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ISOLATION OF INOHANALACTONE, A γ-BUTYROLACTONE, FROM *NOCARDIA INOHANENSIS* IFM0092^{T}

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Abstract – Because research on the genus *Nocardia* has not progressed as compared to the extensively studied genus *Streptomyces*, *Nocardia* is considered as a useful undeveloped resource for exploring natural products. In this study, a new γ-butyrolactone derivative, named inohanalactone (**1**), was isolated from *Nocardia inohanensis* IFM0092^{T}. Inohanalactone possesses an aliphatic side chain containing 8 carbons, a vicinal diol, and a *cis* double bond. Inohanalactone was produced by *N. inohanensis* IFM0092^{T} in the modified Czapek–Dox medium, whereas it was not produced in other media such as Nutrient broth, Waksman, and Yeast-Malt-Glucose media.

Actinomycetes of the genus *Nocardia* are Gram-positive bacteria widely present in nature. Many species in this genus possess weak virulence for human and some species are known to infect other animals and plants. Although several secondary metabolites have been isolated from the genus *Nocardia*, such as nocardicin A, a monocyclic *β*-lactam, from *Nocardia uniformis* subsp. *tsuyamanensis* ATCC21806[1] and nocardialactone, a *β*-lactone, from *Nocardia brasiliensis* IFM0406,[2] research on the genus *Nocardia* has not progressed when compared to the genus *Streptomyces*.

Recently, we have focused on the secondary metabolites of the genus *Nocardia*,[3,4] and the search for new compounds was initiated by culturing strains of the genus *Nocardia* in several culture-media, such as Nutrient broth,[5] modified Czapek–Dox,[6] Waksman,[7] and Yeast-Malt-Glucose,[8] to obtain small-scale culture extracts for HPLC analysis. In our previous report,[9,10] three characteristic peaks in the extract of *Nocardia abscessus* IFM10029^{T} grown in the modified Czapek–Dox (mCD) medium were observed and identified as new aminocyclitol derivatives, nabscessins A, B, and C. In the present study, we describe the isolation and structural elucidation of a new compound (**1**) obtained from *Nocardia inohanensis* IFM0092^T grown in the mCD medium (Figure 1). HPLC examination revealed that compound **1** was preferentially produced in the mCD medium, while it was not produced in the other three media studied (Figure 2).

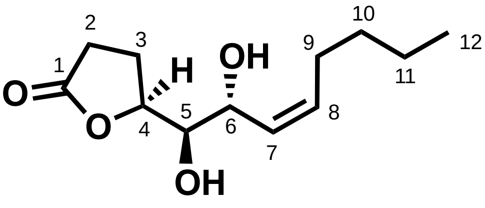

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Figure 1. Structure of inohanalactone (**1**)

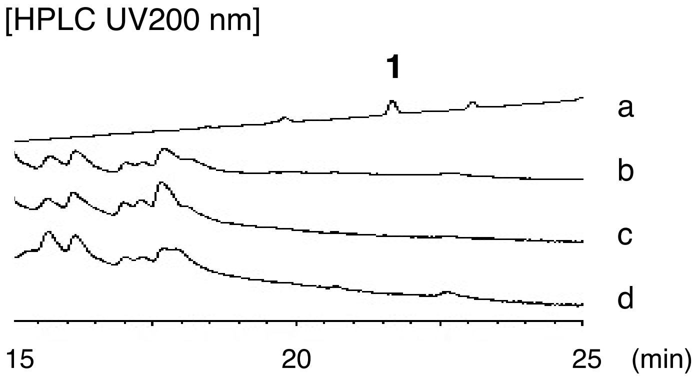

Figure 2. Comparison of extracts of culture broth prepared in
(a) modified Czapek–Dox, (b) Waksman, (c) NB, and (d) YMG media

A large-scale culture (2.0 L) of *Nocardia inohanensis* IFM0092^T was performed by growing the strain in the mCD medium for 2 weeks at 28 °C in ambient air as a shaking culture (160 rpm) in four 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 ODS column chromatography (H_2O–MeOH system) to obtain new compound (**1**), designated inohanalactone (Figure 1).

The molecular formula of compound **1** was determined to be $C_{12}H_{20}O_4$ using ESI-MS [*m/z* 479 $[2M+Na]^+$ and 273 $[M+HCOO]^-$] as well as NMR spectroscopy (Table 1). The ^{1}H NMR spectrum of **1** recorded in CD_3OD shows the presence of one *Z*-olefin [δ_H 5.60 (1H) and 5.44 (1H); J = 10.5 Hz], three sp^3 methines (δ_H 4.67–3.68), five methylenes (δ_H 2.52–1.35), and one methyl (δ_H 0.90). The ^{13}C NMR spectrum of **1** exhibited 12 peaks (Table 1), which were assigned to one quaternary carbon, one olefin, three sp^3 methines, five methylenes, and one methyl upon analysis of the HMQC spectrum. The cross-peaks observed in the ^1H-^1H COSY spectrum of **1** suggests the presence of two partial structures (A and B), as indicated in Figure 3. The HMBC spectrum of **1** shows a correlation between the methyl protons (H_3-12, δ_H 0.90) in partial structure B and one methylene carbon (C-10, δ_C 33.0) in partial structure A, suggesting that partial structure A was connected to partial structure B (Figure 4). The HMBC correlations observed between the two methylene protons [H-2 (δ_H 2.52) and H-3 (δ_H 2.29)] and the carbonyl carbon [C-1 (δ_C 180.5)], which was also correlated to the sp^3 oxymethine proton [H-4 (δ_H 4.67)], suggest that a γ-butyrolactone moiety constructs the planar structure of **1** (Figure 4). The stereochemistry of **1** was elucidated by comparing the coupling constants of H-4 (J = 5.5 and 6.9 Hz), H-5 (J = 5.5 Hz), and H-6 (J = 9.3 and 5.5 Hz) recorded in $CDCl_3$ (Supporting Information, Table S1) with those of pseudonocardide A,[11] which has a related structure (Table S1). The absolute configuration of pseudonocardide A was established using X-ray analysis of its 4-bromobenzoate derivative.[11] Compound **1** is likely to have the same absolute configuration as pseudonocardide A (4S, 5S, 6R) since they exhibit similar optical rotation values (**1** $[\alpha]_D$ –23.3; pseudonocardide A $[\alpha]_D$ –27.8). In the genus *Streptomyces*, many γ-butyrolactones have been isolated and are known to act as signal compounds for bacterial communication.[12] However, there are no reports of a γ-butyrolactone compound from the genus *Nocardia* reported to date. Compound **1** showed no cytotoxicity to human gastric adenocarcinoma (AGS) cells at 50 µg/mL (96% cell viability).

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

	1 (inohanalactone)	
position	δ_H (J in Hz)	δ_C
1		180.5
2	2.45 m 2.52 m	29.4
3	2.22 m 2.29 m	23.0
4	4.67 ddd (6.9, 4.1, 1.4)	82.3
5	3.68 dd (6.6, 4.1)	75.9
6	4.35 dd (9.0, 6.6)	68.8
7	5.44 ddt (10.5, 9.0, 2.1)	129.8
8	5.60 dt (10.5, 6.9)	135.2
9	2.13 m	28.6
10	1.35 m	33.0
11	1.35 m	23.4
12	0.90 t (6.9)	14.3

Measured in CD_3OD, 600 MHz (^1H), 150 MHz (^{13}C) δ in ppm, J in Hz.

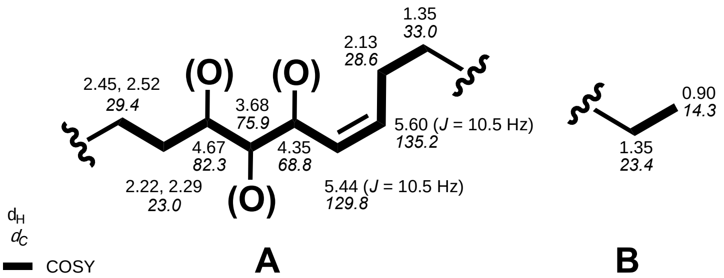

Figure 3. Partial structures A and B

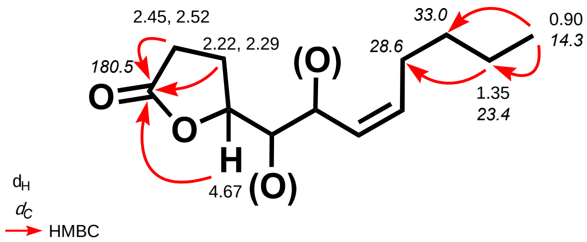

Figure 4. HMBC correlations of **1**

In the present study, a new natural product, inohanalactone (**1**) was isolated from *Nocardia inohanensis* IFM0092^T, and its structure was elucidated using spectroscopic analysis and a comparison of the coupling constants and optical rotation with those of a related compound.

EXPERIMENTAL

General experimental procedures

The following instruments were used in the present study: A P-2200 polarimeter (JASCO) was used to determine the optical rotation; a UV mini 1240 UV-vis spectrophotometer (Shimadzu) for UV-vis spectroscopy; an ECZ-600 spectrometer (JEOL) for NMR spectroscopy (the chemical shift of the NMR solvent was used as an internal standard); and a JMS-T100LP (JEOL) for HR-ESIMS. 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, and LCMS 2020 for ESIMS was used and an N$_2$ Supplier Model 24F was used for N$_2$ generation; chromatographic data were collected and processed using LabSolution software (version 5.42 SP4, Shimadzu). The conditions used for LCMS analysis were as follows: 0–100% MeOH in 0.1% HCO$_2$H, 0–30 min, linear gradient, and 100% MeOH in 0.1% HCO$_2$H, 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 (φ 1.5 × 10 mm, Nomura Chemical); column: COSMOSIL 5C$_{18}$-AR-II (φ 2.0 × 150 mm, Nacalai Tesque). The following adsorbents were used for purification: Silica gel 60 F254 (0.25 mm, Merck) and silica gel 60 RP-8 F254 S (0.25 mm, Merck) for analytical TLC; Chromatrex ODS (Fuji

Silysia Chemical) for column chromatography. The following instruments were used for cultivation and extraction: FMC 1000 (EYELA) and SOFT INCUBATOR SLI-450ND (EYELA) as incubators; M150-IVD (Sakuma), HIMAC CENTRIFUGE (Hitachi), KS-5000 (Kubota), and Avanti centrifuge HP-60XP (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. These strains were re-identified by sequencing the 16S rRNA gene.

Fermentation for mCD medium and isolation

An aliquot (500 µL each) of the stock strain of *Nocardia inohanensis* IFM0092^T was added to 25 mL of BHI+ liquid medium in a 50 mL Erlenmeyer flask and cultured at 160 rpm for 5 d at 28 °C. The resulting culture broth was transferred to four 50 mL tubes. The supernatant was removed after centrifugation at 3000 rpm, 1674 ×g at 20 °C for 2 min. A strain suspension was prepared upon adding 25 mL of mCD medium to the tube. The strain suspensions (25 mL each) were added to 500 mL of mCD medium in five 1 L Erlenmeyer flasks and the resulting cultures were incubated with shaking at 28 °C for 2 weeks. The resulting culture broth (2 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 L × 2). The MeOH extract was combined with the supernatant obtained above and the combined materials partitioned between EtOAc (2 L × 3) and water. The combined EtOAc layers were concentrated in vacuo and the resulting residue (244.9 mg) subjected to ODS column chromatography (φ 17 × 230 mm, MeOH–H_2O system) to give fractions 1A–1L. Compound **1** was obtained in fraction 1F (MeOH–H_2O = 1:1, 3.7 mg).

*Inohanalactone (**1**)*

Yellow oil. $[\alpha]^{26}_{D}$ –23.3 (*c* 0.1, MeOH). ESIMS *m/z* 479 $[2M+Na]^+$ and 273 $[M+HCOO]^-$. ^1H and ^{13}C NMR data (Table 1).

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