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Ascochytatin, a Novel Bioactive Spirodioxynaphthalene Metabolite Produced by the Marine-derived Fungus, *Ascochyta* sp. NGB4

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Abstract Ascochytatin, a new spirodioxynaphthalene metabolite produced by a marine-derived fungus, was found from a screening program focused on the bacterial two-component regulatory system. The structure of ascochytatin was determined by spectroscopic methods, including NMR and MS. The relative stereochemistry was determined by an X-ray crystallographic analysis, and the absolute stereochemistry was determined by the modified Mosher's method.

Keywords marine-derived fungus, ascochytatin, two-component regulatory system, modified Mosher's method

Introduction

The two-component regulatory system (TCS) of bacteria, which consists of two proteins, a histidine kinase (HK) and a response regulator (RR), has received increasing attention for its potential as a novel antibacterial drug target [1], and a number of TCS autophosphorylation inhibitors with inhibitory activity against multi-drug-resistant bacteria have been reported [2～4]. TCS is a fundamental system of bacterial response to environmental stress in both Gram-negative and Gram-positive bacteria, and most TCSs are not necessarily essential for steady-state growth without stress. However, a small number of TCS-encoding genes have been found to be essential; for instance, a set of YycG (HK) and YycF (RR) is the essential TCS in *Bacillus subtilis* and *Staphylococcus aureus*. A sensitive screening method for antibacterial agents that inhibit YycG/YycF has been developed. This utilizes a temperature-sensitive *yycF* mutant (CNM2000) of *B. subtilis*, and aranorosinol B was found to be a YycG inhibitor by this screening method in an earlier study [5]. Continued screening for new YycG/YycF inhibitors enabled us to find ascochytatin (**1**), a new spirodioxynaphthalene metabolite produced by a marine-derived fungus. The producing fungus, isolation, structural determination, including the absolute stereochemistry, and some biological activities of **1** are described in this paper.

Materials and Methods

Microorganism

The marine-derived fungus, *Ascochyta* sp. NGB4, was isolated from a floating scrap of festering rope that had been collected at a fishing port in Nagasaki prefecture, Japan (N32°47′31″, E129°46′55″). The fungus was

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cultured and kept on potato dextrose agar (Difco) prepared in 50% natural seawater. This strain has been deposited as NITE AP-488 at the NITE Patent Microorganisms Depositary (NPMD), Japan.

Taxonomic Studies

The following media were used to identify the producing fungus: potato dextrose agar (PDA), 2.0% malt agar (MA), oatmeal agar (OA), Miura's medium (LcA), and corn meal agar (CMA). The formed colonies were observed after a two-week incubation at 25°C. The color names used in this study were taken from Kornerup and Wanscher [6].

Bioassay Using *B. subtilis* 168 and CNM2000 [5]

In order to search for the TCS inhibitors, each sample (*i.e.* the Me_2CO extract of a cultured fungus) was spotted on trypticase soy (0.75%) agar (1.5%) plates that had been overlaid with 3.0 ml of top agar (0.5%) containing 30 μl of an overnight culture of *B. subtilis* 168 or CNM2000. After 24 hours of the incubation at 37°C, the diameter of inhibition zone of each strain was measured. In screening for TCS inhibitors, those samples that inhibited the growth of CNM2000 more strongly than that of 168 were selected.

Culture and Isolation

Ascochyta sp. NGB4 as the seed culture was cultured at 25°C at a rotation speed of 100 rpm for 7 days in potato dextrose broth (Difco) that had been prepared with 50% seawater. One-ml samples of the seed culture were inoculated on the production agar medium (400 ml) in stainless steel plates (320×190 mm). The composition of the agar medium is as follows: glucose, 0.5%; glycerol, 2.0%; yeast extract, 0.2%; Pharmamedia® (Traders Protein), 2.0%; NaCl, 0.25%; and agar, 1.5%; and adjusted to pH 6.5 before autoclaving. After incubating at 25°C for 14 days, the cultured agar medium (8.0 liters) was extracted with Me_2CO. The extract was evaporated to remove the Me_2CO, and the aqueous residue (*ca.* 2.0 liters) was extracted twice with an equal volume of EtOAc. The extract was concentrated *in vacuo* and chromatographed on a silica gel column by a stepwise elution with mixtures of $CHCl_3$/MeOH : MeOH 0, 1, 10 and 100%. The 1.0% MeOH - $CHCl_3$ fraction, which exhibited stronger inhibitory activity toward *B. subtilis* CNM2000 than 168, was further purified by a silica gel column chromatography using hexane/EtOAc (1 : 1) as the eluent. The active fractions were collected and evaporated *in vacuo* to yield 80 mg of **1** as a white powder. Half of this powder was crystallized from MeOH for an X-ray crystallographic analysis, yielding colorless needles (12 mg).

General for Structural Determination

UV spectra were recorded with a Beckman DU 640 spectrometer and IR spectra with a JASCO FT/IR-430 instrument. Melting points were measured on Yanagimoto apparatus. Optical rotations were measured on a Horiba SEPA-300. The ^{1}H- and all 2D NMR spectra were recorded with a Varian Unity INOVA 750 instrument at 750 MHz, and the ^{13}C-NMR spectrum was recorded with a Varian Unity INOVA 500 instrument at 125 MHz. Chemical shifts are referenced to the solvent peaks of δ_H 3.31 and δ_C 49.15 for CD_3OD, and δ_H 7.25 and δ_C 77.0 for $CDCl_3$. Low- and high-resolution FAB-MS data were obtained with a JEOL JMS700 spectrometer, and ESI-MS data were measured with a ThermoFinigan LCQ Advantage instrument.

X-Ray Crystallographic Analysis

The crystal used for the X-ray crystallographic analysis was obtained by crystallization from MeOH. Crystal data for **1**: $C_{40}H_{28}O_{14}$ (for two molecules), colorless needle, M_r=732.62, monoclinic, $P2(1)$, a=12.8150 (14) Å, b=8.2858 (9) Å, c=15.6756 (18) Å, α=90°, β=109.9230 (10)°, γ=90°, V=1564.9 (3) Å^3, Z=2, $\rho_{calcd.}$=1.555 Mg m^{-3}, μ=0.158 mm^{-1}, T=100 K, 8976 measured reflections, 4549 [R(int)=0.0434] independent reflections, 495 parameters, GOF=1.013, and $R1$ ($wR2$)=0.0611 (0.1581). The measurements were made with a Bruker-AXS SMART Apex II CCD system with MoK_α radiation (λ= 0.71073 Å) at 100 K. The crystal structure was solved by direct methods and refined by a full matrix least squares operation on F^2 with SHELXTL. Absorption corrections were applied with SADABS. CCDC 669952 contains supplementary crystallographic data to those reported here. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif.

Trimethylascochytatin (2)

To a solution of **1** (5.0 mg, 13.6 μmol) in a $CHCl_3$/MeOH (5 : 1) mixture (2.0 ml) was added an excess amount of trimethylsilyldiazomethane (Tokyo Chemical Industry Co.). After stirring at ambient temperature for 36 hours, the reaction mixture was evaporated to dryness, and the residue was chromatographed on a silica gel column (i.d. 10×150 mm), eluting with hexane/EtOAc (2 : 1) to afford **2** (3.5 mg). Compound **2**: white solid; TLC Rf value 0.7 ($CHCl_3$/MeOH (10 : 1)) and 0.2 (hexane/EtOAc (1 : 1)); ^{1}H-NMR (750 MHz, $CDCl_3$): 3.71 (1H, t, 3.8, H-3), 3.67 (1H, d, 3.8, H-2), 3.69 (3H, s, 8-O–CH_3), 3.92 (3H, s, 5-O–CH_3), 3.98 (3H, s, 4′-O–CH_3), 4.26 (1H, br s, 4-OH), 5.38 (1H, br s, H-4), 6.80 (1H, d, 9.0, H-3′) 6.95 (1H, d, 8.3, H-7′), 6.98 (1H, d, 9.0, H-2′), 6.99 (2H, s, H-6 and H-7

overlapping), 7.41 (1H, t, 8.3, H-6′), 7.82 (1H, d, 8.3, H-5′); ^{13}C-NMR (125 MHz, $CDCl_3$): 53.12 (C-2), 54.21 (C-3), 55.86 (4′-O–CH_3), 56.26 (5-O–CH_3), 57.86 (8-O–CH_3), 63.71 (C-4), 96.73 (C-1), 105.53 (C-3′), 108.82 (C-2′), 109.67 (C-7′), 113.11 (C-8a′, interchangeable with C-6), 113.19 (C-6, interchangeable with C-8a′), 115.29 (C-7), 115.39 (C-5′), 121.97 (C-8a), 125.41 (C-4a), 125.89 (C-4a′), 126.49 (C-6′), 140.74 (C-1′), 147.37 (C-8′), 150.05 (C-4′), 151.83 (C-5), 154.32 (C-8).

(*R*)-MTPA Ester (3)

To a solution of **2** (1.5 mg, 3.7 μmol) in pyridine (100 μl) was added (+)-(*S*)-MTPACl (15 μl, Kanto Kagaku Co.). After stirring at ambient temperature for 14 hours, the reaction mixture was diluted with $CHCl_3$ and then successively washed with aqueous $NaHCO_3$ and saturated NaCl. The organic layer was dried with Na_2SO_4 and evaporated to dryness, and the residue was chromatographed on a silica gel column (i.d. 10×200 mm), eluting with hexane/EtOAc (2 : 1) to afford **3** (1.3 mg). Compound **3**: white solid; TLC Rf value 0.4 (hexane/EtOAc (1 : 1)); ^{1}H-NMR (750 MHz, $CDCl_3$): 3.50 (3H, s, 5-O–CH_3), 3.65 (3H, br s, –O–CH_3 of MTPA), 3.69 (1H, d, 4.5, H-2), 3.69 (3H, s, 8-O–CH_3), 3.90 (1H, t, 4.5, H-3), 3.99 (3H, s, 4′-O–CH_3), 6.64 (1H, d, 4.5, H-4), 6.81(1H, d, 8.3, H-3′), 6.85 (1H, d, 9.0, H-6), 6.97 (1H, d, 7.5, H-7′), 7.00 (1H, d, 8.3, H-2′), 7.03 (1H, d, 9.0, H-7), 7.39 (3H, m, phenyl group of MTPA), 7.43 (1H, t, 7.5, H-6′), 7.71 (2H, m, phenyl group of MTPA), 7.84 (1H, d, 7.5, H-5′).

(*S*)-MTPA Ester (4)

A sample of **2** (1.5 mg, 3.7 μmol) was treated with (−)-(*R*)-MTPACl (15 μl) (Kanto Kagaku Co.), and the reaction mixture was processed by the same procedure as that just described to afford **4** (1.0 mg). Compound **4**: white solid; TLC *Rf* value 0.4 (hexane/EtOAc (1 : 1)); ^{1}H-NMR (750 MHz, $CDCl_3$): 3.52 (3H, br s, –O–CH_3 of MTPA), 3.64 (1H, d, 4.5, H-2), 3.71 (3H, s, 8-O–CH_3), 3.76 (3H, s, 5-O–CH_3), 3.79 (1H, t, 4.5, H-3), 3.98 (3H, s, 4′-O–CH_3), 6.74 (1H, d, 4.5, H-4), 6.78 (1H, d, 8.3, H-3′), 6.95 (1H, d, 8.3, H-2′), 6.96 (1H, d, 9.0, H-6), 6.96 (1H, d, 7.5, H-7′), 7.07 (1H, d, 9.0, H-7), 7.37 (3H, m, phenyl group of MTPA), 7.42 (1H, t, 7.5, H-6′), 7.62 (2H, m, phenyl group of MTPA), 7.82 (1H, d, 7.5, H-5′).

Antimicrobial Activity

A MeOH soln of **1** (200 or 20 μg/ml) was added to paper disks (15 μl on a 6-mm diameter paper disk), and the disks were dried for 30 minutes in a clean bench. The disks were then placed on agar media that had been inoculated with each of the test microorganisms (*Arthrobacter paraffineus* ATCC21220, *Brevibacterium* sp. JCM6894, *Staphylococcus aureus* IFO12732, *Bacillus subtilis* IFO3134, *Cytophaga marinoflava* IFO14170, *Pseudovibrio* sp. MBIC3368, *Escherichia coli* IFO3301, *Pseudomonas aeruginosa* IFO3446, *Candida albicans* IFO1060, and *Saccharomyces cervisiae* ATCC27202). Halo formation was observed after 48 hours of incubation at 30°C.

Cytotoxicity

A549 cells (a human lung cancer cell line) and Jurkat cells (a human leukemia cell line) were purchased from Dainippon Sumitomo Pharma Co. The A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The cells were seeded in a flat-bottomed 96-well microplate at a density of 4,000 cells/200 μl/well, and then cultured in an incubator (5.0% CO_2 - air) at 37°C for 14 hours. Serially diluted samples were added to each well, and the cells were cultured for a further 48 hours. The number of living cells was counted by the Alamar Blue™ assay according to the provided procedure. The IC_{50} value was determined as the concentration of a test compound that inhibited the number of living cells by 50% compared to the blank control. The Jurkat cells were cultured in an RPMI 1640 medium containing 10% fetal bovine serum. The IC_{50} value for the Jurkat cells was determined by the same procedure as that used for the A549 cells, except that the seeded density of the Jurkat cells was 2,000 cells/200 μl/well.

Results

Taxonomy

Colonies on PDA plates reached 47～60 mm diameter, floccose to deplanate with radiately sulcate, brown to greyish brown (5E-4 to 5E-3). Colonies on MA reached 53～56 mm diameter, floccose to plane, olive (3E-F-8). Colonies on OA reached 60～65 mm diameter, floccose, light orange to orange white (5A-5 to 5A-2). Colonies on LcA reached 55～65 mm diameter, floccose, light orange to orange white (5A-5 to 5A-2). Colonies on CMA reached 35～37 mm diameter, deplanate, white (1A-1). Conidiomata are pycnidial, superficial and immersed in the center of colonies on PDA and CMA, globose, dark brown. Conidiogenous cells are phialidic, doliiform to ampulliform, hyaline, 7～10×2～3 μm. Conidia are ellipsoidal to oblong, mainly two-celled (rarely three-celled), hyaline, 3～5×1.5～2 μm. On these characteristics, strain NGB4 was identified as anamorphic genera Ascochyta [7], the fungus being tentatively designated as *Ascochyta* sp. NGB4.

Table 1 Physico-chemical properties of ascochytatin (**1**)

Appearance	Colorless needle
Melting point	227~229°C (decomp.)
Molecular weight	366
Molecular formula	$C_{20}H_{14}O_7$
ESI-MS (positive)	384 $(M+H_2O)^+$
(negative)	365 $(M-H)^-$
HRFAB-MS (negaitive)	found: 365.0658
	calcd : 365.0661 ($C_{20}H_{13}O_7$)
$[\alpha]_D^{25}$	−153° (*c* 0.2, MeOH)
UV λ_{max} nm (log ε) in MeOH	211 (4.71), 311 (4.10), 339 (3.85)
IR ν_{max} (KBr) cm^{-1}	3481, 3430, 3328, 1611, 1519, 1478, 1419
TLC[a] Rf value	0.30 [$CHCl_3$/MeOH (10 : 1)]
	0.41 [hexane/EtOAc (1 : 2)]

[a] Silica gel 60 F254, Merck.

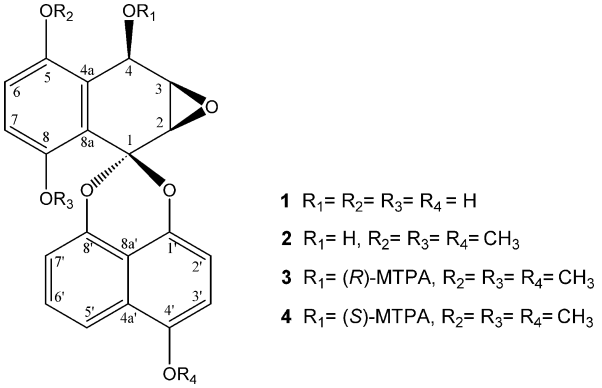

Fig. 1 Structures of ascochytatin (**1**) and its derivatives (**2**, **3** and **4**).

Structural Determination

Ascochytatin (**1**) was obtained from the culture extract of the marine-derived fungus, *Ascochyta* sp. NGB4. The physico-chemical properties of **1** are summarized in Table 1. The molecular formula was determined to be $C_{20}H_{14}O_7$ from the HRFAB-MS and ^{13}C-NMR data. The ^{13}C-NMR spectrum showed twenty resolved signals, of which sixteen aromatic carbons and three O-bearing aliphatic carbons were observed, while the remaining one (δ_C 100.29) could be an acetal carbon. The ^{1}H-NMR spectrum showed ten signals corresponding to ten protons. The remaining four protons were exchangeable and must be hydroxyl group protons (–O*H*). All direct ^{1}H–^{13}C connections were determined by an HSQC experiment. An analysis of ^{1}H–^{1}H-COSY data indicated four spin systems; three aromatic parts (Fig. 2a, c and d) and an aliphatic moiety (Fig. 2b) were obvious. An epoxide group in the aliphatic moiety was presumed from the ^{1}H- and ^{13}C- chemical

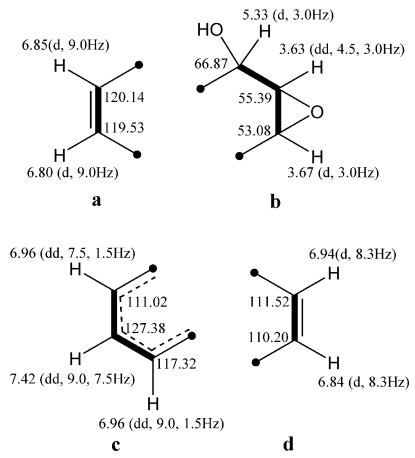

Fig. 2 Partial structures determined from COSY.

Table 2 NMR spectral data of **1** in CD_3OD.

Position	δ_C[a]	δ_H (*J* in Hz)[a]	HMBC (H→C)
1	100.29		
2	53.08	3.67 (1H, d, 4.5)	1, 3, 4, 8, 8a
3	55.39	3.63 (1H, dd, 4.5, 3.0)	2, 4, 4a
4	66.87	5.33 (1H, d, 3.0)	4a, 5, 8a
4a	122.09		
5	151.00		
6	120.14	6.85 (1H, d, 9.0)	4, 4a, 8, 8a
7	119.53	6.80 (1H, d, 9.0)	1, 4a, 5, 8a
8	151.28		
8a	118.45		
1′	140.22		
2′	111.52	6.94 (1H, d, 8.3)	5′, 8′, 8a′
3′	110.20	6.84 (1H, d, 8.3)	4a′, 5′, 8′, 8a′
4′	150.11		
4a′	126.78		
5′	117.32	7.82 (1H, dd, 9.0, 1.5)	1′, 2′, 4a′, 5′, 8′, 8a′
6′	127.38	7.42 (1H, dd, 9.0, 7.5)	1′, 2′, 4′, 4a′, 8a′
7′	111.02	6.96 (1H, dd, 7.5, 1.5)	1′, 4′
8′	149.02		
8a′	115.19		

[a] Residual solvent signals are used as internal standards (δ_H 3.31 for CHD_2OD, δ_C 49.15 for CD_3OD)

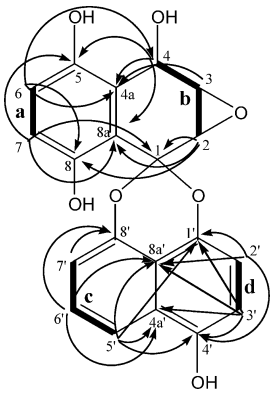

Fig. 3 Planar structure of **1** deduced from HMBC.

Bold lines show spin systems from COSY, which correspond to the partial structures a, b, c and d in Fig. 2, and arrows indicate HMBC signals.

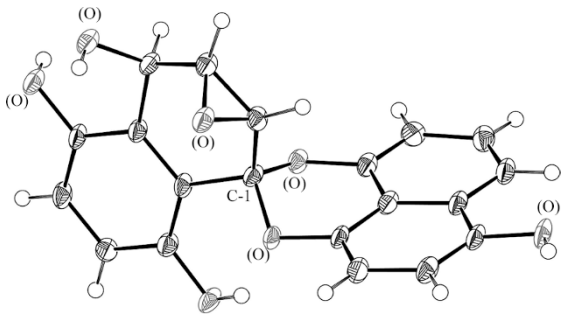

Fig. 4 ORTEP drawing of **1**.

shifts. The planar structure of **1** shown in Fig. 3 was deduced from the HMBC signals, and the NMR data are summarized in Table 2. Compound **1** was a 1′,4′,8′-trihydroxynaphthalene unit connected by a spiroacetal (*via* 1′ and 8′ hydroxy groups) to another partially reduced and functionalized naphthalene unit.

An X-ray crystallographic analysis was conducted to confirm the structure and determine the relative stereochemistry. A suitable crystal, whose crystal parameters and refinement parameters are described in the Materials and Methods section, was obtained by crystallization from MeOH. An ORTEP drawing is shown in Fig. 4, the X-ray analysis validating the structure deduced by NMR experiments and also allowing us to establish the relative configuration.

The modified Mosher's method [8] was employed to determine the absolute stereochemistry of **1**. The three

phenolic and naphtholic hydroxy groups (5-OH, 8-OH, and 4′-OH) of **1** were initially methylated by trimethylsilyldiazomethane to avoid signal complexity, and then the trimethylated derivative (**2**) was further converted to the (*R*)- or (*S*)-MTPA ester (**3** or **4**, respectively). The protons of each derivative were assigned by ^{1}H-NMR, COSY, HSQC and HMBC data. The key $\Delta\delta$ (δ_S–δ_R) values are shown in Fig. 5, indicating that the absolute configuration of C-4 was *R*. The absolute stereochemistry was therefore determined as shown in Fig.1.

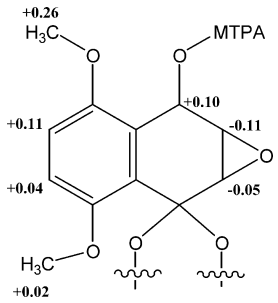

Fig. 5 $\Delta\delta$ ($\delta_{(S)\text{-MTPA ester}} - \delta_{(R)\text{-MTPA ester}}$) values for the MTPA esters of **2**.

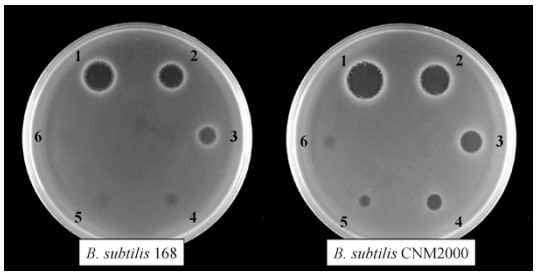

Fig. 6 Sensitivity of *Bacillus subtilis* 168 and CNM2000 to **1**.

The differential inhibitory activity of **1** against *B. subtilis* 168 and CNM2000 was investigated. One microliter of the DMSO solution of **1** at six concentrations (μg/ml) was spotted on Trypticase Soy agar plates containing *B. subtilis* 168 or CNM2000: 1000 (1), 500 (2), 250 (3), 125 (4), 62.5 (5), 31.3 (6).

Sensitivity of *B. subtilis* 168 and CNM2000 to 1

The sensitivity of *B. subtilis* 168 and CNM2000 to **1** was examined. As shown in Fig. 6, **1** exhibited stronger activity against *B. subtilis* CNM2000 than against wild-type strain 168, as aranorosinol B did [5]. The difference in sensitivity to **1** between *B. subtilis* 168 and CNM2000 suggests that **1** inhibited the function of TCS (YycG/YycF) in *B. subtilis*.

Antimicrobial Activity and Cytotoxicity

The antimicrobial activity was investigated by the paper disk method, the result being presented in Table 3. Compound **1** exhibited relatively strong and specific activity against Gram-positive bacteria and *C. albicans*. The cytotoxicity of **1** to mammalian cancer cells was also investigated, compound **1** exhibiting cytotoxicity to both A549 and Jurkat cells with respective IC_{50} values of 4.8 and 6.3 μM.

Discussion

Ascochytatin (**1**) was found by a screening method using *B. subtilis* 168 and its temperature-sensitive mutant, CNM2000, which could differentiate the TCS inhibitors

Table 3 Antimicrobial activity of (**1**)

Taxon	Tested strain	Halo diameter (mm)[a]	
	Strain	3 μg/disk	0.3 μg/disk
Actinobacteria	*Arthrobacter paraffineus* ATCC21220	8	—
	Brevibacterium sp. JCM6894	18	7
Firmicutes	*Staphylococcus aureus* IFO 12732	11	7
	Bacillus subtilis IFO 3134	17	10
Bacteroidetes	*Cytophaga marinoflava* IFO 14170	—	—
Alphaproteobacteria	*Pseudovibrio* sp. MBIC3368	—	—
Gammaproteobacteria	*Escherichia coli* IFO 3301	—	—
	Pseudomonas aeruginosa IFO3446	—	—
Yeast	*Candida albicans* IFO 1060	16	11
	Saccharomyces cerevisiae ATCC27202	—	—

[a] — : No activity.

[5]. The structure of **1** was determined by spectroscopic methods, including MS and NMR. The relative stereochemistry was determined by an X-ray crystallographic analysis, and the absolute configuration was determined by the modified Mosher's method. Compound **1** was shown to be a 1′,4′,8′-trihydroxy-naphthalene unit connected by a spiroacetal to another partially reduced and functionalized naphthalene unit as shown in Fig. 1. Although more than twenty structurally related compounds having a spirodioxynaphthalene unit in the molecule, *e.g.* cladospirone bisepoxide [9], CJ-12,371 and CJ-12,372 [10], Sch 49210 and its related compounds [11], palmarumycins [12], diepoxins [13], and decaspirones [14], have been reported, **1** is the sole compound being oxidized at 4′ position of the naphthalene unit.

The earlier study found that aranosinol B exhibited differential activity between *B. subtilis* 168 and CNM2000, and completely inhibited YycG autophoshorylation at 50 μg/ml. Although **1** exhibited almost the same differential activity againt *B. subtilis* 168 and CNM2000 as aranosinol B did, the inhibitory activity of **1** for YycG autophosphorylation was quite week (unpublished result). Studies on the detailed mechanism of action of **1** are being undertaken.

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