacuo*, and the residue was separated by reversed-phase HPLC [COSMOSIL Cholester (ϕ 10.0 × 250 mm); eluent: 80% MeOH; flow rate: 4 mL/min; UV detection: photodiode array (190–400 nm)] to give **5** (3.8 mg, 0.011 mmol, 26% yield, t_R 8.2 min).

^{1}H-NMR (600 MHz, $CDCl_3$) δ: 11.20 (1H, d, J = 11.3 Hz), 7.60 (2H, d, J = 8.6 Hz), 7.46 (1H, dd, J = 11.3, 9.0 Hz), 6.95 (2H, d, J = 8.6 Hz), 5.55 (1H, s), 5.19 (1H, d, J = 9.0 Hz), 4.23 (1H, s), 3.83 (3H, s), 3.73 (2H,

m), 3.68 (3H, s), 1.17 (3H, s), and 1.11 (3H, s). ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 168.4, 168.0, 160.3, 135.9, 130.0, 127.6, 113.7, 101.3, 98.0, 83.7, 78.4, 55.3, 51.2, 33.4, 21.6, and 19.2. $[\alpha]_D^{20}$ − 2.3 (*c* 0.1, MeOH). IR (ATR) cm^{-1}: 2970, 2369, 2350, 2318, 1737, 1364, and 1217. HRESIMS *m/z*: 372.1413 $[M + Na]^+$ (Calcd for $C_{18}H_{23}NO_6Na$: 372.1423).

(Z)-3-[(4R)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido] acrylic acid (6)

A solution of **5** (3.4 mg, 9.7 μmol) in 0.5 *N* aq. NaOH (6 mL) was stirred at room temperature for 37 h. The solution was neutralized by the addition of 1 *N* HCl, and the mixture was extracted with EtOAc 3 times. The organic layer was evaporated *in vacuo* to give **6** (2.5 mg, 7.5 μmol, 77% yield).

^{1}H-NMR (600 MHz, CD_3OD) δ: 7.53 (2H, d, J = 8.6 Hz), 7.44 (1H, dd, J = 9.0 Hz), 6.20 (2H, d, J = 8.6 Hz), 5.60 (1H, s), 5.20 (1H, d, J = 9.0 Hz), 4.32 (1H, s), 3.79 (3H, s), 3.77 (1H, d, J = 11.0 Hz), 3.68 (1H, d, J = 11.0 Hz), 1.10 (3H, s), and 1.06 (3H, s). ^{13}C-NMR (150 MHz, CD_3OD) δ: 171.3, 169.8, 161.6, 136.6, 131.6, 128.7, 114.5, 102.5, 100.0, 84.7, 79.1, 55.7, 34.3, 21.9, and 19.6. $[\alpha]_D^{20}$ -11.0 (*c* 0.1, MeOH). IR (ATR) cm^{-1}: 2970, 2369, 2350, 2318, 1738, 1737, 1364, and 1217. HRESIMS *m/z*: 358.1230 $[M + Na]^+$ (Calcd for $C_{17}H_{21}NO_6Na$: 358.1267).

(4R)-2-(4-methoxyphenyl)-5,5-dimethyl-N-[(Z)-3-oxo-3-(propylamino)prop-1-en-1-yl]-1,3-dioxane-4-carboxamide (7)

EDC·HCl (17 mg, 0.09 mmol, TCI), 1-hydroxybenzotriazole monohydrate (HOBt·H_2O, 14 mg, 0.09 mmol, TCI), and propylamine (5 mL, 0.06 mmol, Wako) were added to a solution of **6** (10 mg, 0.03 mmol) in dry DMF (3 mL, Wako), which was then stirred at room temperature for 24 h. The reaction mixture was quenched with saturated aq. $NaHCO_3$ and then extracted with hexane/EtOAc = 1/4 3 times. The organic layer was evaporated *in vacuo* and the residue was separated by reversed-phase HPLC [COSMOSIL Cholester (ϕ 10.0 × 250 mm); eluent: 80% MeOH; flow rate: 4 mL/min; UV detection: photodiode array (190–400 nm)] to give **7** (5.0 mg, 0.013 mmol, 44% yield, t_R 6.8 min).

^{1}H-NMR (600 MHz, $CDCl_3$) δ: 12.05 (1H, d, J = 11.0 Hz), 7.66 (2H, d, J = 8.9 Hz), 7.46 (1H, dd, J = 11.0, 9.0 Hz), 6.95 (2H, d, J = 8.9 Hz), 5.54 (1H, s), 5.40 (1H, t, J = 5.5 Hz) 5.00 (1H, d, J = 9.0 Hz), 4.19 (1H, s), 3.82 (3H, s), 3.17 (2H, s), 3.28 (1H, m), 3.25 (1H, m), 1.55 (2H, m), 1.16 (3H, s), 1.10 (3H, s), and 0.94 (3H, t, J = 7.6 Hz); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 168.0, 167.7, 160.1, 133.3, 130.1, 127.7, 113.7, 101.2, 100.8, 83.9, 78.5, 55.3, 40.9, 33.4, 22.8, 21.7, 19.3, and 11.5. $[\alpha]_D^{20}$ +7.6 (*c* 0.1, MeOH). IR (ATR) cm^{-1}: 2970, 2368, 2350, 2319, 1737, 1364, and 1217. HRESIMS *m/z*: 399.1847 $[M + Na]^+$ (Calcd for $C_{20}H_{28}NO_5Na$: 399.1896).

Dehydropropylpantothenamide (1, synthetic)

A solution of **7** (2.6 mg, 6.9 μmol) in CH_3COOH/H_2O = 4/1 (80% CH_3COOH, 2 mL) was stirred at room temperature for 14 h. The reaction mixture was evaporated *in vacuo* and the residue was separated by reversed-phase HPLC [COSMOSIL Cholester (ϕ 10.0 × 250 mm); eluent: 60% MeOH; flow rate: 4 mL/min; UV detection: photodiode array (190–400 nm)] to give **1** (0.7 mg, 2.7 μmol, 39% yield, t_R 6.0 min).

$[\alpha]_D^{20}$ +12.5 (*c* 0.1, MeOH). IR (ATR) cm^{-1}: 2970, 2368, 2350, 2319, 1737, 1364, and 1217. CD λ_{nm} (MeOH) nm ($\Delta\varepsilon$): 236 (-1.5), and 264 (1.3). HRESIMS *m/z*: 539.3060 $[2 M + Na]^+$ (Calcd for $C_{24}H_{44}N_4O_8Na$: 539.3057).

Fermentation for DMEM with 10% FBS medium and isolation

The strain *N. tenerifensis* IFM 10554^T was cultured at 37 °C for 24 h under 5% CO_2 on DMEM with 10% FBS medium in cell culture flasks (40 mL × 200) in the presence of J774.1 (the cell number ratio J774.1: *N. tenerifensis* = 1:5). The culture broth (8.0 L) was centrifuged at 5000 rpm for 20 min to give the supernatant and mycelial cake; the mycelial cake was extracted with MeOH (4 L × 2). The MeOH extract was combined with the supernatant obtained above, and the combined materials were partitioned with EtOAc (8 L × 3) and water. A part of the EtOAc-soluble fraction (319 mg) was evaporated and MeOH (32 mL) was added to the residue. A MeOH-soluble fraction was separated by reversed-phase HPLC [COSMOSIL 5C_{18}-AR-II (ϕ 10.0 × 250 mm); eluent: 0–100% MeOH with 0.1% HCOOH in H_2O, 0–30 min, linear gradient; flow rate: 4 mL/min; UV detection: photodiode array (190–400 nm)] to give 1,4-androstadine-16α, 17β-diol-3-one (**8**, 0.3 mg, t_R 19.0 min, colorless amorphous solid, $[\alpha]_D^{25}$ +61.1 (*c* 0.05, MeOH), HRESIMS *m/z*: 325.1803 $[M + Na]^+$ (Calcd for $C_{19}H_{28}O_2Na$: 325.1804)), 4-androstene-16α, 17β-diol-3-one (**9**, 1.1 mg, t_R 20.5 min, colorless amorphous solid; $[\alpha]_D^{25}$ +69.7 (*c* 0.05, MeOH), HRESIMS *m/z*: 327.1951 $[M + Na]^+$ (Calcd for $C_{19}H_{28}O_2Na$: 327.1960)), 1,4-androstadine-3,17-dione (**10**, 0.3 mg, t_R 22.0 min, colorless amorphous solid, $[\alpha]_D^{25}$ +84.0 (*c* 0.05, MeOH), HRESIMS *m/z*: 307.1674 $[M + Na]^+$ (Calcd for $C_{19}H_{24}O_2Na$: 307.1674)), and 4-androstene-3,17-dione (**11**, 0.2 mg, t_R 23.7 min, colorless amorphous solid, $[\alpha]_D^{25}$ +96.8 (*c* 0.05, MeOH), HRESIMS *m/z*: 309.1834 $[M + Na]^+$ (Calcd for $C_{19}H_{26}O_2Na$: 309.1830)). A MeOH-insoluble compound (10.9 mg) in the EtOAc soluble fraction was identified as cholesteryl oleate (**12**, 10.9 mg, colorless amorphous solid, $[\alpha]_D^{25}$ − 13.6 (*c* 0.1, $CHCl_3$), HRESIMS *m/z*: 673.5939 $[M + Na]^+$ (Calcd for $C_{45}H_{78}O_2Na$: 673.5899).

Transcriptional analysis

RNA was extracted from the strain *N. tenerifensis* IFM 10554^T cultured under **10**- and **11**-producing and non-producing conditions. DMEM with 10% FBS medium was used for the **10-** and **11**-producing conditions, and DMEM medium without FBS was used for the non-producing conditions. The strain *N. tenerifensis* IFM 10554^T pre-cultivated on BHI + medium at 37 °C for 5 days was inoculated into **10**- and **11**-producing and non-producing media and then incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. Following the incubation, the strain *N. tenerifensis* IFM 10554^T was collected and suspended in ISOGEN RNA extraction solution (Nippon Gene Co.). The resuspended strain *N. tenerifensis* IFM 10554^T was homogenized using a MagNA Lyser (Roche Diagnostics) at 7000 rpm for 20 s, and RNA was extracted according to the ISOGEN protocol. Following the DNase treatment, 2 μg of total RNA was treated with a Ribo-Zero Magnetic Kit (Epicentre) to remove ribosomal RNA. In the construction of the RNA-seq library, the mRNA samples obtained were treated with a KAPA Standard RNA-seq Library Preparation Kit (illumina) according to the manufacturer's protocol. Constructed libraries were sequenced with the MiSeq system (Illumina) using Miseq Reagent Kit v2 (Illumina). The resulting data were mapped against the sequenced genome data of *N. tenerifensis* IFM10554^T using CLC GenomicsWorkbench.

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