Coculture of Marine Streptomyces sp. With Bacillus sp. Produces a New Piperazic Acid-Bearing Cyclic Peptide

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Microbial culture conditions in the laboratory, which conventionally involve the cultivation of one strain in one culture vessel, are vastly different from natural microbial environments. Even though perfectly mimicking natural microbial interactions is virtually impossible, the cocultivation of multiple microbial strains is a reasonable strategy to induce the production of secondary metabolites, which enables the discovery of new bioactive natural products. Our coculture of marine Streptomyces and Bacillus strains isolated together from an intertidal mudflat led to discover a new metabolite, dentigerumycin E (1).

Dentigerumycin E was determined to be a new cyclic hexapeptide incorporating three piperazic acids, N-OH-Thr, N-OH-Gly, β-OH-Leu, and a pyran-bearing polyketide acyl chain mainly by analysis of its NMR and MS spectroscopic data. The putative PKS-NRPS biosynthetic gene cluster for dentigerumycin E was found in the Streptomyces strain, providing clear evidence that this cyclic peptide is produced by the Streptomyces strain. The absolute configuration of dentigerumycin E was established based on the advanced Marfey's method, ROESY NMR correlations, and analysis of the amino acid sequence of the ketoreductase domain in the biosynthetic gene cluster. In biological evaluation of dentigerumycin E (1) and its chemical derivatives [2-N,16-N-deoxydenterigumycin E (2) and dentigerumycin methyl ester (3)], only dentigerumycin E exhibited antiproliferative and antimetastatic activities against human cancer cells, indicating that N-OH and carboxylic acid functional groups are essential for the biological activity.

Keywords: marine microorganism, coculture, natural product, dentigerumycin, cyclic peptide

INTRODUCTION

Genomic analysis of microbes strongly suggests that microorganisms have more potential gene clusters that would allow them to produce more secondary metabolites than are currently known (Omura et al., 2001). Even if it is poorly understood, microorganisms are presumed to communicate with each other physically or/and chemically (Hogan and Kolter, 2002; Straight et al., 2006). Coculturing has been shown to alter individual cellular physiology and induce the production of microbial secondary metabolites that are genetically encoded but not produced under conventional laboratory culture conditions (Ueda and Beppu, 2017). In this context, coculturing different microbes to elicit the production of bioactive microbial compounds not previously observed when microbes are cultured independently could be a promising strategy to access microbial
chemical diversity. A thorough literature search indicated that chemical studies of cocultures initially focused on mixed cultures of fungi and bacteria. The first new natural product from a coculture, parentone, was discovered by cocultivation of the marine fungus Pestalotia sp. and the marine bacterium Thalassosira sp. (Cueto et al., 2001). A subsequent coculture experiment with the Thalassosira strain with the marine fungus Libertella sp. resulted in the production of new pimarane-type diterpenoids (Oh et al., 2005). In addition, the interaction between the fungus Aspergillus fumigatus and the bacterium Sphingomonas affored glnonitrin A (Park et al., 2009). Relatively recently, cocultures of two different bacterial strains have also successfully contributed to the discovery of new bioactive natural products. Cocultivating various Streptomyces strains with Tsukamurella pulmonis elicited the production of new butanolides (Hoshino et al., 2015). Ecologically relevant cocultures of marine invertebrate-associated Rhodococcus and Micromonomospora strains produced the antibiotic bis-nitroglycosylated anthracline (Adnani et al., 2017). As part of our efforts to discover new bioactive molecules from marine bacteria, we adopted the coculture strategy. Chemical analysis of a coculture of two marine bacterial strains isolated together from an intertidal mudflat in Wando, Republic of Korea, showed the induction of the formation of a bacterial metabolite. This result prompted us to scale up the coculture and subsequently characterize the metabolite. Here, we report the production, structure elucidation, putative biosynthetic gene cluster (BGC), and biological activity of the new bacterial metabolite, dentigerumycin E (1).

MATERIALS AND METHODS

General Experimental Procedures

Specific rotations were obtained using a JASCO P-2000 polarimeter with a 1-cm cell at 25°C. UV spectra were obtained using an Applied Photophysics Chirascan™-plus spectrometer with a 1-cm quartz cell at 25°C. IR spectral data were obtained using a JASCO FTIR-4200 spectrometer. NMR spectra were recorded on an 800 MHz Bunker Avance III HD spectrometer with a 5-mm TCI cryoprobe and a Bunker Avance 600 MHz spectrometer at the National Center for Inter-university Research Facilities (NCIRF). LC-MS and low-resolution electrospray ionization mass spectroscopic (LR-ESI-MS) data were obtained using an Agilent Technologies 1200 series high performance liquid chromatography (HPLC) coupled with an Agilent Technologies 6130 quadrupole MS. High-resolution fast atom bombardment MS (HR-FAB-MS) data were obtained using a JEOL JMS-700 high-resolution MS at NCIRF.

Bacterial Isolation

A mud sample was collected from the intertidal mudflat in Wando (34°18′55.5″N 126°45′21.8″E), Republic of Korea in September 2014. For bacterial isolation from the sample, 1 g of the mud sample was diluted in 10 mL of sterilized artificial seawater, and the mixture was spread onto A4 medium, actinomycete isolation agar medium, starch casein medium, chitin-based medium, Czapek-Dox agar medium, Bennet's agar medium, YPM agar medium, YPG agar medium, and K agar medium (all agar media were made with artificial seawater and 100 mg/L cycloheximide). These isolation agar plates were incubated at 25°C for 3 weeks. The actinobacterial strain JB5 and the Bacillus strain GN1 were isolated on the same plate together from actinomycete isolation agar medium. The strain JB5 was identified as Streptomyces sp. (99% identical to Streptomyces albogriseolus strain B24) on the basis of 16S rRNA gene sequence analysis (GenBank accession No. MH656702). The strain GN1 was identified as Bacillus sp. (99% identical to Bacillus cereus) by 16S rRNA gene sequence analysis (GenBank accession No. MH656703).

Coculture Experiment

Streptomyces sp. strain JB5 and various strains in different phyla were cultivated separately in 50 mL of YEME liquid medium (4 g of yeast extract, 10 g of malt extract, and 4 g of glucose as a 40% solution in 1 L of artificial seawater) in a 125-mL Erlenmeyer flask. After 4 days of cultivation in a rotary shaker at 200 rpm at 30°C, equal volumes of the liquid cultures of JB5 and other strains were mixed (10 mL to 10 mL) and inoculated into a 500-mL baffled Erlenmeyer flask containing 200 mL of YEME liquid medium. Mixed strains were cocultivated over 8 days in a rotary shaker at 200 rpm at 30°C, and the chemical profiles of the cocultures were monitored by LC-MS every 2 days. The strains cocultivated with JB5 were Bacillus sp. (GN1), Streptomyces sp. (SD53; isolated from the gut of Bombys mori), Paenibacillus sp. (CC2; isolated from the gut of Meloe proscarabaeus), Brevibacillus sp. (PTH23; isolated from the excreta of Onthophagus lenzii), Streptomyces sp. (UTZ13; isolated from the Nicrophorus concolor parasitic mites), Mycobacterium sp. (Myco6; isolated from the vegetable mold formed by Oligochaeta), Hafnia sp. (CF1; isolated from the Meloe proscarabaeus), and Bacillus sp. (HR1; isolated from the gut of Pseudopyrochroa rufula).

Genome Sequencing and Analysis

The genome of the JB5 strain was constructed de novo using Pacbio sequencing data. Genome sequencing of the JB5 strain was performed using the PacBio RS II by Chunlab, Inc. (Seoul, Republic of Korea), and sequencing data were assembled with PacBio SMRT Analysis 2.3.0 using the HGAP2 protocol (Pacific Biosciences, USA). Nucleotide sequences with 122.76-fold coverage of the Streptomyces sp. JB5 genome (~7.72 Mbp) were generated. Gene prediction was performed using Prodigal 2.6.2, and sequences were annotated with Chunlab's in-house pipeline with EggNOG 4.5, Swissprot, KEGG, and SEED as references. The dentigerumycin E BGC was identified using antiSMASH (Medema et al., 2011; Weber et al., 2015).

Production and Extraction of Dentigerumycin E

JB5 and GN1 strains were cultivated separately in 50 mL of YEME liquid medium in 125-mL Erlenmeyer flasks. After 4 days of cultivation in a rotary shaker at 200 rpm at 30°C, 10 mL of the JB5 culture and 1 mL of the GN1 culture (based on a serial dilution, approximately 4.9 × 10⁷ cells were estimated to be in 1 mL of
Bacillus sp. GN1 culture) were inoculated together into a 500-
ml baffled Erlenmeyer flask containing 200 mL of YEME liquid
medium. The Streptomyces sp. JB5 and Bacillus sp. GN1 were
cocultivated for 6 days in a rotary shaker at 200 rpm at 30°C.
A total of 120 L of the coculture was extracted twice with 180 L
of EtOAc by using a separation funnel. The EtOAc layer was
separated from the aqueous phase, and the residual water in the
organic layer was removed by adding anhydrous sodium sulfate.

Isolation of Dentigerumycin E
The crude extract was filtered through a 25HP045AN syringe
filter unit and directly injected onto a semipreparative reversed-
phase HPLC column (YMC-Pack ODS-A, 250 × 10 mm, C18,
5 µm) and was separated with a gradient solvent system (flow
rate: 2 mL/min; UV detection: 210 nm; 35% to 50% CH3CN/H2O
with 0.1% formic acid over 50 min). Dentigerumycin E (1)
was eluted at a retention time of 27 min and was further purified
with 0.1% formic acid over 50 min). Dentigerumycin E (1)
eluted at a retention time of 27 min and was further purified
by semipreparative HPLC using gradient solvent conditions
(column: YMC-Pack ODS-A, 250 × 10 mm, C18, 5 µm, flow
rate: 2 mL/min; UV detection: 230 nm; 66 to 88% gradient MeOH/H2O with 0.1% formic acid over 20 min). Pure
dentigerumycin E (34 mg) was obtained at a retention time of
38 min under the final purification conditions.

Dentigerumycin E (1): white, amorphous powder; [α]D25
= −3.1 (c 0.01, MeOH); UV (MeOH) λmax (log ε) 204 (3.36)
nm; IR (neat) νmax 3401, 2929, 1741, 1644, 1507, 1445, 1248,
1196 cm−1; HR-FAB-MS [M+Na]+ m/z 936.4290 (calcd for
C39H63N9O14Na, 936.4290); For 1H and 13C NMR spectral data,
Table 1.

Reduction of N-OHs in Dentigerumycin E
Dentigerumycin E (1, 10 mg) was dissolved in 4 mL of THF
(tetrahydrofuran). Aqueous ammonium acetate (2 mL, 4.5 M)
and 1 mL of 12% TiCl3 solution in 20-30 wt. % HCl were added
to the solution. The mixture was stirred at room temperature
for 2 h, and the product was extracted with 20 mL of EtOAc.
The organic layer was concentrated in vacuo to a small volume,
and its molecular formula was confirmed as C39H63N9O14 by
HR-FAB mass spectroscopic analysis. The 1H and 13C NMR
chemical shifts of 2-N,16-N-deoxycigentigerumycin E (2) were
assigned based on 1H NMR, HSQC, COSY, HMBC, ROESY, and
TOCSY analyses.

2-N,16-N-deoxycigentigerumycin E (2): white, amorphous powder; [α]D19 = −2.6 (c 0.01, MeOH); UV (MeOH) λmax (log ε)
200 (3.31) nm; IR (neat) νmax 3385, 2935, 1745, 1640, 1511, 1314,
1246, 1201 cm−1; HR-FAB-MS [M+Na]+ m/z 904.4399 (calcd for
C39H63N9O14Na, 904.4392); For 1H and 13C NMR spectral
data, Table S1.

Application of Advanced Marfey’s Method
Dentigerumycin E (1, 0.6 mg) was hydrolyzed in 0.5 mL of
6 N HCl at 115°C for 1 h with stirring, and the reaction was
quenched by cooling in an ice bath for 3 min. The HCl was
evaporated in vacuo. Then, 0.5 mL of water was added to the
dry material and dried in vacuo three times to remove the
residual HCl. The hydrolysate was lyophilized for 24 h to ensure
complete removal of the acid. The hydrolysate containing free
amino acids was divided into two vials. Each hydrolysate was
dissolved in 200 µL of 1 N NaHCO3, and 100 µL of 1-FDAA
(1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) or D-FDAA
in acetone (10 mg/mL) was added to each vial. Both vials were
incubated at 80°C for 3 min, and 100 µL of 2 N HCl was added
to neutralize the reactions. Each reaction mixture was diluted
with 300 µL of 50% CH3CN/H2O solution, and 20-µL aliquots of
each reaction product were analyzed by LC-MS using a gradient
solvent system (flow rate: 0.7 mL/min; UV detection: 230 nm; 10
to 60% CH3CN/H2O with 0.1% formic acid over 40 min)
with a reversed-phase column (Phenomenex Luna C18(2), 100
× 4.6 mm, C18, 5 µm). To determine the stereochemistry of
N-OH Thr, the reduction product (2, 1.5 mg) was hydrolyzed with
6 N HCl at 115°C for 1 h with stirring. The hydrolysate of 2 was
derivatized with Marfey reagents (1-FDAA and D-FDAA), and
the FDAA-adducts were analyzed by LC-MS in the same manner
as described above.

GITC (2,3,4,6-Tetra-O-Acetyl-β-D-
Glucopyranosyl Isothiocyanate)
Derivatization
Authentic standards of L-allo-Thr and L-Thr (0.5 mg) along
with the hydrolysate of the reduction product (2) were
dissolved in 200 µL of water in 4-ml vials. Then, 200 µL of
6% trimethylamine and 1% GITC (2,3,4,6-tetra-O-acetyl-β-D-
glucopyranosyl isothiocyanate) in acetone were added to each
vial. The reaction mixtures were stirred at 25°C for 15 min, and
the derivatization was quenched by adding 100 µL of 5% acetic
acid. Aliquots of each reaction mixture (40 µL) were analyzed by
LC-MS under gradient HPLC conditions (flow rate: 0.7 mL/min;
UV detection: 254 nm; 10 to 100% CH3CN/H2O with 0.1% formic acid over 50 min, column: Phenomenex Luna C18(2), 100
× 4.6 mm, 5 µm).

Methylation of Dentigerumycin E
Dentigerumycin E (1, 10 mg) was dissolved in 3 mL of anhydrous
MeOH. AcCl (2.5 µL) was added to the solution, and the
mixture was stirred at room temperature for 9 h with LC-MS
monitoring of the reaction progress. The reaction mixture was
concentrated in vacuo and purified by reversed-phase HPLC
(YMC-Pack ODS-A, 250 × 10 mm, C18, 5 µm) with an isocratic
solvent system (flow rate: 2 mL/min; UV detection: 210 nm; 38%
CH3CN/H2O with 0.1% formic acid). Dentigerumycin E methyl
ester (3, 1.5 mg) eluted at 41.5 min under these HPLC conditions.
Its molecular formula was confirmed as C40H62N10O16 by HR-
FAB mass spectroscopic analysis. The 1H and 13C NMR chemical
shifts of dentigerumycin E methyl ester (3) were assigned by
1H NMR, HSQC, COSY, HMBC, ROESY, and TOCSY spectral
analyses.

Dentigerumycin E methyl ester (3): white, amorphous powder; [α]D25 = −1.3 (c 0.012, MeOH); UV (MeOH) λmax (log ε) 205

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TABLE 1 | $^1$H and $^{13}$C NMR spectral data of dentigerumycin E (1) in pyridine-$d_5$.

| Position | δC | Type | δH | J (in Hz) | Position | δC | Type | δH | J (in Hz) |
|----------|----|------|----|--------|----------|----|------|----|--------|
| 1        | 170.6 | C  | 20a | 21.3 | CH$_2$  | 1.68 | m  |
| 2        | 66.9  | CH  | 20b | 1.37 | m          |
| 2-N-OH   | n.d. |   | 21a | 47.4 | CH$_2$  | 3.11 | m  |
| 3        | 68.8  | CH  | 21b | 2.95 | br. d (15.5) |
| 3-OH     | n.d. |   | 21-NH | 5.69 | br. d (13.0) |
| 4        | 19.0  | CH$_3$ | 22 | 169.3 | C          |
| 5        | 169.6 | C  | 23 | 57.0 | CH          |
| 6        | 52.7  | CH  | 23-NH | 9.13 | d (10.0) |
| 7a       | 24.4  | CH$_2$ | 24 | 77.8 | CH          |
| 7b       | 1.62  | m  | 25 | 30.6 | CH          |
| 8a       | 22.0  | CH$_2$ | 26 | 15.5 | CH$_3$  |
| 8b       | 1.06  | br. d (13.0) | 27 | 20.4 | CH$_3$ |
| 9a       | 47.3  | CH$_2$ | 28 | 178.2 | C          |
| 9b       | 2.69  | m  | 29 | 77.8 | C          |
| 9-NH     | 4.90  | br. d (13.0) | 29-OH | 6.97 | s |
| 10       | 176.3 | C  | 30 | 100.0 | C          |
| 11       | 43.7  | CH  | 30-OH | 7.03 | s          |
| 12a      | 25.2  | CH$_2$ | 31a | 28.3 | CH$_2$  |
| 12b      | 1.84  | br. d (13.0) | 31b | 2.06 | m |
| 13a      | 20.4  | CH$_2$ | 32a | 26.0 | CH$_2$  |
| 13b      | 2.02  | m  | 32b | 2.10 | m          |
| 14a      | 47.6  | CH$_2$ | 33 | 36.9 | CH          |
| 14b      | 2.93  | br. d (14.5) | 34 | 75.2 | CH          |
| 14-NH    | 5.59  | dd (12.5, 1.0) | 35 | 22.7 | CH$_3$ |
| 15       | 167.4 | C  | 36a | 38.0 | CH$_2$  |
| 16a      | 52.6  | CH$_2$ | 36b | 2.62 | dd (15.0, 4.5) |
| 16b      | 4.82  | d (17.5) | 37 | 175.2 | C          |
| 16-N-OH  | n.d. |   | 37-OH | n.d. |   |
| 17       | 174.1 | C  | 38a | 25.7 | CH$_2$  |
| 18       | 48.7  | CH  | 38b | 1.72 | m          |
| 19a      | 25.8  | CH$_2$ | 39 | 9.7  | CH$_3$ |
| 19b      | 1.92  | m  |   | 0.98 | t (7.5) |

$^1$H and $^{13}$C NMR data were recorded at 800 and 200 MHz, respectively.

(3.65) nm; IR (neat) $\nu_{\text{max}}$ 3263, 2935, 1738, 1643, 1508, 1442, 1355, 1247, 1195 cm$^{-1}$; HR-FAB-MS [M+Na]$^+$ m/z 950.4442 (calcld for C$_{40}$H$_{65}$N$_5$O$_{16}$Na, 950.4447); For $^1$H and $^{13}$C NMR spectral data, Table S1.

Cell Culture

Human cancer cells (A549, HCT116, MDA-MB-231, SK-HEP-1) and human breast epithelial cell (MCF-10A) were obtained from the American Type Culture Collection (Manassas, VA, USA) and a stomach cancer cell (SNU-638) was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in an appropriate medium (Dulbecco’s modified Eagle’s medium for MDA-MB-231 and SK-HEP-1; Roswell Park Memorial Institute 1640 for A549, HCT116 and SNU-638 cells; Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 for MCF-10A) supplemented with antibiotics-antimycotics (PSF: 100 units/mL sodium penicillin G, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B) and 10% fetal bovine serum (FBS) in an incubator containing 5% CO$_2$ at 37°C. All reagents were purchased from Gibco® Invitrogen Corp. (Grand Island, NY, USA).

Cell Proliferation Assay

Cell proliferation was measured by a sulforhodamine B (SRB) assay. Briefly, cells were seeded in 96-well plates and incubated for 30 min (for zero day controls) or treated with 1-3 for the indicated times. After incubation, the cells were fixed, dried and stained with 0.4% SRB in 1% acetic acid. Unbound dye was removed by washing, and the stained cells were suspended in 10 mM Tris (pH 10.0). The absorbance was measured at 515 nm, and the cell proliferation was determined. IC$_{50}$ values were calculated by nonlinear regression analysis using TableCurve 2D v5.01 software (Systant Software Inc., Richmond, CA, USA). All reagents were purchased from Sigma-Aldrich.
Wound Healing Assay
MDA-MB-231 cells were grown to 80–90% confluence in a 6-well plate. A confluent monolayer of MDA-MB-231 cells was artifically wounded with a 200 µL pipette tip, and the detached cells were washed with phosphate-buffered saline (PBS, Invitrogen Corp.) and then incubated with 1% FBS in medium containing various concentrations of 1–3 for 24 h. The wounds were photographed at 0 and 24 h using an inverted microscope (Olympus, Tokyo, Japan). The wound area was quantified using ImageJ Software (National Institutes of Health) and presented as wound healing (%) relative to the area of the wound at 0 h.

Cell Invasion Assay
Twenty-four-well transwell membrane inserts 6.5-mm in diameter with 8 µm pores (Corning, Tewksbury, MA, USA) were coated with 10 µL of type I collagen (0.5 mg/mL, BD Biosciences, San Diego, CA, USA) and 20 µL of a 1:20 mixture of Matrigel (BD Biosciences)/PBS. After treatment with the test compounds for 24 h, MDA-MB-231 cells were harvested, resuspended in serum-free medium, and plated (1 × 10⁶ cells/chamber) in the upper chamber of the Matrigel-coated transwell insert. Medium containing 30% FBS was used as the chemottractant in the lower chambers. After 24 h of incubation, the cells that had invaded the outer surface of the lower chambers were fixed and stained using a Diff-Quik Staining Kit (Sysmex, Kobe, Japan) and imaged. Representative images from 3 separate experiments are shown, and the number of invaded cells was counted in 5 randomly selected microscopic fields (200× magnification) (Kim et al., 2018).

RESULTS AND DISCUSSION

Coculture Experiments for the Production of Dentigerumycin E
The marine Streptomyces sp. JB5, isolated from an intertidal mudflat in Wando, Republic of Korea, was cocultivated with the marine Bacillus sp. GN1, which was associated with the Streptomyces strain JB5 (Figure S1). The production of secondary metabolites was monitored by LC-MS every 2 days. The coculture with marine Bacillus sp. GN1, which was isolated from the intertidal mudflat along with Streptomyces sp. JB5, displayed a significantly different chemical profile compared with those of the pure cultures of the strains JB5 and GN1. In particular, on the sixth day cocultivation, LC-MS analysis of the coculture of JB5 and GN1 showed a newly produced metabolite at a retention time of 12.8 min that was not virtually detected in the cultures of the individual strains (Figure 1, Figure S2). The observed compound, later identified as a new cyclic depsipeptide, dentigerumycin E (1), showed a UV absorption maximum at 204 nm and a molecular ion [M+H]+ at m/z 914. To optimize the production of dentigerumycin E, the two bacterial strains were mixed in various ratios. After 4 days of individual cultivation, the two pure cultures of JB5 and GN1 were mixed in ratios of 1:1, 2:1, 5:1, and 10:1. LC-MS analysis of the cocultures indicated that a 10:1 ratio of JB5/GN1 provides the best yield of dentigerumycin E (1).

The Streptomyces sp. JB5 was also cocultivated with phylogenetically diverse but ecologically irrelevant bacterial strains including Bacillus sp. HR1, Paenibacillus sp. CC2, Brevibacillus sp. PTH23, Streptomyces sp. SD53, Streptomyces sp. UTZ13, Hafnia sp. CF1, and Mycobacterium sp. Myc06. Most of the cocultures of Streptomyces sp. JB5 with these bacterial strains did not show the production of dentigerumycin E or the induction of other metabolites, the coculture with Bacillus sp. HR1, which is phylogenetically close to Bacillus sp. GN1, produced dentigerumycin E (Figure S3). Although the mechanism triggering the biosynthesis of dentigerumycin E by Bacillus strains remains unclear, these coculture experiment results implied that Bacillus strains, which are most closely related to B. cereus, possibly have common ability to induce the production of dentigerumycin E from Streptomyces sp. JB5.

Structure Elucidation of Dentigerumycin E
Dentigerumycin E (1) was isolated as an amorphous white powder with a molecular formula of C₃₉H₅₄N₂O₁₆ based on HR-FAB-MS analysis (obsd. [M+Na]+ at m/z 936.4290, calcd. 936.4290). The molecular formula indicated that this molecule possesses 13 degrees of unsaturation. The ¹³C NMR spectrum of 1 indicated eight carbonyl carbons (δC: 178.2, 176.3, 175.2, 174.1, 170.6, 169.4, 169.3, and 167.4), one dioxygenated carbon (δC: 100.0), 13 N/O-bound carbons (δC: 77.8, 77.8, 75.2, 68.8, 66.9, 57.0, 52.7, 52.6, 48.7, 47.6, 47.4, 47.3, and 43.7), and 18 alkyl carbons (δC: 38.0–9.7) (Table 1). The ¹H and HSQC NMR spectra of 1 indicated six exchangeable protons (δH: 9.13, 7.03, 6.97, 5.69, 5.59, and 4.90), three oxygenated methines (δC/δH: 77.8/5.95, 75.2/4.03, and 68.8/6.00), five methines at the α-positions of amino acids (δC/δH: 66.9/4.19, 57.0/5.83, 52.7/5.44, 48.7/6.22, and 43.7/6.55), four nitrogenous methylenes (δC/δH: 52.6–5.21 and 4.82, 47.6/2.93 and 2.59, 47.4/3.11 and 2.95, and 47.3/3.03 and 2.69), two alkyl methines, 10 alkyl methylenes, one singlet methyl (δC/δH: 22.71/75), three doublet methylys (δC/δH: 20.4/1.25, 19.0/1.89, and 15.5/1.35), and one triplet methyl (δC/δH: 9.7/0.98) (Table 1).

The COSY, TOCSY, and HMBC NMR spectra indicated that dentigerumycin E (1) possesses six unusual amino acid residues and one polyketide-derived substructure (Figure 2, Figures S4–S10). First, an array of COSY/TOCSY correlations constructed a spin system from H-6 to 9-NH. The HMBC correlation from H-6 to the C-5 carbonyl carbon and from 9-NH to C-6 demonstrated that this spin system includes a piperazic acid (Pip-1). Two additional piperazic acid units (Pip-2 and Pip-3) were similarly assigned based on the corresponding COSY, TOCSY, and HMBC correlations. Further analysis of COSY and TOCSY spectroscopic data revealed another discrete spin system from 23-NH to two doublet methyl groups (H₃-26 and H₃-27). The α-amino proton H-23 displayed an HMBC correlation with the carbonyl carbon at C-22, indicative of a β-hydroxy leucine (β-Ø-Leu). In addition, a N-hydroxy threonine unit (N-OH-Thr) was proposed based on the COSY/TOCSY correlations among H-2, H-3, and H₃-4. An independent α-amino methylene signal correlated with the C-15 carbonyl carbon in the HMBC NMR spectrum indicated the presence of N-hydroxy glycine (N-OH-Gly).

The final structural fragment was assigned as a pyran-bearing polyketide-derived moiety. A terminal triplet methyl group (C-39) was connected to C-38 by the Hₐ-39/H₂-38 COSY...
correlation. The 3-bond $^1$H-$^1$H couplings between H-23 and H-24 placed the C-39-C-38 ethyl group next to C-34. Subsequently, the COSY and TOCSY correlations among H-34, H-33, H-32, and H-31 constructed an alkyl chain spin system from C-39 to C-31. H-23 displayed a COSY coupling with H-33, indicating C-33 was a branch point. The 3-bond $^1$H-$^1$C correlations from H-23 to the C-37 carbonyl carbon connected C-37 to C-36. The alkyl chain was further extended from C-31 to C-30, a dioxygenated carbon, based on the H-23/C-30 HMBC correlation. A singlet methyl signal (H-35) exhibited strong HMBC correlations to C-30, C-29, and C-28, which finally constructed a C$_9$ backbone chain with a C$_2$ branch and a branch methyl group. 29-OH and 30-OH, which were visible in the $^1$H NMR spectrum, were assigned at C-29 and C-30, respectively, based on their HMBC correlations. Further analysis of the HMBC spectrum revealed the presence of a pyran ring by the H-34/C-30 3-bond correlation. Therefore, the polyketide-derived partial structure was determined to be a C$_{12}$ alkyl moiety bearing a pyran ring and a branching C$_2$ chain and a branch methyl group (Figure 3).

The six amino acids and the polyketide-derived acyl chain were assembled by analysis of the HMBC spectrum. N-OH-Thr was located adjacent to Pip-1 based on the H-2/C-5 and H-2/C-5 $^1$H-$^1$C correlations. The HMBC correlations from H-6 and H-12 to C-10 connected Pip-1 and Pip-2. The sequence from Pip-2 to N-OH-Gly was established by heteronuclear couplings from 14-NH and H-2 to C-15. H-2 also displayed an HMBC correlation to C-17. The connectivity between N-OH-Gly and Pip-3 was supported by the H-2/C-17 HMBC coupling. β-OH-Leu was located next to Pip-3 based on the 3-bond correlations from 21-NH and H-23 to C-22. The polyketide-derived moiety was connected to β-OH-Leu by the 28-NH/C-28 HMBC correlation. Therefore, the sequence of the partial structures of 1 was determined to be N-OH-Thr-Pip-1-Pip-2-N-OH-Gly-Pip-3-β-OH-Leu-polyketide-derived acyl chain (Figure 3).

The carbonyl groups explained 8 of the 13 double bond equivalents, indicating that dentigerumycin E (1) must be a pentacyclic compound. The three piperazic acids and pyran ring accounted for another four of the double bond equivalents. Thus, dentigerumycin E must possess an additional ring. The 3-bond HMBC correlation between H-24 and C-1 constructed a macrocycle (Figure 3). After elucidating most of the structure of 1, the C-37 carbonyl carbon was proposed to be a carboxylic acid based on the molecular formula, which allowed the planar structure of dentigerumycin E (1) to be proposed as shown.

The uncertainty in the planar structure of 1 due to the invisibility of the N-OH and carboxylic OH moieties in the $^1$H NMR spectrum was further clarified by utilizing chemical reactions and NMR spectroscopic analyses of the products. To confirm the presence of two N-OH groups, the N-OHs were converted to NHs using TiCl$_3$ (Pennings et al., 1983). The structure of the reduction product (2, N,16-N-deoxygenentigerumycin E, 2) (Figure 2) was elucidated based on the $^1$H NMR, COSY, HMBC, HSQC, TOCSY, and ROESY spectra (Table S1, Figures S11–S16) and HRMS data, confirming the presence of the N-OH groups of N-OH-Thr and N-OH-Gly in dentigerumycin E (1). On the other hand, the only HMBC correlation to the C-37 carbonyl carbon ($^1$C 175.2) was from H-36, not fully proving the proposed structure.
carboxylic acid functionality. To validate the presence of the carboxylic acid group in the acyl side chain, we performed an AcCl-mediated methylation and confirmed the structure of the methylated product (dentigerumycin E methyl ester, 3) (Figure 2) by $^1$H NMR, COSY, HMBC, HSQC, TOCSY, and ROESY (Table S1, Figures S17–S22) as well as HRMS analysis. The protons of the methoxy group displayed a clear HMBC correlation to the carbonyl carbon in 3, confirming the carboxylic acid functionality in 1. Therefore, the planar structure of dentigerumycin E (1) was unequivocally elucidated.

The relative configuration of the two congruent stereogenic centers in β-hydroxy leucine were established through J-based configuration analysis (Figure 4A). The large $^1$H-$^1$H coupling between H-23 and H-24 ($J_{2324} = 10.0$ Hz) indicated that these two protons have anti-relationship. Further ROESY correlations allowed the assignment of 23$^S$ and 24$^S$ (Matsumori et al., 1999). The relative configuration of the pyran ring in the acyl side chain was determined by ROESY correlations (Figure 4B).

To establish the absolute configuration of dentigerumycin E (1), acid hydrolysis was performed using 6 N HCl at 115°C for 1 h. The hydrolysate was then derivatized with Marfey’s reagents (L-FDAA and D-FDAA) and analyzed by LC-MS (Fujii et al., 1997). The L-FDAA and D-FDAA derivatives of β-hydroxy leucine were detected at retention times of 15.2 min and 18.2 min, determining its $\alpha$-(S)-configuration at the α-amino position (Figure S23). Based on the advanced Marfey method and the previously determined relative stereochemistries, the absolute configuration of the chiral centers in the β-hydroxy leucine were determined to be 23$S$ and 24$S$. The L-FDAA and D-FDAA derivatives of the three piperazic acid units eluted at retention times of 11.9 min and 14.1 min in a ratio of 2:1. Based on Marfey analysis of synthetic (S) and (R)-piperazic acids, the L-FDAA derivative of piperazic acid elutes faster than the D-FDAA derivative when piperazic acid has an R-configuration (Oh et al., 2009). Therefore, dentigerumycin E contains two R-Pip and one S-Pip units.

Because the free N-OH-Thr does not react with Marfey’s reagents, 2-N,16-N-deoxydentigerumycin E (2), which possesses Thr converted from N-OH-Thr, was hydrolyzed and derivatized with Marfey’s reagents. In the LC-MS analysis of the derivatives,
the L-FDAA and D-FDAA derivatives of threonine eluted at 18.2 min and 21.4 min, respectively, indicating the α-position of threonine was in the L (S) configuration (Figure S23). Due to the presence of the additional stereogenic center at the β-carbon in threonine, GITC (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) derivatization was performed (Hess et al., 2004). The retention times of the GITC derivatives of 2 and authentic standards of L-allo-Thr and L-Thr were compared. The GITC derivatives of L-allo-Thr, L-Thr, and Thr in 2 eluted at retention times of 14.7 min, 15.1 min, and 15.1 min, respectively (Figure S24). Therefore, the N-hydroxy threonine of dentigerumycin E (1) was determined to be N-OH-L-Thr, and thus the absolute configurations of its stereogenic centers were determined to be 2S and 3R.

Analysis of the Putative Biosynthetic Pathway of Dentigerumycin E for Stereochemical Assignment

Complete assignment of the sequence of the two R- and one S-piperazic acid moieties was accomplished through analysis of the putative biosynthetic gene cluster (BGC) (Table S2). Full genome sequencing of Streptomyces sp. JB5 allowed the identification of the BGC of dentigerumycin E (1). Even though its origin was reasonably inferred as Streptomyces sp. JB5 based on previous reports of dentigerumycin class compounds (Oh et al., 2009; Wyche et al., 2017), identifying the BGC added clearer evidence of its Streptomyces origin because sometimes the same class of compounds can be discovered together from phylogenetically diverse bacteria (Blodgett et al., 2010). As predicted based
on the structure, the BGC was composed of a multimodular hybrid polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS) (Figure 5). The feature of NRPS involving six modules in the four open reading frames (ORFs 6590, 6606, 6605, and 6604) showed high degree similarity to that of dentigerumycins B-D (Wyche et al., 2017). Based on the antiSMASH analysis, these modules produce a cyclic peptide with the sequence Leu-Pip-3-Gly-Pip-2-Pip-1-Thr, which is
consistent with the structure elucidated by spectroscopic analysis. Epimerase domains, which determine the absolute configurations of the amino acid residues in NRPS, were incorporated in the modules for Pip-3 and Pip-2 but not Pip-1. The 2:1 ratio of R- and S-piperazic acids determined by advanced Marfey’s analysis (vide supra) established that the absolute configuration of the three piperazic acids is 6S, 11R, and 18R (S-Pip-1, R-Pip-2, and R-Pip-3) (Figure 5).

The absolute configuration of the acyl side chain was also assigned through analysis of the BGC. Within the BGC of dentigerumycin E, four type I PKS modules (ORFs 6594, 6593, 6592, and 6591) were involved in the biosynthesis of the C12 acyl side chain (Figure 5). Both module 6594 and 6591 are composed of a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP). Because the KS of 6594 was identified as KSQ with the active site cysteine (C) being replaced by glutamine (Q) (Kuhstoss et al., 1996), 6594 was identified as the loading module of dentigerumycin E BGC (Figure S25). Module 6593 involves KS, AT, dehydratase (DH), ketoreductase (KR), and ACP. In modular polyketide synthase (PKS), certain amino acid motifs in the KR domain are correlated with the stereochemistry of the hydroxyl groups in the product (Caffrey, 2003; Reid et al., 2003). KR domains lead to A-type alcohol stereocentre (3S when C-2 has higher priority than C-4 and 3R when C-4 has higher priority than C-2) if amino acid residue 141 is tryptophan (W) and B-type alcohol stereocentre (3R when C-2 has higher priority than C-4 and 3S when C-4 has higher priority than C-2) if an LDD motif is present in the region between 88 and 103. In addition, B-type KR domains typically contain proline (P) or/and asparagine (N) at residue 144 and 148, respectively. During the reaction with PKS, it was hypothesized that the tryptophan motif guides the polyketide into the active site of A-type KR, while the LDD motif guides polyketides into the active site of B-type KR (Zheng et al., 2010). The KR domain of module 6593, which determines the absolute configuration of C-34 hydroxy group, was aligned with other KR domains reported by Caffrey (2003), and the residues were compared. Based on the LDD motif, the KR domain of module 6593 was identified as B-type, indicating the product should have a 34R configuration (Figure 6). R-configuration is also supported by the evidence that residue 141 was not a tryptophan and the presence of asparagine at residue 148. Based on the relative configuration previously determined by ROEY correlations, the absolute configuration of the stereogenic centers in the acyl side chain should be 29S, 30R, 33R, and 34R (Figure 2).

On the other hand, because the hydroxy group at C-34 was preserved rather than eliminated, the DH domain in module 6593 is assumed to be nonfunctional, similar to what is seen with polyoxypeptin A (Du et al., 2014), which has a BGC that closely resembles that of the dentigerumycin class of compounds. Although this DH domain of dentigerumycin E has all three key amino acids residues of the conserved motif HxxxGxxxxP, the motif is not always universally conserved (Joshi and Smith, 1993). In addition, the two tyrosine (Y) residues in the GYxYGpxF motif were altered to phenylalanine (F) and histidine (H), respectively. Such a significant alteration in these conserved motifs could cause the domain to be nonfunctional (Keatinge-Clay, 2008), and these alterations in the GYxYGpxF motif could explain the nonfunctional DH in module 6593. (Figure S26).

**Biological Activity of Dentigerumycin E and Its Derivatives**

To explore the biological activities of dentigerumycin E (1) and its derivatives (2 and 3), cytotoxicity was evaluated against various human cancer cells. Dentigerumycin E (1) showed moderate cytotoxicity against the tested cancer cell lines including A549 (lung cancer), HCT116 (colorectal cancer), MDA-MB-231 (breast cancer), SK-HEP-1 (liver cancer), and SNU638 (stomach cancer) whereas 2N16N-deoxydentigerumycin E (2) and dentigerumycin E methyl ester (3) did not inhibit the proliferation of these cancer cell lines (Table S3). To determine the cancer cell-specific cytotoxicity, cytotoxicity of 1-3 against a normal human breast epithelial cell line (MCF-10A) was also evaluated. As shown in Table S3, in comparison to cancer cell lines, dentigerumycin E (1) did not demonstrate a significant cytotoxicity against normal epithelial cells with IC50 value of over 50 µM. Furthermore, the antimetastatic activity of dentigerumycin E was evaluated with metastatic breast cancer cells (MDA-MB-231) by wound healing and cell invasion assays. For each assay, cells were treated with 20 µM or 40 µM dentigerumycin E for 24 h. Compared to the vehicle-treated group, dentigerumycin E inhibited cell migration in the wound healing assay by 20 and 48% at 20 and 40 µM, respectively (Figure 7). In the cell invasion assay, dentigerumycin E exhibited inhibitory activity by 10 and 34% at 20 and 40 µM, respectively (Figure 8). These results indicated the antimetastatic potential of dentigerumycin E (1) against breast cancer cells. However, 2N16N-deoxydentigerumycin E (2) and dentigerumycin E methyl ester (3) displayed no significant activity in the wound healing and cell invasion assays (Figures S27, S28), suggesting that 2-N-OH, 16-N-OH, and 37-OH (carboxylic acid) in 1 are essential for its antiproliferative and antimetastatic activities.

**CONCLUSION**

A new cyclic peptide, dentigerumycin E (1), was discovered from a coculture of marine Streptomyces sp. JB5 and Bacillus sp. GN1 strains isolated from an intertidal mudflat. Cocultivating Streptomyces sp. JB5 and a Bacillus strain (either GN1 or HR1), most closely related B. cereus, was required because dentigerumycin E was virtually detectable only in cocultures of these bacterial strains, not in single cultures of these bacteria. Dentigerumycin E was determined to be a new member of the dentigerumycin nonribosomal peptide class with three piperazic acid units and an acyl chain of PKS origin (Oh et al., 2009). Dentigerumycin E (1) is most similar to dentigerumycin B, previously reported from the Pseudonocardia symbiont of a fungus-growing ant (Wyche et al., 2017), but the N-hydroxy threonine moiety in 1 has a different configuration from that in dentigerumycin B (L-Thr for dentigerumycin E whereas L-allo-Thr for dentigerumycin B). Moreover, the polyketide-derived acyl chain of 1 has a carboxylic acid group in the
branched C2 chain, which has not been reported in the other dentigerumycin-type compounds. Our biological evaluation of the antiproliferative and antimetastatic activities revealed that the 2-N-OH, 16-N-OH, and 37-OH groups are essential for the activities of dentigerumycin E. Genomic analysis of the Streptomyces sp. JB5 strain allowed the identification of the putative BGC of dentigerumycin E (1) and thus confirmed that 1 was produced by the Streptomyces strain. In conjunction with chemical derivatizations, comprehensive analysis of the biosynthetic modules allowed the absolute configurations of the piperazic acid residues and PKS-derived acyl chain to be proposed. Induction of dentigerumycin E production by a couple of phylogenetically close Bacillus strains raised an interesting question about the mechanism activating the biosynthetic gene cluster for dentigerumycin E in Streptomyces sp. JB5, which might lead a more comprehensive mechanism study. The discovery of dentigerumycin E (1) in the marine bacterial coculture highlights that marine microorganisms are prolific chemical sources of bioactive natural products and that the coculture strategy could be a promising method for exploring hidden microbial chemical diversity.

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AUTHOR CONTRIBUTIONS

DS, WB, KM, YK, MB, SU, SL, and D-CO designed the experiments. YK and MB collected intertidal mud samples. KM isolated the bacterial strains. DS performed the coculture experiments, chemical experiments, and analyzed the data. WB and SL performed the bioassay. DS, WB, KM, SU, SL, and D-CO wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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