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Bacterial Production of the Tunicate-Derived Antitumor Cyclic Depsipeptide Didemnin B

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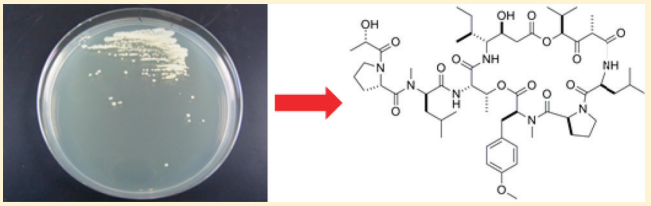
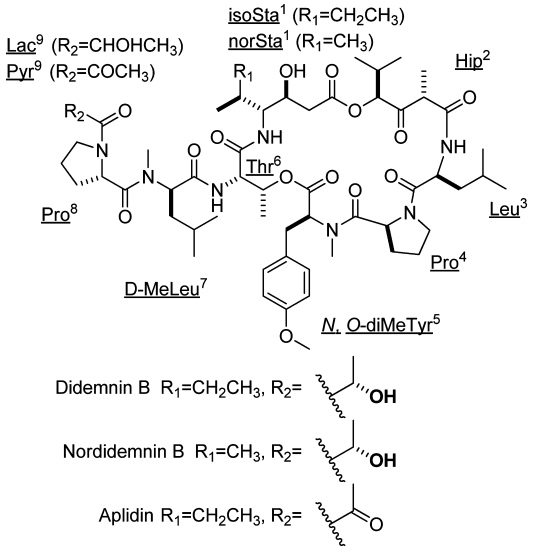
Supporting Information

ABSTRACT: Natural products obtained from marine invertebrates such as sponges and tunicates are attractive sources of drugs. However, a critical obstacle in the development of these compounds is the problem of supply. In most cases, neither chemical synthesis nor mariculture of invertebrates is economically feasible. Due to structural similarities, many marine natural products are suspected to be produced by associated microorganisms. A favorable strategy for the production of such compounds is to use culturable microorganisms. Here we report that didemnin B, a tunicate-derived depsipeptide, has been isolated from a culturable bacterium, *Tistrella mobilis* YIT 12409.



Didemnins, a class of depsipeptides, have been isolated from several species of tunicates (sea squirts).[1] These compounds show significant *in vitro* cytotoxicity and *in vivo* antitumor activity. Didemnin B demonstrated the most potent antitumor activity, and its development was initiated by the National Cancer Institute (NCI).[2] Didemnin B was the first marine natural product to enter clinical trials as an anticancer agent. Phase II clinical trials revealed the anticancer activity of didemnin B against various types of cancer; however, the NCI discontinued developmental efforts because of its significant toxicity. Aplidin, which has a pyruvic acid unit in place of the lactic acid unit in didemnin B, is now being developed by PharmaMar S. A., and clinical trials are ongoing.[3]



Didemnin B was first isolated from Caribbean tunicates of the family *Didemnidae* by Rinehart et al.[1a] They suggested that didemnins are products of symbiosis between tunicates and cyanobacteria because of their occurrence in taxonomically distant species of tunicates and the presence of the *N,O*-dimethyltyrosine residue, which was also found in metabolites of the cyanobacterium *Lyngbya majuscula*.[4] Even though a wide variety of peptides have been isolated from cyanobacteria, none of them are structurally related to the didemnins. Therefore, no direct evidence to determine the primary producer of the didemnins is available.

In our search for antitumor antibiotics from marine bacteria, we found activity in an isolate of the α-proteobacterium *Tistrella mobilis,* obtained from marine sediment collected at a depth of 3 m off the Tateyama cove, Chiba, Japan. Molecular analysis of the bacterium based on the 16S rRNA gene permitted its identification. Members of the genus *Tistrella* have been isolated from wastewater, marine sediment, and soil as polycyclic aromatic hydrocarbon-degrading bacteria.[5]

Fractionation of a 4 L culture extract of this strain by silica gel column chromatography, followed by reversed-phase HPLC separation, yielded cytotoxic constituents **1** (12.8 mg) and **2** (1.9 mg) as colorless noncrystalline solids. The identities of **1** and **2** in the bacterial extract were confirmed by MS and NMR data and specific rotation.[1c,6]

The molecular formula of **1** was deduced as $C_{57}H_{89}N_7O_{15}$ on the basis of HRESIMS data. The ^{13}C NMR spectrum indicated the presence of 57 carbons, which were classified into 12 nonprotonated carbons (of which 10 were carbonyls), 20

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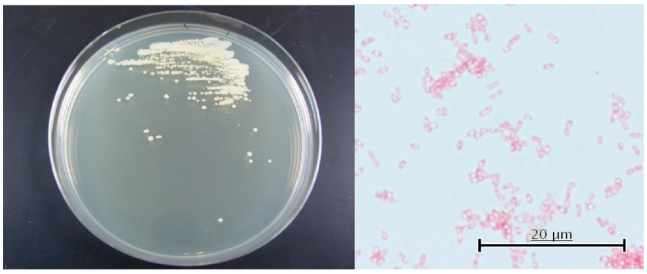

Figure 1. (a) Photograph and (b) microscopic view of *Tistrella mobilis* YIT 12409.

methines, 11 methylenes, and 14 methyls (of which two were *N*-methyls and one was an *O*-methyl) on the basis of the DEPT spectrum. Analysis of 2D NMR data established a planar structure identical to that of didemnin B (Figure 2). A detailed

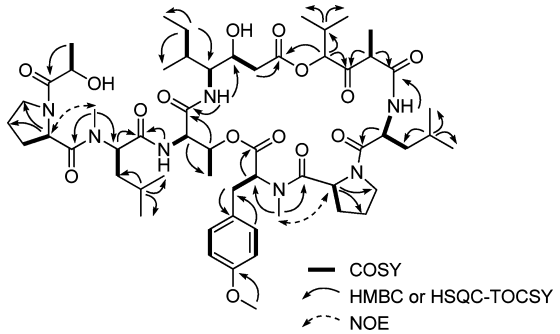

Figure 2. Key 2D NMR and NOE correlations for determination of the planar structure of **1**.

comparison of NMR data for **1** with values reported in the literature is shown in Table S1.[1c] Specific rotation of **1** was also close to the value in the literature (Table 1).[6] As the molecular

Table 1. Comparison of Specific Rotation and Cytotoxicity[a]

		cytotoxicity, IC_{50} (ng/mL)	
	$[\alpha]_D^{25}$ in CH_2Cl_2 (*c*)	A549	HT-29
didemnin B	−78 (6.91)	2	2
1	−83 (0.3)	2	2
2	−77 (0.3)	7	10

[a]The specific rotation and cytotoxicity of didemnin B were cited through ref 6.

formula, NMR data, and specific rotation were identical to those reported for didemnin B in the literature, we concluded that **1** was didemnin B.

The molecular formula of **2** was deduced as $C_{56}H_{87}N_7O_{15}$ on the basis of HRESIMS data, which was smaller than that of **1** by a CH_2 unit. ^1H NMR and ^{13}C NMR spectra of **2** were very similar to those of **1**, except for the isostatine unit of **1** (Table S2). A detailed comparison between the NMR data for **1** and **2** indicated that **2** had a norstatine unit in place of the isostatine unit in **1**. We identified **2** as nordidemnin B.

Cytotoxic activity of **1** and **2** was tested using an *in vitro* cytotoxicity assay against human cancer cell lines (A549 for lung cancer and HT-29 for colorectal cancer). IC_{50} values for these cell lines were similar to those in the literature (Table 1).[6]

We have discovered a didemnin-producing bacterium from the marine environment and isolated didemnin B and nordidemnin B from the extract of culture broth. Optimization of fermentation conditions may enable environmentally friendly and economical production of didemnin B. Didemnin B is an important reagent in biochemistry because of its diverse and potent bioactivity.[7] Additionally, didemnin B can be utilized as a precursor molecule of aplidin. Studies on the biosynthetic gene cluster associated with didemnin production, along with engineering of the cluster, could lead to direct production of aplidin. Most marine natural products are difficult to develop because of the problem of supply.[8] Exploring culturable producers may lead to the effective use of marine natural products. Meanwhile, using the DNA sequence as a probe, it may be possible to identify the didemnin-producing organism in the tunicate. Our discovery suggests a possible role of an α-proteobacterium in the secondary metabolism of tunicates, in which the association of symbiotic cyanobacteria has been chiefly emphasized.[4,9]

EXPERIMENTAL SECTION

General Experimental Procedures. Specific rotation was determined using a Jasco DIP-360 digital polarimeter. ^1H and ^{13}C NMR spectra were measured on a JCA-500 (JEOL) spectrometer at 500 and 125 MHz, respectively. HRESIMS data were measured on an LCT-Premier XE (Waters Corp.) time-of-flight instrument.

Collection and Phylogenetic Analysis of Strain YIT 12409. The marine-derived bacterium, strain YIT 12409, was isolated from marine sediment collected at a depth of 3 m off the Tateyama cove, Chiba, Japan. The 16S rRNA gene sequence (1416 bases) of strain YIT 12409 was identical to that of *Tistrella mobilis* (DDBJ accession no. AB071665) at 99.9% similarity, and the strain was therefore identified as *T. mobilis*.

Cultivation and Extraction. The bacterium was cultured in a 5 L conical flask containing 4 L of seawater-based medium (4 g galactose, 4 g gelatin peptone, 4 g glycerol, 6 g yeast extract, 20 g peptone) containing XAD-7 resin and shaken at 140 rpm at 25 °C. After 14 days of cultivation, the resin was washed with water and eluted with MeOH. The MeOH was concentrated *in vacuo*. The resulting MeOH extract was partitioned between water and EtOAc, and the EtOAc fraction was dried *in vacuo* to yield 127 mg of extract.

Isolation of Constituents 1 and 2. The extract (127 mg) was separated by silica gel column chromatography, eluted with a step gradient of $CHCl_3$ and MeOH. The $CHCl_3$/MeOH (98:2) fraction was further fractionated by reversed-phase chromatography (InertSep RP-1, 1 g), eluted with a step gradient of MeOH and water. The 85% MeOH fraction was purified by reversed-phase HPLC (YMC-Pack ODS-AM C_{18}, 10 × 250 mm, 60% MeCN, 2 mL/min, PDA) to afford **1** (12.8 mg) and **2** (1.9 mg) as colorless noncrystalline solids.

*Didemnin B (**1**):* $[\alpha]_D^{25}$ −83 (*c* 0.3, CH_2Cl_2); HRESIMS $[M + H]^+$ *m/z* 1112.6497 (calcd for $C_{57}H_{90}N_7O_{15}$, 1112.6495); ^1H and ^{13}C NMR (500 MHz, 125 MHz, $CDCl_3$), see Table S2.

*Nordidemnin B (**2**):* $[\alpha]_D^{25}$ −77 (*c* 0.3, CH_2Cl_2); HRESIMS $[M + H]^+$ *m/z* 1098.6342 (calcd for $C_{56}H_{88}N_7O_{15}$, 1098.6338); ^1H and ^{13}C NMR (500 MHz, 125 MHz, $CDCl_3$), see Table S2.

Cells and Culture. Human lung cancer cell line A549 and colorectal cancer cell line HT-29 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). A549 and HT-29 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Sigma) and D-MEM/F-12 medium (Sigma), respectively, with 10% heat-inactivated fetal bovine serum and 5 mg/mL gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2.

Growth-Inhibition Assay. Exponentially growing cells (density, 1 × 10^4 cells per 1.9 mL; volume, 190 μL) were seeded into a 96-well microtiter plate, and test compounds (dissolved in 10 μL of DMSO) were added at various concentrations 24 h after the tumor cells were seeded. After incubation for 96 h at 37 °C, 10 μL of Tetra Color ONE

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(Seikagaku Biobusiness Corporation, Tokyo, Japan) was added to each well, and the plates were incubated for a further 1 h at 37 °C. After incubation, the optical density was measured at 450 nm with a microplate reader (Spectra Max Plus, Molecular Devices, CA, USA), and the IC_{50} values were calculated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR data for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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