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Coculture of a Pathogenic Actinomycete and Animal Cells To Produce Nocarjamide, a Cyclic Nonapeptide with Wnt Signal-Activating Effect

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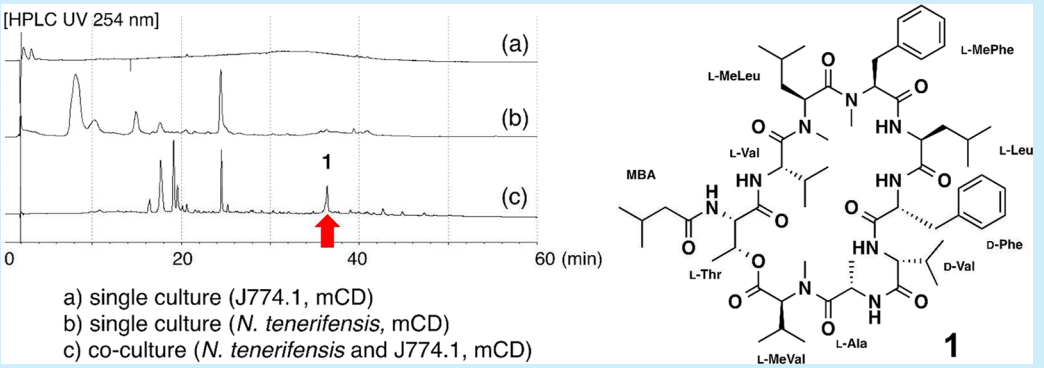
Supporting Information



ABSTRACT: A coculture method with a pathogenic actinomycete of the genus *Nocardia* and an animal cell line was designed to reconstruct and emulate the initial infection state, and a new cyclic nonapeptide, named nocarjamide (**1**), was obtained by coculture of *Nocardia tenerifensis* IFM 10554[T] and the mouse macrophage-like cell line J774.1 in a modified Czapek−Dox medium. Nocarjamide (**1**) exhibited Wnt signal-activating effects.

Actinomycetes of the genus *Nocardia* are Gram-positive bacteria that are widely present in natural environments such as soil and water. Many of these species possess pathogenicity and infect the human lungs, skin, brain, and other organs, causing nocardiosis. The study of the genus *Nocardia* began with the isolation of the first *Nocardia* actinomycete, *Nocardia farcinica*, by French veterinarian Edmond Nocard in 1888.[1] Although there are some reports on secondary metabolites of *Nocardia* spp., such as nacardicin A,[2] nargenicins,[3] and others, research on members of the genus *Nocardia* has not progressed as rapidly as that on members of the genus *Streptomyces*.

Microbial coculture is a method of culturing two or more kinds of microorganisms in the same environment, thereby potentially activating the biosynthetic genes responsible for the production of secondary metabolites.[4] This microbial coculture is inspired by naturally occurring microbial communities, where microbial interactions through secondary metabolites are related to chemical defense and various other phenomena. Coculture can be conducted in a solid or liquid medium and has been widely used since the 1990s for research on the interaction between microorganisms in nature and in the search for novel, biologically active metabolites. Coculture in a liquid medium has been performed using various combinations of microorganisms such as bacteria−fungi, bacteria−bacteria, archaea−fungi, and fungi−fungi.[4] Recently, we described a new method for producing secondary metabolites by "coculture" using pathogenic bacteria in combination with animal cells to reconstruct and emulate the initial infection state.[5] An actinomycete, *Nocardia tenerifensis* IFM 10554[T], was used as the pathogenic bacteria, while a mouse macrophage-like cell line, J774.1, was used as the animal cell. In the present study, nocarjamide (**1**), a new cyclic nonapeptide with a Wnt signal-activating effect, was obtained in a modified Czapek−Dox (mCD)[6] medium by performing this new coculture method using *N. tenerifensis* IFM 10554[T] and J774.1 cells.

The bacterial strain for the coculture study was initially selected from among 76 strains, all belonging to the genus *Nocardia*, that were obtained from the Medical Mycology Research Center at Chiba University. A phylogenetic tree was prepared using members of the group that harbored the

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nocobactin-related biosynthetic gene cluster[7] as an index, yielding classification of the strains into six 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. otitidiscaviarum* IFM 0239^T, and *N. vulneris* NBRC 108936^T) were selected for further study. A mouse macrophage-like cell line, J774.1, was selected for use as the animal cell line for coculturing in an effort to reconstruct the initial infection state. Based on LC−UV analysis of the culture broth extract obtained under various conditions, we focused on the extract of *Nocardia tenerifensis* IFM 10554^T cultured in the presence of J774.1; this coculture extract yielded several specific peaks (Figure 1). Thus, this bacterial strain was selected for further study.

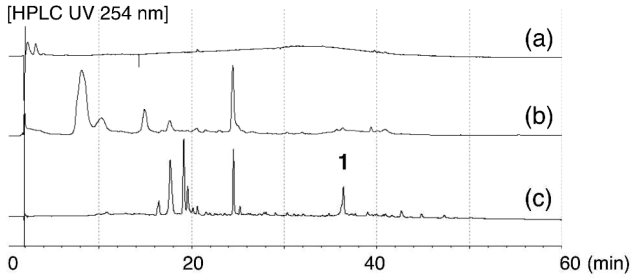

Figure 1. Comparison of extracts of culture broth: (a) single culture (J774.1, mCD), (b) single culture (*N. tenerifensis*, mCD), (c) coculture (*N. tenerifensis* and J774.1, mCD).

A large-scale (0.8 L) coculture of *N. tenerifensis* IFM 10554^T and J774.1 in an mCD medium at a cell number ratio of 10:1, respectively, 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 a methanol extract of the mycelia and animal cells were combined and subjected to partitioning between ethyl acetate (EtOAc) and water. The EtOAc fraction was subjected to fractionation by ODS column chromatography (the MeOH−H_2O system), followed by reversed-phase HPLC separation (85% MeOH) to yield a new compound **1**, named nocarjamide (Figure 2). LC−MS analysis revealed that compound **1** was produced only under coculture of *N. tenerifensis* IFM 10554^T

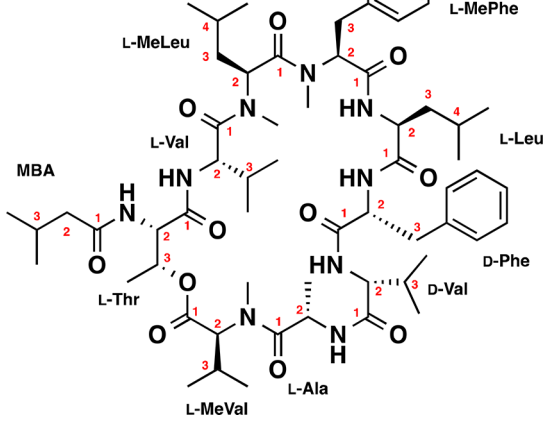

Figure 2. Structure of **1**.

and J774.1, while compound **1** was not detected under single culture conditions (i.e., with *N. tenerifensis* IFM 10554^T or J774.1 only) (Figure 1).

Nocarjamide (**1**) was revealed to have the molecular formula $C_{60}H_{93}N_9O_{11}$ by high-resolution ITTOFMS (obsd m/z 1138.6897 $[M + Na]^+$, calcd for $C_{60}H_{93}N_9O_{11}Na$, 1138.6892).

The ^{1}H NMR spectrum of nocarjamide (**1**) measured in DMSO-d_6 (Figure S5, Supporting Information (SI)) showed six NH [δ_H 8.80 (1H), 8.55 (1H), 8.42 (1H), 7.82 (1H), 7.58 (1H), 7.32 (1H)] and three NCH_3 [δ_H 3.09 (3H), 2.69 (3H), 2.57 (3H)]. The cross-peaks observed in the 2D TOCSY (Figure S8−S10, SI) and HMBC (Figure S12, SI) data of **1** suggested the presence of one alanine (Ala), one leucine (Leu), one phenylalanine (Phe), one threonine (Thr), two valines (Val^1 and Val^2), one *N*-methyl leucine (MeLeu), one *N*-methyl phenylalanine (MePhe), one *N*-methylvaline (MeVal), and one 3-methylbutanoic acid (MBA) residue (Figure S22, Table S1, SI).

As shown in Figure 3, the HMBC spectrum of **1** in DMSO-d_6 showed correlations from MeVal-NCH_3 (δ_H 2.57) to Ala-

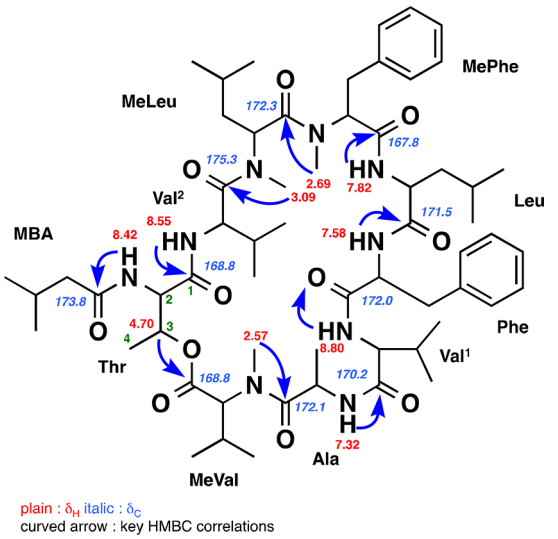

Figure 3. HMBC correlations of **1**.

CO (δ_C 172.1), Ala-NH (δ_H 7.32), and Val^1-CO (δ_C 170.2) from Val^1-NH (δ_H 8.80) to Phe-CO (δ_C 172.0), from Phe-NH (δ_H 7.58) to Leu-CO (δ_C 171.5), from Leu-NH (δ_H 7.82) to MePhe-CO (δ_C 167.8), from MePhe-NCH_3 (δ_H 2.69) to MeLeu-CO (δ_C 172.3), from MeLeu-NCH_3 (δ_H 3.09) to Val^2-CO (δ_C 175.3), and from Val^2-NH (δ_H 8.55) to Thr-CO (δ_C 168.8), suggesting that all nine constituent amino acids were connected by eight amide bonds to yield a sequence of Thr-Val^2-MeLeu-MePhe-Leu-Phe-Val^1-Ala-MeVal. HMBC correlations observed from Thr-NH (δ_H 8.42) to MBA-CO (δ_C 173.8) suggested that an *N*-terminal Thr was connected to MBA by an amide bond, whereas HMBC correlations observed from Thr-3 (δ_H 4.70) to MeVal-CO (δ_C 168.8) implied that a *C*-terminal MeVal was connected to the Thr by an ester bond (Figure 3).

The NOESY spectrum of **1** in DMSO-d_6 (Figure S13, SI) showed correlations between MeVal-2 (δ_H 3.96) and Ala-2 (δ_H 4.91); Ala-NH (δ_H 7.32) and Val^1-2 (δ_H 5.09); Ala-NH (δ_H 7.32) and Val^1-4, 5 (δ_H 0.89); Val^1-NH (δ_H 8.80) and Phe-2 (δ_H 5.06); Val^1-NH (δ_H 8.80) and Phe-3 (δ_H 2.59, 3.03); Phe-NH (δ_H 7.58) and Leu-2 (δ_H 4.22); Phe-NH (δ_H 7.58) and

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Leu-3 (δ_H 1.34); Leu-NH (δ_H 7.82) and MePhe-2 (δ_H 4.36); Leu-NH (δ_H 7.82) and MePhe-NCH_3 (δ_H 2.69); MePhe-2 (δ_H 4.36) and MeLeu-2 (δ_H 4.98); MePhe-5 (δ_H 7.17) and MeLeu-3 (δ_H −0.62); MePhe-5 (δ_H 7.17) and MeLeu-5 (δ_H 0.48); MeLeu-NCH_3 (δ_H 3.09) and Val^2-2 (δ_H 4.62); Val^2-NH (δ_H 8.55) and Thr-2 (δ_H 4.58); Val^2-NH (δ_H 8.55) and Thr-3 (δ_H 4.70); Thr-NH (δ_H 8.42) and MBA-2 (δ_H 1.95, 2.05), suggesting the planar structure of nocarjamide (**1**) (Figure 4). This planar structure was also consistent with 2D NMR analysis in $CDCl_3$ (Figures S15−S21, Table S2, SI).

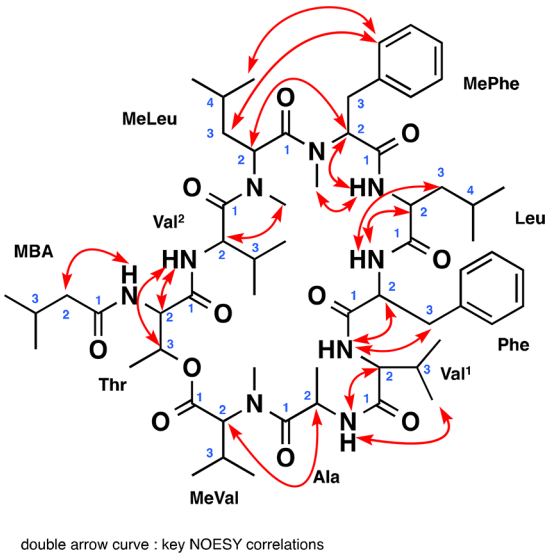

Figure 4. NOESY correlations of **1**.

Furthermore, when MS/MS analysis was carried out using a positive ion peak m/z 1138 $[M + Na]^+$ as a precursor ion, all of the product ions were observed as sodium adducts. The product ions of **1** observed at m/z 1025, 954, 855, and 708 indicated the sequential losses of the MeVal, Ala, Val^1, and Phe from the molecule of **1**, respectively. The product ions at m/z 908, 809, and 662 were assigned to be the result of sequential losses of MeVal-Ala, Val, and Phe coupled with the elimination of CO and H_2O from **1**, respectively. The product ions at m/z 577 and 416 were assigned to be the dehydrated fragments of MBA-Thr-Val^2-MeLeu-MePhe and MBA-Thr-Val^2-MeLeu, respectively. Also, when MS^3 analysis was carried out using a product ion at m/z 954 in MS/MS as the precursor ion, the product ion at m/z 307 indicated the fragment of MBA-Thr-Val^2. Additionally, the above-mentioned characteristic dehydrated product ions at m/z 908, 809, 662, 577, and 416 suggested the presence of the Thr residue in the molecule (Figure 5 and Table S3, SI). These results were also consistent with the planar structure of nocarjamide (**1**) as suggested by NMR analysis.

The absolute configuration of amino acids of compound **1** was determined by the advanced Marfey's method.[9] Based on the results of LC−MS, compound **1** was revealed to consist of L-Ala, L-Leu, D-Phe, L-Thr, L-MeLeu, L-MePhe, and L-MeVal (Figure S1, SI). It also was found that the two valines (Val^1 and Val^2) consist of one D-Val and one L-Val, although which one is D or L remains undefined. The absolute configurations of two valines were firmly established by an X-ray crystallographic analysis of **1** (Figure 6), implying that Val^1 and Val^2 are D and L, respectively. X-ray analysis of **1** revealed that two conformers

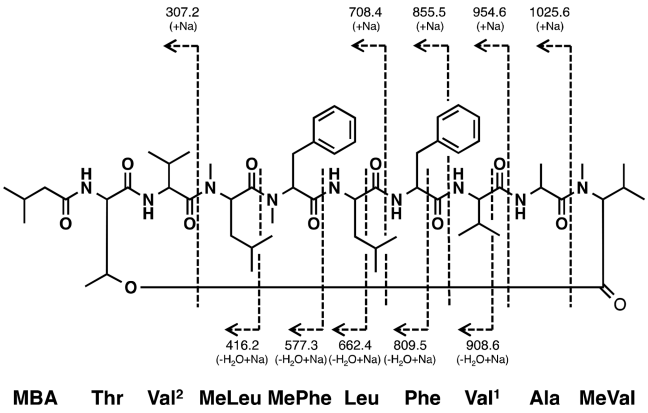

Figure 5. MS/MS and MS^3 fragment analysis of **1**.

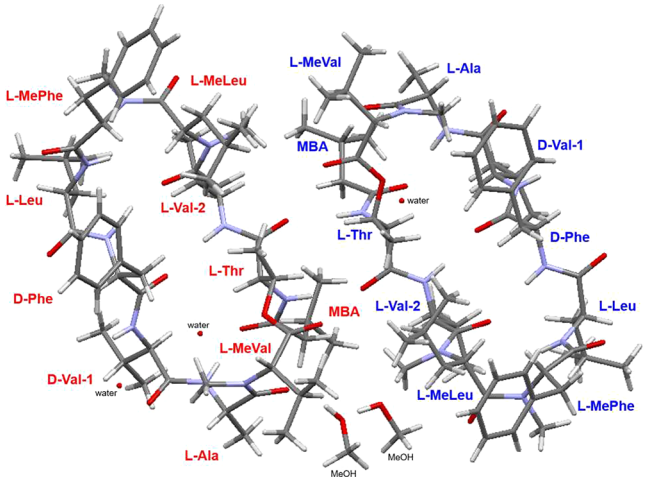

Figure 6. X-ray structure of nocarjamide (**1**).

are present in a 1:1 ratio in the crystal state of **1** (Figures S24−S27, SI).[8] As a result, the whole structure of nocarjamide was concluded to be **1**, as shown in Figure 2. In the ^1H and ^{13}C NMR in $CDCl_3$ solution of **1**, the presence of two conformers was also observed (Figures S15 and S16, Table S2, SI). The ratio of conformers (1:1) was consistent between crystal and solution ($CDCl_3$) states, while the structure of two conformers of the solution ($CDCl_3$) state remained undefined.

The production conditions of compound **1** were next examined using six kinds of media (Δsucrose, $\Delta NaNO_3$, ΔKH_2PO_4, ΔKCl, $\Delta FeSO_4$, and $\Delta MgSO_4$, Figure S2, SI) to reveal that the presence of $NaNO_3$ and $FeSO_4$ is important for production of compound **1**.

Compound **1** exhibited low cytotoxicity against several cell lines (Figures S3 and S4, SI), while **1** exhibited a Wnt signal-activation effect by using the TOPFlash luciferase assay system;[10] TCF/β-catenin transcriptional activity (TOPFlash activity) was 1.8- and 2.2-fold higher in the presence of 20 and 40 μM compound **1**, respectively (Figure 7). The FOPFlash acitivity,[10] using a construct with a mutated site to exclude false-positive signals, was also examined to demonstrate that TOPFlash activity was 1.5-fold higher than FOPFlash activity at both 20 and 40 μM compound **1**. Furthermore, we carried out Western blot analysis using compound **1** in HEK293 cells. β-Catenin is a key molecule in the Wnt pathway,[11] and an increase of β-catenin leads to upregulation of the Wnt signal activity. c-Myc is a target protein of the Wnt pathway, and upregulation of the Wnt pathway leads to an increase of the c-

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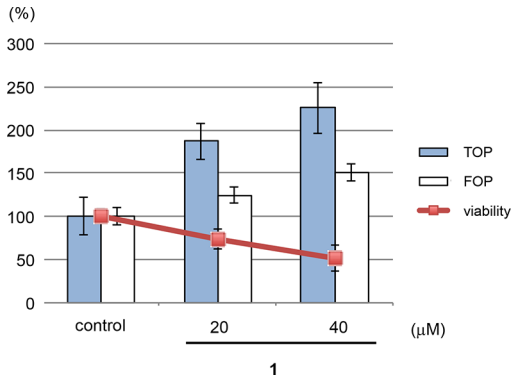

Figure 7. Effect of nocarjamide (**1**) on TCF/β-catenin transcriptional activity. Data are presented as mean ± SD of $n = 3$ independent experiments, and the data in 0.1% DMSO aqueous solution are taken as 100%. TOP: TOPFlash luciferase activity. FOP: FOPFlash luciferase activity. Viability: cell viability.

myc protein. As shown in Figure 8, compound **1** increased protein levels of not only β-catenin but also c-myc. This result

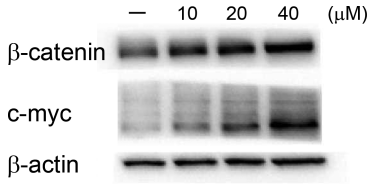

Figure 8. Effects of nocarjamide (**1**) on the expression of β-catenin and c-myc in HEK293 cells.

clearly reveals that compound **1** activated Wnt signal activity and was consistent with the result of the TOPFlash luciferase activity test. The Wnt signal is known to play an important role in various processes of vital phenomena such as the formation of various tissues and the differentiation/proliferation of eukaryotic cells.[12] Regarding human pathology, suppression of Wnt signaling has been implicated in osteoporosis–pseudoglioma syndrome,[13] Parkinson's disease,[14] and other diseases. It is therefore important to identify compounds that exhibit Wnt signal-modulating activity such as **1**.

In the present study, we demonstrated the effectiveness of a method employing coculture of a pathogenic actinomycete of the genus *Nocardia* with animal cells. This technique succeeded in generating nocarjamide (**1**), a novel bioactive natural product.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b02522.

Experimental procedures, spectral data, and X-ray crystal analysis data of **1** (PDF)

Accession Codes

CCDC 1851304 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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