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Anmindenols A and B, Inducible Nitric Oxide Synthase Inhibitors from a Marine-Derived *Streptomyces sp.*

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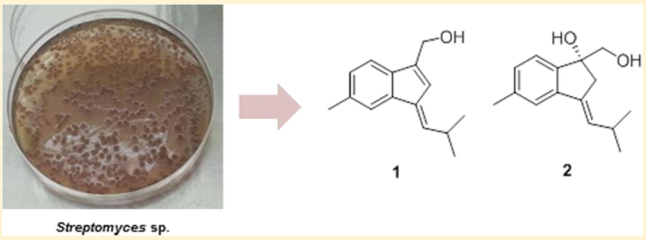
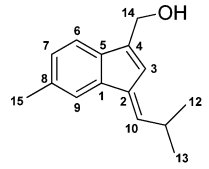
Supporting Information

ABSTRACT: Anmindenols A (**1**) and B (**2**), inhibitors of inducible nitric oxide synthase (iNOS), were isolated from a marine-derived bacterium *Streptomyces sp*. Their chemical structures were elucidated by interpreting various spectroscopic data, including IR, MS, and NMR. Anmindenols A and B are sesquiterpenoids possessing an indene moiety with five- and six-membered rings derived from isoprenyl units. The absolute configuration of C-4 in anmindenol B was determined by electronic circular dichroism (ECD) of a dimolybdenum complex. Anmindenols A (**1**) and B (**2**) inhibited nitric oxide production in stimulated RAW 264.7 macrophage cells with IC_{50} values of 23 and 19 μM, respectively.

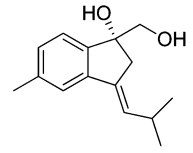


Tidal flats are spread widely over the west and southwest coasts of the Korean peninsula.[1] The physical and chemical properties of these tidal flats are unpredictable due to water exchange caused by tidal cycles.[2] The shallow water column in tidal flats with strong tidal currents and high winds stimulates transportation, dispersion, and mixing of nutrients for biological production.[3] These physical vectors, along with the variability of salinity, temperature, pH, and nutrient composition, contribute to the great diversity of microbial communities in the tidal flats.[2,4] The phylogenetic analysis based on 16S rRNA sequences of bacteria from tidal flat sediments collected at Dongmak, located on the west cost of Korea, showed an average sequence similarity to bacterial strains with sequences in GenBank of only 88.4%, ranging from 74.9 to 97.6%.[5] These data suggested that tidal flat sediments could be a great resource for discovering new microorganisms that produce unique secondary metabolites.

During the course of our screening program designed to discover bacterial secondary metabolites as inhibitors of inflammation, inducible nitric oxide synthase (iNOS) was targeted. Nitric oxide (NO) is a free radical gas with diverse physiological and pathological functions for neurotransmission, host defense, and cardiovascular function in mammals.[6] It is also important for regulating immune cell function and as an activator in the cell-mediated rejection of allergenic transplants.[7] NO is produced by three isoforms of NO synthases (NOS), neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), which catalyze the conversion of arginine to citrulline.[6] However, NO overproduction leads to numerous human diseases, such as asthma, diabetes, inflammation, septic shock, and chronic inflammatory diseases.[6] Thus, the specific control of NO production offers great therapeutic value. In particular, the use of selective iNOS inhibitors could be beneficial in the inflammatory process.[7]



anmindenol A (**1**)



anmindenol B (**2**)

We investigated marine-derived bacterial strains isolated from tidal flat sediments at Anmyeon Island on the west coast of Korea to discover novel iNOS inhibitors, by evaluating the strain CMDD10D111. This strain shares 97.4% 16S rRNA gene sequence identity with *Streptomyces phaeopurpureus*, indicating it could be a new *Streptomyces sp.* LC-MS analysis of an extract of CMDD10D111 growing in Mar4 media to which was added 3% DMSO revealed the presence of *m*/*z* peaks at 215.1 and 237.0 with an unusual chromophore. Large-scale fermentation and flash chromatography followed by HPLC yielded

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Table 1. NMR Data for Anmindenols A and B in $CDCl_3$

	Anmindenol A[a]				Anmindenol B[c]	
number	δ_C, mult.[b]	δ_H, (J in Hz)	COSY	HMBC	δ_C, mult.[b]	δ_H, (J in Hz)
1	138.1, C				134.6, C	
2	137.1, C				141.5, C	
3	120.6, CH	6.67, s	10	1, 2, 4, 5, 14	41.3, CH_2	3.00, d (16.6), 2.62, d (16.9)
4	144.4, C				81.1, C	
5	138.5, C				142.9, C	
6	118.9, CH	7.18, d (7.6)		1, 2, 4, 7, 8	123.7, CH	7.24, d (7.4)
7	127.8, CH	7.03, d (7.6)	6, 9	1, 5, 6, 9, 15	129.0, CH	6.99, d (7.2)
8	135.3, C				139.1, C	
9	120.1, CH	7.38, s		1, 2, 5, 7, 15	120.5, CH	7.19, d (3.1)
10	138.8, CH	6.36, d (10.0)		1, 3, 11, 12, 13	129.0, CH	5.75, d (9.3)
11	29.8, CH	3.00, m	10	2, 5, 10, 12, 13	29.0, CH	2.48, m
12	23.5, CH_3	1.13, d (6.7)	11	10, 11	22.9, CH_3	0.98, d (4.8)
13	23.5, CH_3	1.13, d (6.7)	11	10, 11	22.8, CH_3	0.98, d (5.5)
14	60.2, CH_2	4.73, s	3, 10	1, 3, 4, 5	69.1, CH_2	3.67, d (10.7), 3.53, d (10.2)
15	21.8, CH_3	2.37, s	7, 9	1, 7, 8, 9	21.5, CH_3	2.29, s

[a]600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR. [b]Multiplicity was determined by the analysis of 2D NMR spectroscopic data. [c]700 MHz for ^{1}H NMR and 175 MHz for ^{13}C NMR.

anmindenols A (**1**, 3.2 mg) and B (**2**, 1.5 mg). We herein describe the isolation and structure elucidation of anmindenols A (**1**) and B (**2**) and their inhibitory activity against iNOS.fx1.

Anmindenol A (**1**) was isolated as a yellow amorphous solid. Its molecular formula was determined to be $C_{15}H_{18}O$ based on HRFABMS with seven degrees of unsaturation. The ^{1}H NMR spectrum of **1** displayed three aromatic protons [δ_H 7.18 (d, J = 7.6 Hz), 7.03 (d, J = 7.6 Hz), 7.38 (s)], two olefinic protons [δ_H 6.67 (s), 6.36 (d, J = 10.0 Hz)], and one methyl singlet [δ_H 2.37]. The ^{1}H NMR spectrum also showed a methyl doublet integrating for six hydrogens [δ_H 1.13, (d, J = 6.7 Hz)]. The COSY cross peaks of H_3-12 and H_3-13 to H-11 indicated that these methyls were coupled to a methine proton (Table 1). In addition, the ^{13}C NMR and HSQC spectroscopic data revealed five olefinic carbons and five downfield shifted quaternary carbons. These data and the molecular formula indicated that **1** contained two rings. The presence of 15 carbon signals, including of one methylene carbon [δ_C 60.2], one upfield shifted quaternary carbon [δ_C 29.8], and three methyl carbon signals [δ_C 23.5, 23.5, 21.8], in the ^{13}C NMR spectrum suggested that **1** was a sesquiterpenoid.

The COSY correlations of H-9 and H-6 to H-7, and the long-range HMBC correlations from a methyl singlet H_3-15 to carbons C-7, C-8, and C-9 allowed the establishment of a trisubstituted-benzene moiety bearing a methyl group at position C-8. The remaining unknown part of the structure with the indene moiety was identified by interpreting the COSY and HMBC spectroscopic data. An olefinic proton H-10 coupled to the methine proton H-11 in the COSY spectrum was observed to exhibit HMBC correlations to the carbons C-1, C-2, and C-3, which permitted the C-1/C-2/C-3 attachment. Lastly, HMBC correlations from the methylene protons H_2-14 to the carbons C-3, C-4, and C-5 and from H-3 to the carbons C-1, C-2, and C-4 allowed the assignment of anmindenol A to be completed, as shown in Figure 1. The 10*E* double-bond geometry was assigned by NOESY correlations between H-3 and H-11.

Anmindenol B (**2**) was isolated as a yellow amorphous solid and the molecular formula of **2** was determined to be $C_{15}H_{20}O_2$ based on HRFABMS. The ^{1}H NMR spectrum of **2** was almost identical to that of **1** except for the presence of one additional

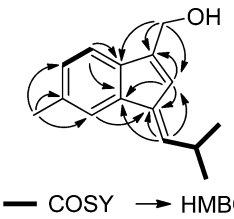

Figure 1. COSY and key HMBC correlations of anmindenol A (**1**).

methylene resonance. The ^{13}C NMR data of **2** were also similar to those of **1** except for the upfield-shifted carbon signals for C-3 [δ_C 41.3] and C-4 [δ_C 81.1]. HMBC correlations from H-3 to carbons C-4, C-5, and C-14 and the carbon chemical shift for C-4 [δ_C 81.1] indicated that **2** had a hydroxy group at C-4. The interpretation of 2D NMR spectroscopic data permitted the identification of the structure of **2**.

To determine the absolute configuration for C-4, we conducted an ECD experiment by using the $Mo_2(OAc)_4$ circular dichroism (CD) method.[8] A metal complex of a vicinal diol (cyclic and acyclic 1,2-diols) and $Mo_2(OAc)_4$ acts as an auxiliary chromophore. As a consequence, the conformational freedom of the flexible molecule gives an induced CD curve at 305 nm. The observed sign of the Cotton effect induced by the O−C−C−O torsion angle allows to assign the absolute configuration.[9] On the basis of the empirical rule, an (*R*)-1,2-diol with $Mo_2(OAc)_4$ gives rise to a negative CD band at 305 nm, whereas a complex having the *S* configuration gives a positive CD band at 305 nm.[8] The negative Cotton effect at 305 nm observed in the CD spectrum of the metal complex of **2** (2-$Mo_2(OAc)_4$) establishes the *R*-configuration for C-4 (Supporting Information).

Dehydration of **2** will form the more highly conjugated **1**. It is possible that **1** is an artifact formed during the extraction and isolation procedure, although **1** was detected in the original extract by LC-MS and there was no indication that **2** was converted to **1** during characterization of **2**.

Anmindenols A and B are sesquiterpenoids possessing a unique indene moiety. Terpenoids consist of important cell components such as steroids, carotenoids, and vitamins, and also have been reported to possess a broad variety of biological

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activities with a diversity of structures.[10] Traditionally, the producers of terpenoid secondary metabolites are plants, insects, fungi, and some marine invertebrates.[11] Actinomycetes are also now known as a source of producing terpenoid natural products including sesquiterpenoids. In particular, nitropyrrolins A–E[12] and neomarinone[13] are meroterpenoids bearing a sesquiterpenoid moiety isolated from marine-derived actinomycetes and caryolane-1,7α-diol,[14] 1,6,11-eudesmanetriols,[14] and 11-eudesmene-1,6-diol[14] are sesquiterpenoids discovered from *Streptomyces sp.* However, indene-containing sesquiterpenoids, such as gloeophyllols A, B, and C isolated from the mushroom *Gloeophyllum sp.* 97022., are rare.[15] In addition, there are other reported sesquiterpenoids with a saturated 6,5 ring system. 2-Octahydro-3a,7,7,7a-tetramethyl-1-methylene-1-*H*-indenemethanol, its ferulic ester and 3b,4,4,7a-tetramethyl-1-*H*-decahydroindeno[1,2-*c*]furan-3-ol are hydroindenes that have been isolated from the plant *Thapsia villosa*.[16] Calenzanol[17] and illudins S[18] and M[18] were discovered from the red alga *Laurencia microcladia* and from the mushroom *Clitocybe illudens*, respectively. Anmindenols A and B are the first sesquiterpenoid natural products containing an indene moiety discovered from actinomycetes.

Anmindenols A and B were tested for their effects against NO production in lipopolysaccharide (LPS)-activated mouse macrophage RAW264.7 cells. Anmindenols A and B inhibited NO production with IC_{50} values of 23 μM and 19 μM, respectively. Anmindenols A and B did not display any significant cytotoxicities against a human renal cancer cell line (A498) and two human pancreatic cancer cell lines (MIA-paca and PANC-1) up to a compound concentration of 100 μM. Therefore, anmindenols A and B can be novel skeletons offering a new platform for iNOS inhibitors.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured using a Rudolph Research Autopol III polarimeter with a 5 cm cell. The UV spectrum was recorded in a Scinco UVS-2100 with a path length of 1 cm. CD spectra were collected in an Applied Photophysics Chirascan plus CD spectrometer with a 0.5 mm path-length rectangular cuvette. Infrared spectra were recorded on a Thermo Electron Corporation spectrometer. NMR spectral spectroscopic data of anmindenols A and B were obtained using Bruker Avance 600 and 700 MHz spectrometers, respectively [$CDCl_3$ (δ_H 7.26; δ_C 77.0) was used as an internal standard]. EI-MS and FAB-MS spectra were measured on a JEOL, JMS-AX505WA mass spectrometer. Low resolution LC-MS data were measured using an Agilent Technologies 6120 quadrupole LC/MS system with a reversed-phase C_{18} column (Phenomenex luna 5u (2), 4.6 mm × 50 mm, 5 μm) at a flow rate of 1.0 mL/min. The extracts were separated by HPLC WATERS 1525 binary HPLC pump, WATERS 2489 UV/visible detector using an MG2 C_{18} (250 mm × 10 mm, 5 μm) reversed-phase HPLC column.

Collection and Phylogenetic Analysis of Strain CMDD10D111. Strain CMDD10D111 was isolated from marine sediment from Anmyeon Island, Chungcheongnam-do, South Korea in 2010. The 16S rRNA gene sequence using primers 27f and 1492r for this strain has been deposited with GenBank (accession number KC136293). It shares 97.4% sequence identity with the type strain for *Streptomyces phaeopurpureus* (EU274371.1).

Cultivation and Extraction. Strain CMDD10D111 was cultured in 40 4-L Pyrex flasks each containing 1 L of the medium Mar 4 (2 g of kelp meal, 2 g of D-mannitol, 1 g of fish meal, 20 g/L of KBr, 8 g/L of $Fe_2(SO_4)_3 \cdot 4H_2O$, and 30 mL of DMSO dissolved in 970 mL natural seawater) at 25 °C with shaking at 150 rpm. After 10 days, the broth was extracted with EtOAc and evaporated to yield an organic extract of CMDD10D111 (3.8 g).

Isolation of Anmindenols A and B. The extract (3.8 g) was subjected to silica flash column chromatography using step-gradient elution of MeOH in CH_2Cl_2 (0%, 1%, 2%, 5%, 10%, 50%, 100%) to afford seven fractions (Fr 1–Fr 7). Fr 1 (740.9 mg), which contained the mixture of anmindenols, was further purified by C_{18} HPLC using 55% CH_3CN in H_2O to obtain anmindenols A (**1**, 3.2 mg) and B (**2**, 1.5 mg).

*Anmindenol A (**1**).* yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 202 (3.07), 262 (3.16), 305 (2.21) nm; IR (film) ν_{max} 3444, 1633 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 1; LRMS m/z 215.1 $[M + H]^+$; HRFABMS m/z 213.2179 $[M - H]^-$ calcd for $C_{15}H_{17}O$, 213.1279.

*Anmindenol B (**2**).* yellow, amorphous solid; $[\alpha]_D^{25}$ + 80 (c 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.39), 260 (3.33), 295(2.67) nm; CD (8.62 mM, DMSO), λ_{max} ($\Delta\varepsilon$) 425 (0.52), 303 (−0.47), and 260 (0.32) nm; IR (film) ν_{max} 3418, 2926, 1710, 1607, 1384 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRFABMS m/z 231.1377 $[M - H]^-$ calcd for $C_{15}H_{19}O_2$, 231.0577.

Determinations of the Absolute Configuration of C-4 in Anmindenol B Using $Mo_2(OAc)_4$. Anmindenol B (0.5 mg) was dissolved in 250 μL of DMSO and the solution was divided into two 125 μL aliquots. A 125 μL aliquot of 8.62 mM DMSO solution of $Mo_2(OAc)_4$ was added to 125 μL of prepared anmindenol B solution to make 4.32 mM anmindenol B and $Mo_2(OAc)_4$ mixture solution. The mixture was kept for 30 min to form a stable metal complex, after which the ECD spectrum was recorded for induced CD. The observed band of the ECD curve at 305 nm was used to determine the absolute configuration of C-4.

Nitric Oxide Assay. The NO assay was performed for compounds by measuring NO production in LPS-induced RAW 264.7 mouse macrophage cells according to a previously published protocol.[19]

ASSOCIATED CONTENT

Supporting Information

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

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Notes

The authors declare no competing financial interest.

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