Regular Article

Isolation of Nabscessin C from *Nocardia abscessus* IFM 10029^T and a Study on Biosynthetic Pathway for Nabscessins

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Received June 6, 2018; accepted July 18, 2018

A new aminocyclitol derivative, designated nabscessin C (1), was isolated from *Nocardia abscessus* IFM 10029^T. Nabcessin C is an isomer of nabscessins A (2) and B (3) with different positioning of the acyl group. Absolute configuration of nabscessin A was determined by conversion into the 2-deoxy-*scyllo*-inosamine pentaacetyl derivative (4) by hydrolysis and acetylation of 2. The biosynthetic pathway of nabscessins is proposed based on gene expression analysis.

Key words actinomycete; *Nocardia*; aminocyclitol; biosynthetic pathway

Actinomycetes of the genus *Nocardia* are Gram-positive bacteria widely present in nature. Many species in this genus possess weak virulence. In humans, *Nocardia* infect the lungs and disseminate hematogenously, causing visceral nocardiosis, as well as skin nocardiosis (in the dermis). Many cases of infection occur in humans with reduced immunity. Some species of the genus *Nocardia* are known to infect other animals and plants. Several secondary metabolites have been isolated from the genus *Nocardia*. Nocardicin A, a monocyclic *β*-lactam, was isolated from *Nocardia uniformis* ssp. Tsuyamanensis ATCC 21806,[1] and the nargenicins were isolated from *Nocardia argentinensis* Huang ATCC 31306.[2] Although these metabolites have been isolated, research on the genus *Nocardia* has not progressed as enough as that on the genus *Streptomyces*. The genome sequences of several *Nocardia* strains have been reported, but members of this genus have not yet been the subject of detailed analyses of gene function.[3]

In our previous report,[4] new aminocyclitol derivatives, nabscessins A and B, were isolated from *Nocardia abscessus* IFM 10029^T grown in modified Czapek-Dox (mCD)[5] medium. In the present study, we describe the isolation and structural elucidation of a new compound (**1**) obtained from *Nocardia abscessus* IFM 10029^T grown in mCD medium. We extend that work by determining the absolute configurations of the nabscessins, and additionally use molecular genetic analyses to propose the pathway for the biosynthesis of this class of compounds.

Results and Discussion

Strains for this study were initially selected from among 76 strains belonging to the genus *Nocardia*; all of these strains were obtained from the Medical Mycology Research Center, Chiba University, Japan. A phylogenetic tree for these strains was constructed based on 16S ribosomal RNA (rRNA) sequence analysis using two analytical software programs, Clustal X[6] and MEGA.[7] This tree classified the strains into nine clades. Biosynthetic gene clusters were examined in a gene analysis of the genus *Nocardia* using antiSMASH.[8] Based on these clades and the number of biosynthetic gene clusters, thirteen strains (*Nocardia abscessus* IFM 10029^T, *Nocardia africana* IFM 10147^T, *Nocardia anaemiae* IFM 0323^T, *Nocardia arthritidis* IFM 10035^T, *Nocardia asiatica* IFM 0245^T, *Nocardia exalbida* IFM 0803^T, *Nocardia inohanensis* IFM 0092^T, *Nocardia kruczakiae* IFM 10565^T, *Nocardia sienata* IFM 10088^T, *Nocardia terpenica* IFM 0706^T, *Nocardia transvalensis* IFM 0333^T, *Nocardia vinacea* IFM 10175^T, and *Nocardia yamanashiensis* IFM 0265^T) were selected for growth as small-scale cultures. Metabolite production in the thirteen selected strains was examined using four different media [mCD, nutrient broth (NB),[9] Waksman,[10] and Yeast-Malt-Glucose (YMG)[11]] and incubating the cultures at 28°C in ambient air with rotary shaking at 160 rpm in 50-mL Erlenmeyer flasks.

The LC-MS analysis of the culture broth extracts obtained under various conditions led us to focus our attention on the extract of *Nocardia abscessus* IFM 10029^T cultured in the mCD medium. In the corresponding extract, three characteristic peaks were observed; the UV absorption spectra and the MS spectra of the 3 peaks largely coincided with each other, even though the compounds represented by the 3 peaks exhibited distinct retention times. A large-scale culture (2.0 L) of *Nocardia abscessus* IFM 10029^T was performed by growing the strain in mCD medium for 1 week at 28°C in ambient air as a shaking culture (160 rpm) in five 1-L Erlenmeyer flasks. After centrifugation of the culture, the supernatant and a methanol extract of the mycelia were combined and subjected to partitioning between ethyl acetate (EtOAc) and water. The EtOAc-soluble fraction was subjected to fractionation using silica gel column chromatography (the $CHCl_3$–MeOH system), followed by reverse-phase HPLC separation (40% MeOH) to obtain a new compound (**1**), designated nabscessin C (Fig. 1), along with the previously identified nabscessins A (**2**) and B (**3**).

High resolution (HR) electrospray ionization (ESI)-MS revealed that the molecular formula of compound **1** is $C_{22}H_{24}N_2O_9$ (obsd. *m/z* 483.1426 $[M+Na]^+$, Calcd for $C_{22}H_{24}N_2O_9Na$, 483.1380). The ^{1}H-NMR spectrum of **1** measured in acetone-d_6 showed seven aromatic hydrogens (δ_H

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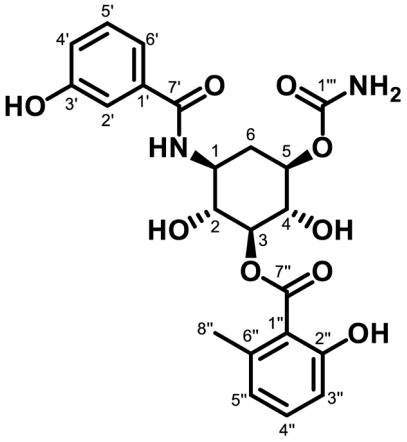


Fig. 1. Structure of **1**

7.44–6.75), five sp^3 methines (δ_H 5.34–3.81), one methylene (δ_H 2.42 and 1.71), one methyl (δ_H 2.56), and one broad signal [δ_H 7.72 (1H)]. The ^{13}C-NMR spectrum of **1** exhibited 21 peaks (Table 1), which were assignable to seven aromatic methines, seven quaternary carbons, five sp^3 methines, one methylene, and one methyl, based on analysis of the ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectrum. The cross-peaks observed in the ^{1}H–^{1}H correlation spectroscopy (COSY) data of **1** suggested the presence of three partial structures A, B, and C as indicated in Fig. 2. Based on COSY and heteronuclear multiple bond correlation (HMBC) correlations (Fig. 2) and comparison to the ^{13}C-NMR spectra for **2** and **3**, partial structures A, B, and C were assigned to 3-hydroxybenzoic acid (3-HBA), 6-methylsalicylic acid (6-MSA), and a cyclohexane ring moiety, respectively; all three correspond to structures that are shared with those of **2** and **3**. The difference in structure of **1** from those of **2** and **3** was revealed to be the position of the 6-MSA group. The HMBC correlation observed from H-3 (δ_H 5.34) in partial structure C to the C-7″ carbonyl (δ_C 171.3) in the 6-MSA moiety suggested that the 6-MSA ester group is located on C-3. Although the spectral data elucidating connection between partial structures A and C as well as the connection between partial structure C and the carbamoyl group (position 1″) were not obtained, the positions of partial structure A and the carbamoyl groups were deduced as the same as those of nabscessins A (**2**) and B (**3**) based on the comparison of the ^{1}H-NMR chemical shifts (Fig. S1, supplementary materials) as well as the observation of interconversion of compound **1** into **2** and **3** (Fig. S2, supplementary materials). The structure of nabscessin C thus was revealed to be **1**, as shown in Fig. 1.

To elucidate the absolute stereochemistry of nabscessins A–C, nabscessin A (**2**) was converted to a 2-deoxy-*scyllo*-inosamine pentaacetyl derivative (**4**), since the specific rotations of both enantiomers of **4** have been reported in the literature.[12] Compound **4** was obtained by hydrolysis and acetylation of **2**, and the spectral data of the resulting pentaacetyl compound **4** derived from **2** was identical with those of literature data,[12] including the sign of the specific rotation (levorotatory), which established the absolute configuration of **2** as shown in Fig. 3. Since nabscessins A–C were obtained from the same *Nocardia* strain, the absolute configuration of nabscessins A–C were presumed to be the same; this inference was consistent with the absolute configuration of nabscessin B (**3**) recently reported by total synthesis.[13]

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts for Nabscessin C (**1**)

Position	δ_H	δ_C
1	4.20 (m)	50.9
2	3.99 (dd, 9.6, 9.6)	73.8
3	5.34 (dd, 9.6, 9.6)	79.6
4	3.81 (dd, 9.6, 9.6)	74.1
5	4.82 (ddd, 12.0, 9.6, 4.6)	73.0
6	1.71 (ddd, 12.0, 12.0, 12.0)	33.9
	2.42 (ddd, 12.0, 4.6, 4.6)	
1-NH	7.72 (d, 8.3)	
1′		137.0
2′	7.44 (dd, 2.7, 1.4)	115.3
3′		158.4
4′	6.95 (ddd, 7.8, 2.7, 0.9)	119.0
5′	7.23 (dd, 7.8, 7.8)	130.0
6′	7.34 (ddd, 7.8, 1.4, 0.9)	119.0
7′		167.8
1″		115.3
2″		162.0
3″	6.77 (d, 8.7)	115.9
4″	7.29 (dd, 8.7, 7.8)	134.3
5″	6.75 (d, 7.8)	123.2
6″		141.6
7″		171.3
8″	2.56 (s)	23.2
1‴-NH_2	5.88 (br s)	n.d.[a]

Measured in acetone-d_6, 600 MHz (^{1}H), 150 MHz (^{13}C) δ in ppm. J in Hz.; [a] n.d.: not determined.

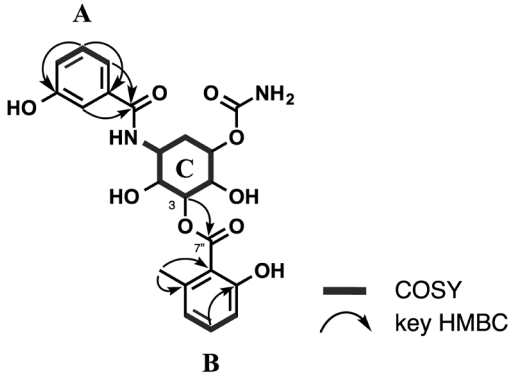


Fig. 2. Partial Structures A–C for **1**

The 2-deoxy-*scyllo*-inosamine moiety contained in the structure of nabscessins A–C is known to be a biosynthetic intermediate of aminoglycoside antibacterial drugs such as kanamycin,[14] and 2-deoxy-*scyllo*-inosose synthase has been reported as a biosynthetic enzyme.[15] The gene encoding this enzyme therefore served as the "bait" in a search for the biosynthetic gene cluster for nabscessins A–C. MiGAP analysis[16] and BLASTP search[17] of the draft genome of *Nocardia abscessus* identified *contig 00601* as a region harboring a gene with sequence similarity to the gene encoding 2-deoxy-*scyllo*-inosose synthase. Analysis of the sequences of *contig 00601* (GeneBank accession no. BAFP01000039.1, BAFP01000256.1, BAFP01000258.1) surrounding the synthase-encoding gene suggested the presence of 6 open reading frames (ORFs) that were possibly involved in the biosynthesis of the nabscessins

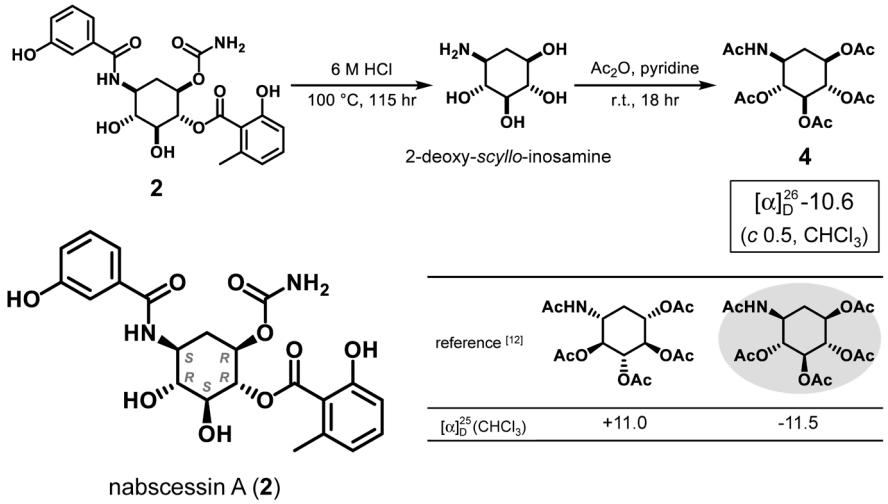
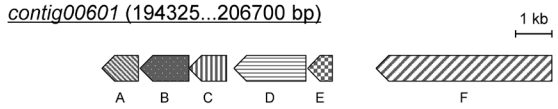


Fig. 3. The Determination of Absolute Configuration of **2**

Table 2. (a) A Schematic of the Putative Cluster and (b) Putative Nabcessin Biosythetic Genes Selectively Expressed in mCD (Production) Medium

a



b

ORF	Size (aa)	Proposed protein function	Closest protein homolog (source organism)	Protein identity (%)
A	331	3-Hydroxybenzoate synthase	3-Hydroxybenzoate synthase (*Streptomyces hygroscopius*)	51
B	450	2-Deoxy-*scyllo*-inosose aminotransferase	L-Glutamine:2-deoxy-*scyllo*-inosose/3-amino-2,3-dideoxy-*scyllo*-inosose aminotransferase (*Saccharopolyspora antimicrobica*)	58
C	347	2-Deoxy-*scyllo*-inosose synthase	2-Deoxy-*scyllo*-inosose synthase (*Streptomyces tenjimariensis*)	62
D	658	Carbamoyltransferase	Carbamoyltransferase (*Streptomyces niveiscabiei*)	42
E	225	AMP ligase	2,3-Dihydroxybenzoate-AMP ligase (*Nocardia terpenica*)	56
F	1611	6-Methylsalicylic acid synthase	6-Methylsalicylic acid synthase (*Streptomyces gandocaensis*)	47

ORF : open reading frame; AMP : adenosine monophosphate.

(Table 2). Based on the structure of this putative gene cluster, the pathway for biosynthesis of the nabscessins is proposed as shown in the Fig. 4. The partial structures of the nabscessin moieties are presumed to be constructed by three separate sub-pathways, as follows: (1) 3-HBA is derived from chorismate by 3-hydroxybenzoate synthase[18]; (2) 2-Deoxy-*scyllo*-inosamine is obtained from glucose-6-phosphate *via* 2-deoxy-*scyllo*-inosose by 2-deoxy-*scyllo*-inosose aminotransferase and 2-deoxy-*scyllo*-inosose synthase[15]; and (3) 6-MSA is generated from acetyl CoA and malonyl CoA by 6-MSA synthase.[19] These three moieties of the nabscessins then are connected by the activities of an AMP ligase[20] and a carbamoyltransferase,[21] yielding nabscessins A–C (**1**–**3**).

The expression levels of these putative biosynthesis genes in cells grown in mCD medium and in Waksman medium then were compared by the RNA-seq method. Notably, nabscessins A–C were not produced in Waksman medium, while these compounds were produced in mCD medium (Fig. 5). Analysis of the RNA-seq data revealed that the expression of the genes coding for the six putative nabscessin biosynthetic enzymes (A–F), was 30-fold or more higher in mCD medium than in Waksman medium (Fig. 6). Enhancement of the expression of these genes in production medium is consistent with the use of the corresponding proteins in the proposed biosynthetic pathway (Fig. 4).

Production conditions for nabscessins A–C were further examined, as shown in Fig. 7. Figure 7c, which corresponds to Fig. 5a, shows the HPLC profile for the extract of *Nocardia abcessus* obtained when the strain was precultured in BHI+ medium[5] before shifting to mCD medium (mCD with BHI+). Figure 7b shows the HPLC profile for the extract of *Nocardia abcessus* obtained when the BHI+ medium used in precultur-

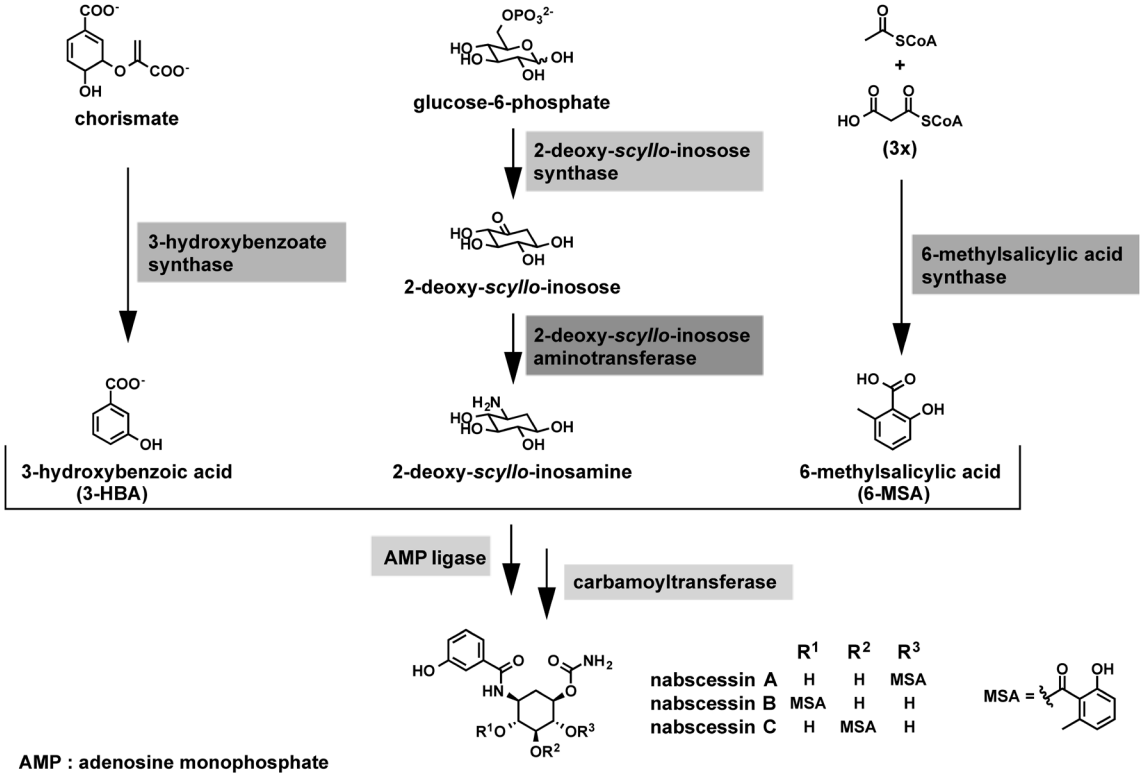


Fig. 4. The Proposed Biosynthetic Pathway for Nabscessins A–C

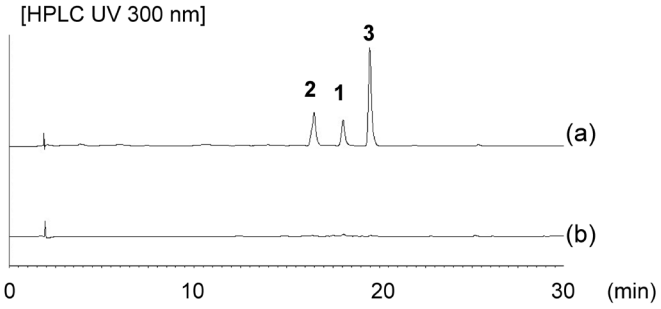


Fig. 5. Comparison of Extracts of Culture Broth Following Growth in mCD or Waksman Medium

a) mCD medium, b) Waksman medium.

ing was removed from the strain (by centrifugation and washing with mCD medium before shifting to mCD medium; mCD without BHI+). Notably, nabscessins A–C were produced in mCD with BHI+ (Fig. 7c), while these compounds were not produced in mCD without BHI+ (Fig. 7b). Thus, the presence of the BHI+ medium used for preculture was essential for the production of nabscessins A–C in mCD medium. On the other hand, when *Nocardia abcessus* was co-cultured with the mouse macrophage-like cell line J774.1[22] in mCD medium without BHI+, nabscessins A–C were produced (Fig. 7d); a culture of the J774.1 cell line alone (single culture) did not yield nabscessins A–C (Fig. 7a). These observations demonstrate that nabscessins A–C were produced by *Nocardia abscessus* only when the bacteria were cultured in mCD medium with BHI+ medium or J774.1.

In the present study, a new molecule, nabscessin C (**1**), was isolated from *Nocardia abscessus* IFM 10029^T, and the absolute structure of nabscessin A was determined by conversion into a tetraacetyl derivative (**4**) through hydrolysis and acetylation. Gene analysis suggested the involvement of six enzymes (A–F) in a proposed pathway for the biosynthesis of nabscessins (Fig. 4); RNA-seq identified a corresponding gene cluster that is selectively expressed in mCD, the growth medium in which biosynthesis of the nabscessins is observed.

Experimental

General Experimental Procedures The following instruments were used in the present study: a P-2200 polarimeter (JASCO) for optical rotations; an ECZ-600 spectrometer (JEOL) for NMR spectroscopy (solvent chemical shifts were used as the internal standard); and a JMS-T100LP (JEOL) for HR-ESI-MS. A HPLC system consisting of a PU1580 (JASCO) pump, along with UV 970 (JASCO) UV and RI 1530 detectors, was used; chromatographic data were collected using a chart recorder (ROSS). A Shimadzu LCMS system (Shimadzu, Japan) consisting of LC-20AD pumps, DGU-12A$_3$ online 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; chromatographic data were collected and processed using LabSolution software (version 5.42 SP4, Shimadzu). The conditions of the LCMS analysis were as 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, Japan); column: COSMOSIL 5C$_{18}$-AR-II (ϕ2.0×150 mm, Nacalai Tesque, Japan). The following adsorbents were used for purification: Silica gel 60 F$_{254}$ (0.25 mm, Merck, Germany) and Silica gel 60 RP-8 F$_{254}$ S

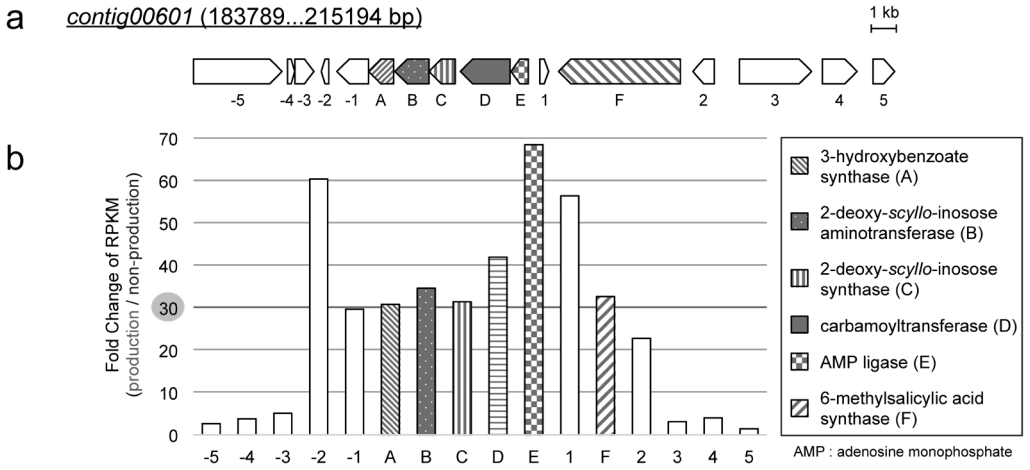


Fig. 6. (a) A Schematic of the Putative Cluster and (b) Expression of Putative Nabscessin Biosynthetic Genes (A–F) in mCD Medium (Production) Compared to That in Waksman Medium (Non-production) Based on Fold Change of RPKM (Reads per Kilobase of Exon per Million Mapped Sequence Read) Values

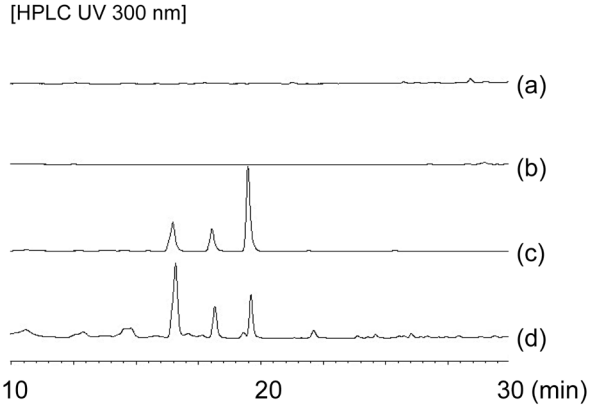


Fig. 7. Comparison of Extracts of Culture and Co-culture Broth in mCD Medium

a) Single culture, J774.1, b) single culture, *Nocardia abscessus* in mCD without BHI+, c) single culture, *Nocardia abscessus* in mCD with BHI+, d) co-culture, *Nocardia abscessus* and J774.1 in mCD without BHI+.

(0.25 mm, Merck) for analytical TLC; Chromatrex ODS (Fuji Silysia Chemical, Japan) for column chromatography; COSMOSIL $5C_{18}$-AR-II (ϕ10.0×250 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), SOFT INCUBATOR SLI-450ND (EYELA), and LTI-1200E (EYELA) for incubation; DOUBLE SHAKER NR-30 (TAITEC) and Bio-Shaker BR-30L (TAITEC) for shaking incubation; BIOLABO BL-171 (Juji Field, Japan) for CO_2 incubation; and M150-IVD (Sakuma, Japan), HIMAC CENTRIFUGE (Hitachi, Japan), KS-5000 (Kubota, Japan), and Avanti centrifuge HP-26XP (Beckman Coulter) for centrifugation.

Microbial Strain Strains of the genus *Nocardia* were stored in a freeze-dried state at the Medical Mycology Research Center, Chiba University, Japan. The GenBank accession number of *Nocardia abscessus* IFM 10029^T was BAFP00000000.1.

Culture of *Nocardia* sp. in Modified Czapek-Dox Medium for LC-MS Analysis Each strain of *Nocardia* sp. was cultured 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, Japan), and glycerol (1 mL/100 mL, Nacalai Tesque) in a 10-mL Erlenmeyer flask at 28°C for 5 d with shaking (160 rpm). Five milliliters of the culture broth were added to 25 mL 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), in a 50-mL Erlenmeyer flask. The strains were cultured at 28°C for 1 week on mCD medium. The culture broth was centrifuged at 3000 rpm, 1674×***g*** for 20 min to give the supernatant and mycelial cake; the mycelial cake was extracted with MeOH (12.5 mL×2). The MeOH extract was combined with the supernatant obtained above, and the combined materials were partitioned with EtOAc (25 mL×3) and water. The EtOAc extracts then were analyzed by LC-MS.

Fermentation for mCD Medium and Isolation An aliquot (100 μL) of the stock of strain *Nocardia abscessus* IFM 10029^T was added to 5 mL of BHI+ liquid medium in a 10-mL Erlenmeyer flask and cultured at 160 rpm for 5 d at 28°C. Aliquots (500 μL each) of the resulting culture broth were added to each of two 25-mL volumes of BHI+ liquid medium in 200-mL Erlenmeyer flasks, and the resulting cultures were incubated with shaking at 28°C for 5 d. Portions (15 mL each) of the resulting culture broth in BHI+ liquid medium were added to each of five 500-mL volumes of mCD medium in 1-L Erlenmeyer flasks, and the resulting cultures were incubated with shaking at 28°C for 1 week. The resulting culture broth (2.0 L) was centrifuged at 3000 rpm, 1674×***g*** for 20 min to give the supernatant and mycelial cake; the mycelial cake then was extracted with MeOH (1.0 L×2). The MeOH extract was combined with the 2-L supernatant obtained above, and the combined materials were partitioned with EtOAc (2.0 L×3) and water. The EtOAc layer (202.9 mg) was subjected to ODS column chromatography (ϕ14×320 mm, MeOH–H_2O system) to give fractions 1A–1O. Fraction 1F (MeOH : H_2O=3 : 2, 6.7 mg) was subjected to reverse-phase HPLC [COSMOSIL $5C_{18}$-AR-II (ϕ10.0×250 mm); eluent: 40% MeOH; flow rate: 1.5 mL/min; UV detection: photodiode array (190−400 nm)] to give compound **1** (0.5 mg, t_R 28.0 min) and **3** (0.8 mg, t_R 40.0 min). Compound **2** was obtained in fraction 1E

(MeOH : H_2O=7 : 3, 9.5 mg).

Nabscessin C (1) Colorless amorphous solid. HR-ESI-MS *m/z*: 483.1426 $[M+Na]^+$ (Calcd for $C_{22}H_{24}N_2O_9Na$: 483.1380). ^1H- and ^{13}C-NMR data are provided in Table 1. As shown in Fig. S2 (Supplementary materials), compound **1** was unstable and other physical data of **1** were not determined.

Transcriptional Analysis RNA was extracted from the strain *Nocardia abscessus* IFM 10029^T cultured under nabscessin-producing and non-producing conditions. mCD medium was used for the nabscessin-producing conditions, while Waksman medium was used for the non-producing conditions. Specifically, the strain *Nocardia abscessus* IFM 10029^T, precultivated on BHI+ medium at 28°C for 3 d, was inoculated into nabscessin-producing and non-producing media. After incubation at 28°C for 7 d, the cells of *Nocardia abscessus* IFM 10029^T cultured in either of the two media were collected and resuspended in RNeasy Mini Kit (Qiagen, U.S.A.) Buffer RLT with β-mercaptoethanol. The resuspended cells were homogenized using a multi-bead beater (Yasui Kikai) at 2500 rpm for 60 s at 4°C, and RNA was extracted according to the RNeasy Mini Kit protocol. Following the deoxyribonuclease (DNase) treatment, total RNA was treated with a Ribo-Zero Gram-positive bacteria Kit (Illumina) to remove rRNA. For construction of the RNA-seq libraries, each of the mRNA samples obtained were treated with a KAPA Stranded RNA-seq Kit (Kapa Biosystems, U.S.A.) according to the manufacturer's protocol. The constructed libraries were sequenced using the HiSeq system (Illumina). The resulting data were mapped against the sequenced genome data of *Nocardia abscessus* IFM 10029^T using CLC GenomicsWorkbench 6.5.1 (CLC bio).

Seed Culture of a Mouse Macrophage-Like Cell Line (J774.1) in a 75-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 15 mL of Dulbecco's modified Eagle's medium (DMEM) (Wako) with 10% fetal bovine serum (FBS, Bio West) in 75-cm^2 cell culture flasks (Violamo), which reached approximately 80–90% confluency after 5–7 d. 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 abscessus* for a Co-culture in mCD Medium *Nocardia abscessus* was cultivated in 5 mL of BHI+ liquid medium in a 10-mL Erlenmeyer flask at 28°C for 5 d 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. 2 mL of mCD medium 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. OD_{600} was measured for counting the number of strain.

Co-culture in mCD Medium in a Cell Culture Flask 25 mL of mCD medium was added to J774.1 in a cell culture flask after the removal of DMEM with 10% FBS medium. A suspension of *Nocardia abscessus* was added to the flasks until the cell number ratio was reached (J774.1: *Nocardia abscessus*.=1 : 10). Flasks were incubated at 28°C for 1 weeks.

(1*R*,2*S*,3*S*,4*R*,5*S*)-5-Acetamidocyclohexane-1,2,3,4-tetrayl Tetraacetate (4) Six mole aq. HCl (1.5 mL) was added to a solution of nabscessin A (**2**, 7.0 mg, 0.015 mmol), which was then stirred at 100°C for approximately 5 d. After addition of DOWEX 2×8, the reaction mixture was evaporated and EtOAc was added to the residue. The EtOAc solution was extracted 3 times with H_2O. After removal of H_2O by evaporation, pyridine (1.0 mL) and acetic anhydride (500 μL) were added to the residue (5.6 mg), and the mixture was then stirred at room temperature for 18 h. After the solvent was removed, H_2O was added and then the H_2O solution was extracted 3 times with EtOAc. The organic layer was evaporated *in vacuo* and the residue was separated by reverse-phase HPLC [COSMOSIL 5C_{18} AR-II (ϕ10.0×250 mm); eluent: 40% MeOH; flow rate: 1.5 mL/min; UV detection: photodiode array (190−400 nm)] to give **4** (1.0 mg, 0.0026 mmol, 18% yield, t_R 18.0 min).

^1H-NMR (600 MHz, $CDCl_3$) δ: 5.62 (1H, d, J=8.3 Hz), 5.17 (1H, t, J=9.6 Hz), 5.10 (1H, t, J=9.6 Hz), 4.98 (1H, ddd, J=12.4, 9.6, and 4.8 Hz), 4.89 (1H, dd, J=11.0 and 9.6 Hz), 4.15 (1H, m), 2.42 (1H, dt, J=12.4 and 4.8 Hz), 2.03 (3H, s), 1.99 (3H, s), 1.90 (9H, s), 1.47 (1H, q, J=12.4 Hz). $[\alpha]_D^{26}$−10.6 (*c* 0.5, $CHCl_3$). ESI-MS *m/z*: 374 $[M+H]^+$.

Acknowledgments This study was supported by JSPS KAKENHI Grant Number 17H03992 and the Strategic Priority Research Promotion Program of Chiba University, titled "Phytochemical Plant Molecular Sciences."

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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