Antarones A and B, Two Polyketides from an Endophytic *Penicillium antarcticum*

Yoshihito Shiono[a], Yumi Seino[a], Takuya Koseki[a], Tetsuya Murayama[a], and Ken-ichi Kimura[b]

[a] Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan
[b] Laboratory of Chemical Biology, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan

Reprint requests to Dr. Y. Shiono. Fax: +81-235-28-2873. E-mail: yshiono@tds1.tr.yamagata-u.ac.jp

Z. Naturforsch. **2008**, *63b,* 909 – 914; received February 5, 2008

Two new polyketides, antarones A and B, were isolated from the endophytic fungus *Penicillium antarcticum*. Their structures were determined by spectroscopic methods. Their general toxicity and cytotoxicity were evaluated, respectively, by brine shrimp lethality and cytotoxicity assay with human cancer cell lines. Neither antarones A nor B exhibited activity at the concentration of 100 μM.

Key words: Antarones A and B, Hamigerone, *Penicillium antarcticum*

Introduction

Endophytes are microorganisms that colonize living inside the healthy plants tissue without causing apparent symptoms of plant disease. Recently, a lot of biologically active secondary metabolites have been reported from endophytes, which emphasized their potential ecological roles in endophyte and host interactions [1]. Endophytes have become an important source of novel active metabolites with potential as agrochemicals and pharmaceutical agents [2]. In our continuing search for biologically active compounds from endophytes [3, 4], an endophytic fungus, *Penicillium antarcticum* A. D. Hocking & C. F. McRae (FH-14) was isolated from the stem of *Cedrus deodara*. From the cultivation of this fungus in unpolished rice, two new polyketides, antarones A (**1**) and B (**2**) were isolated (Fig. 1). This paper describes the structure elucidation of these compounds.

Results and Discussion

P. antarcticum A. D. Hocking & C. F. McRae (FH-14) was cultivated on sterilized unpolished rice at 25 °C for 4 weeks. Purification of the compounds was guided by their characteristic coloration by TLC. A MeOH extract of the fermented unpolished rice was concentrated and partitioned between ethyl acetate and water. The organic layer was subjected to silica gel column chromatography eluting with an *n*-hexane-EtOAc

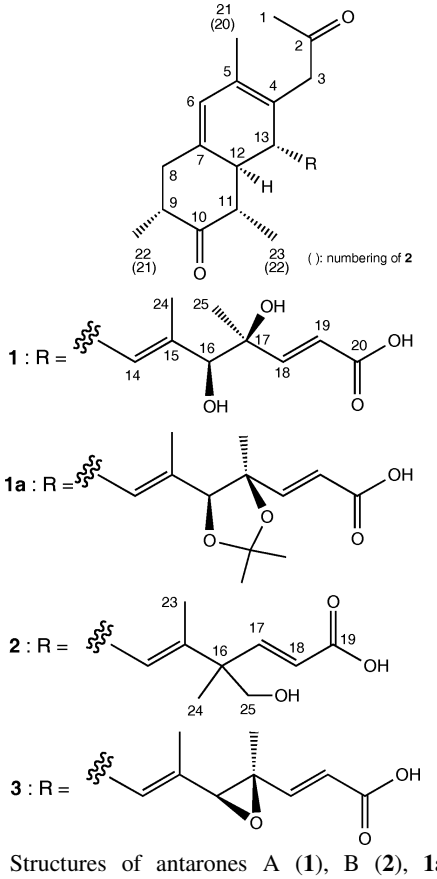


Fig. 1. Structures of antarones A (**1**), B (**2**), **1a**, and hamigerone (**3**).

Table 1. ^{1}H and ^{13}C NMR data of antarone A (**1**)[a].

No	δ_C	δ_H	HMBC
1	29.6 q	2.15 (3H, s)	2, 3
2	207.4 s		
3	47.1 t	3.01 (1H, d, 15.6)	1, 2, 4, 5, 13
		3.32 (1H, d, 15.6)	1, 2, 4, 5, 13
4	122.6 s[b]		
5	127.4 s		
6	122.7 d[b]	5.72 (1H, s)	4, 5, 8, 12, 21
7	137.4 s		
8	42.4 t	2.00 (1H, t, 11.9)	6, 7, 9, 10
		2.64 (1H, dd, 11.9, 6.6)	6, 7, 9, 10
9	48.5 d	2.35 – 2.44[b]	
10	212.6 s		
11	49.1 t	2.35 – 2.44[b]	
12	50.2 d	1.70 (1H, d, 12.6)	4
13	38.9 d	3.11 (1H, d, 10.1)	5, 7, 14, 15
14	132.4 d	5.29 (1H, d, 10.1)	4, 12, 16, 24
15	133.6 s		
16	81.6 d	3.89 (1H, s)	14, 15, 17, 18, 24, 25
17	75.6 s		
18	155.3 d	7.02 (1H, d, 15.6)	17, 20, 25
19	119.2 d	6.06 (1H, d, 15.6)	17, 18, 20
20	170.1 s		
21	18.3 q	1.81 (3H, s)	4, 5, 6
22	14.8 q	1.09 (3H, d, 6.3)	8, 9, 10
23	11.1 q	1.07 (3H, d, 6.3)	10, 11, 12
24	13.4 q	1.74 (3H, s)	14, 15, 16
25	23.5 q	1.18 (3H, s)	16, 17, 18

[a] Solvent: $CDCl_3$; coupling constants in Hz in parentheses; [b] overlapping signals.

gradient. Further chromatographic studies using silica and ODS gel yielded two pure compounds, antarones A (**1**, 22.0 mg) and B (**2**, 10.5 mg).

Antarone A (**1**) was obtained as colorless oil that analyzed for $C_{25}H_{34}O_6$ by a combination of HRFABMS and ^{13}C NMR spectroscopy which indicated nine degrees of unsaturation. Its UV spectrum with an absorption maximum at 283 nm was indicative of a conjugated chromophore. The IR spectrum exhibited bands at 1702 and 1652 cm^{-1}, characteristic of carbonyl groups. The analyses of ^{13}C NMR and DEPT spectra indicated the presence of six methyls, two sp^3 methylens, five sp^3 methines (one of them bearing an oxygen atom), one sp^3 quaternary carbon, four sp^2 methines, four sp^2 quaternary carbons, two ketones, and one carboxylic group. Since seven out of the nine unsaturation equivalents were accounted for by the ^{13}C NMR data, **1** was inferred to have two rings. The ^{1}H NMR spectrum (Table 1) showed signals for four singlet methyls at δ_H = 1.18, 1.74, 1.81, 2.15 and two doublet methyls at δ_H = 1.07 (d, J = 6.3 Hz), 1.09 (d, J = 6.3 Hz), two methylenes at δ_H = 2.00 (d, J = 11.9 Hz, 1H), 2.64 (d, J =11.9, 6.6 Hz, 1H), 3.01 (d, J = 15.6 Hz, 1H), 3.32 (d, J = 15.6 Hz, 1H), an oxygenated methine at δ_H = 3.89 (s, 1H), four methines at δ_H = 1.70 (d, J = 12.6 Hz, 1H), 2.35 – 2.44 (m, 2H), 3.11 (d, J = 10.1 Hz, 1H), and four olefinic methines at δ_H = 5.29 (d, J = 10.1, 1H), 5.72 (s, 1H), 6.06 (d, J = 15.6 Hz, 1H), 7.02 (d, J = 15.6 Hz, 1H). The ^{1}H-^{1}H COSY spectrum of **1** demonstrated that **1** had three partial structures represented by thick lines in Fig. 2. The connectivities of these fragments were determined by an HMBC experiment (Fig. 2, Table 1). In the HMBC spectrum of **1**, the olefinic methyl protons (21-H_3) were correlated with C-4, C-5 and C-6, the methine protons (H-6) with C-8 and C-12, two methyl protons (22-H_3 and 23-H_3) with C-10, and the methine proton (12-H) with C-4. These data suggested the presence of a trimethyl-hexahydro-naphthalen-2-one substructure. These structural features strongly suggested that **1** had the same carbon skeleton with a naphthalene ring system as that of hamigerone (**3**) [5]. Investigation of the HMBC spectrum revealed the presence of a propan-2-one group, which was located at C-4 due to the HMBC correlations from 3-H to C-5 and C-13. The presence of 4,6-dimethyl-4,5-dihydro-hepta-2,6-dienoic acid was established by HMBC correlations from 24-H_3 and 25-H_3 to C-16, 16-H and 25-H_3 to C-18, and 18-H to C-20. The positioning of this group on C-13 was confirmed by HMBC correlations from 13-H to C-15.

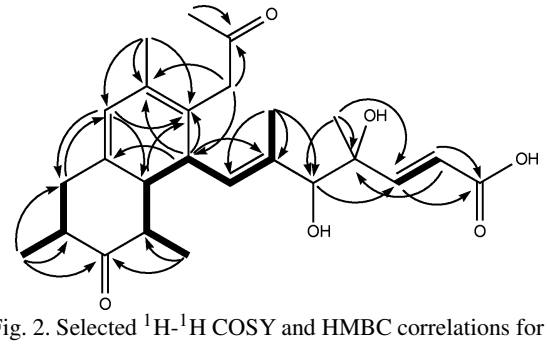


Fig. 2. Selected ^{1}H-^{1}H COSY and HMBC correlations for **1**.

The relative stereochemistry for **1** was determined by a combination of NOE experiments and measurement of the carbon chemical shifts and proton-proton coupling constants. Analysis of the observed coupling constant (J = 0 Hz) by the Karplus relationship suggested that the dihedral angle for 12-H / 13-H was approximately 90°, showing that 13-H is arranged *pseudo-equatorial* and *trans* to 12-H. A coupling constant of J = 12.6 Hz between the 11-H and 12-H signals showed that these two protons have an *axial-axial* con-

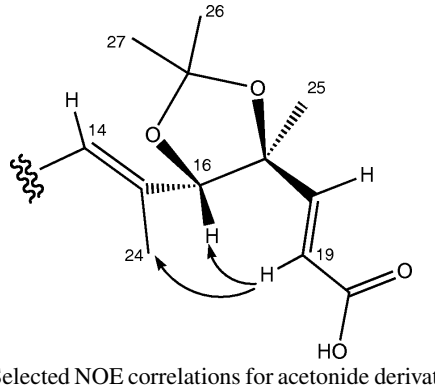
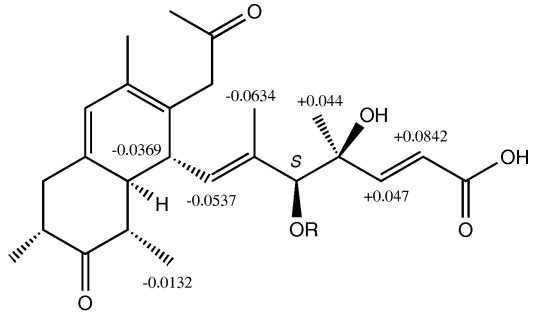


Fig. 3. Selected NOE correlations for acetonide derivative **1a**.



1 b: R : (*S*)-(–)-MTPA
1 c: R : (*R*)-(+)-MTPA

Fig. 4. Chemical shift differences for the (*S*)-(–)-MTPA esters (**1b**) and (*R*)-(+)-MTPA esters (**1c**) in ppm at 400 MHz.

figuration. The relative stereochemistry of the hydronaphthalenone system of **1** was also supported on the basis of the close similarity of the spectral parameters, especially the ^{13}C NMR chemical shifts, with the corresponding values for **1** and **3**. The *E* stereochemistry of the trisubstituted double bond (C-14) is based on the NOE correlations from 14-H to 16-H. The double bond at C-18 was established to be in the *E*-form by the coupling constants ($J_{18,19}$ = 15.6 Hz). To confirm the relative configuration of the two hydroxyl groups in positions 16 and 17, the acetonide derivative **1a** was prepared by treatment of **1** with 2,2-dimethoxypropane and a catalytic amount of *p*-TsOH without the other diastereomeric isomers. The *syn*-relationship of the hydroxy groups at C-16 and C-17 in **1a** was inferred from NOEs between 16-H and 19-H, and 19-H and 24-H_3 (Fig. 3). The absolute stereochemistry of **1** at C-16 was determined by a modified Mosher method. On comparing the ^1H NMR spectroscopic data for the (*S*)-(–)- and (*R*)-(+)-MTPA esters (**1b** and **1c**) (Fig. 4), the *S* configuration was assigned to the chiral C-16,

Table 2. ^1H and ^{13}C NMR data of antarone B (**2**)[a].

No	δ_C	δ_H	HMBC
1	29.5 q	2.15 (3H, s)	2, 3
2	207.4 s		
3	47.5 t	3.07 (1H, d, 15.2)	1, 2, 5
		3.24 (1H, d, 15.2)	1, 2, 5
4	122.7 s[b]		
5	127.5 s		
6	122.7 d[b]	5.73 (1H, s)	5, 8, 20
7	137.4 s		
8	42.5 t	2.04 (1H, t, 12.2)	6, 7, 9, 10
		2.65 (1H, dd, 12.2, 6.6)	6, 7, 9, 10
9	48.4 d	2.35 – 2.41[b]	8, 10
10	212.5 s		
11	49.2 t	2.35 – 2.41[b]	
12	50.2 d	1.69 (1H, d, 12.5)	
13	39.7 d	3.09 (1H, d, 8.9)	5, 7, 14, 15
14	130.0 d	5.14 (1H, d, 8.9)	16, 23
15	135.9 s		
16	48.5 s		
17	155.4 d	7.01 (1H, d, 16.1)	18, 19, 25
18	119.6 d	5.82 (1H, d, 16.1)	16
19	170.4 s		
20	18.4 q	1.83 (3H, s)	4, 5, 6
21	14.9 q	1.09 (3H, d, 6.4)	8, 9, 10
22	11.2 q	1.08 (3H, d, 6.4)	10, 11, 12
23	13.4 q	1.65 (3H, d, 1.2)	14, 15, 16
24	20.5 q	1.14 (3H, s)	15, 16, 17, 25
25	67.2 t	3.56 (1H, d, 11.0)	15, 16, 17, 24
		3.61 (1H, d, 11.0)	15, 16, 17, 24

[a] Solvent: $CDCl_3$; coupling constants in Hz in parentheses; [b] overlapping signals.

but the absolute stereochemistry of **1** was not determined.

Antarone B (**2**) was found to have the molecular formula $C_{25}H_{34}O_5$ from its HRFAB and NMR data. ^1H and ^{13}C NMR data for **2** correspond well to those of **1**, but are characterized by the disappearance of the oxymethine signal (16-H) in **1** and the appearance of the characteristic signals due to an isolated oxymethylene [δ_H = 3.56 (d, *J* = 11.0 Hz, 1H) and 3.61 (d, *J* = 11.0 Hz, 1H)]. In the HMBC spectrum (Table 2) of **2**, correlations from the oxymethylene protons to C-15, C-16, C-17, and C-24 confirmed the placement of the hydroxymethyl group at C-25. The structure of **2** was further supported by HMBC correlations (Table 2). The stereochemistry at C-16 remains uncertain. The absolute stereochemistry of **2** also remains unsettled, such as that of **1** and **3**. Clearly, in order to establish the absolute configurations of **1** and **2** reliable single crystal structure determinations on suitable derivatives of **1** and **2** are needed.

The closest similar microbial metabolites having a heptanoic acid-substituted naphthalenone ring sys-

tem are hamigerone (**3**) and dihydrohamigerone [5] isolated from *Hemigera avellanea*, and embellostatin [6, 7] isolated from *Culvunaria lunata*. Hamigerone (**3**) showed weak antimicrobial activity against *Pyricularia oryzae*, while embellostatin is reported to be a microtubule inhibitor and antiangiogenic agent. Compounds **1** and **2** were tested for antimicrobial activity against Gram-positive and Gram-negative bacteria, yeast, and fungi strains. None of the compounds showed acivity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus clavatus*, *P. oryzae* at the highest concentration (300 μg/disk). Furthermore, we investigated the cytotoxicity of **1** and **2** against human tumor cells by an MTT assay. Neither **1** nor **2** showed cytotoxicity against K562, HL-60, LNCaP and HeLa cells at the tested concentrations ($IC_{50} > 100$ μM). In addition, the isolated compounds **1** and **2** were also assayed for brine shrimp (*Artemia salina)* toxicity, but showed no effective results ($IC_{50} > 100$ μM). Further pharmacological studies of **1** and **2** are currently in progress.

Experimental Section

General experimental procedures

Optical rotation was measured with a Horiba model SEPA-300 polarimeter, IR spectra were recorded with a JASCO J-20A spectrophotometer, and UV spectra were recorded with a Shimadzu UV mini-1240 instrument. Mass spectra were recorded with a JEOL JMS-700 instrument, and ^{1}H and ^{13}C NMR spectra were obtained with a JEOL EX-400 spectrometer. Chemical shifts are given in ppm on a δ scale with TMS as an internal standard. Column chromatography was conducted on ODS (Fuji Silysia, Japan) and silica gel 60 (Kanto Chemical Co., Inc.). TLC was done on a precoated silica gel plate (Merck), and spots were detected by spraying 10 % vanillin in H_2SO_4 followed by heating.

Isolation of the producing strain

The fungal strain FH-14 was isolated from the stem of *Cedrus deodara* collected in April 2004 in the Tsuruoka Park, Yamagata, Japan. This strain was identified as a *Penicillium antarcticum* A. D. Hocking & C. F. McRae by Centraalbureau voor Schimmelcultures (The Netherlands). The strain FH-14 has been deposited at the laboratory of the Faculty of Agriculture, Yamagata University, Yamagata, Japan.

*Cultivation of the endophytic fungus, extraction and isolation of compounds **1** and **2***

P. antarcticum A. D. Hocking & C. F. McRae (FH-14) was cultivated on sterilized unpolished rice (1200 g) at 25 °C for 4 weeks. The moldy unpolished rice was extracted with MeOH, and the MeOH extract was concentrated. The resulting aqueous concentrate was partitioned into *n*-hexane and EtOAc layers. The purification of the EtOAc layer was guided by the intense blue characteristic coloration with vanillin-sulfuric acid solution on TLC plates. The EtOAc layer was chromatographed on a silica gel column using a gradient of *n*-hexane-EtOAc (100 : 0 – 0 : 100) to give fractions 1 – 11 (Fr. 1-1 – 1-11). Fr. 1-8 (*n*-hexane-EtOAc, 30 : 70, 1.2 g) was subjected to silica gel column chromatography by eluting with $CHCl_3$ and an increasing ratio of MeOH. Eleven fractions (Fr. 2-1 – 2-11) were obtained. Fr. 2-7 ($CHCl_3$-EtOAc, 40 : 60, 0.25 g) was subjected to ODS column chromatography using a gradient of MeOH-H_2O (0 : 100 – 100 : 0) to afford crude **1**, which was finally purified by silica gel flash column chromatography with a mixture of $CHCl_3$-MeOH (90 : 10) to yield antarone A (**1**, 22.0 mg). Fr. 1-7 (*n*-hexane-EtOAc, 40 : 60, 1.5 g) was purified by silica gel flash column chromatography eluting with $CHCl_3$ and an increasing ratio of EtOAc. Eleven fractions (Fr. 3-1 – 3-11) were obtained. Fr. 3-7 ($CHCl_3$-EtOAc, 40 : 60, 0.25 g) was subjected to ODS column chromatography using a gradient of MeOH-H_2O (0 : 100 – 100 : 0) to afford antarone B (**2**, 10.5 mg).

*Antarone A (**1**)*

Colorless oil. – $[\alpha]_D^{20} = -207°$ (c = 0.46, MeOH). – UV (MeOH): $\lambda_{max}(\lg \varepsilon)$ = 283 nm (3.6). – IR (KBr): ν = 3465, 1702, 1652, 1070 and 1049 cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) data see Table 1. – HRMS (negative mode, FAB): m/z = 429.2282 (calcd. for $C_{25}H_{33}O_6$, 429.2277, [M–H]$^-$). – MS (negative mode, FAB): m/z = 429 [M–H]$^-$.

*Antarone B (**2**)*

Colorless oil. – $[\alpha]_D^{20} = -256°$ (c = 0.31, MeOH). – UV (MeOH): $\lambda_{max}(\lg \varepsilon)$ = 283 nm (3.6). – IR (KBr): ν = 3421, 1706, 1652, 1162 and 1045 cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) data see Table 2. – HRMS (negative mode, FAB): m/z = 413.2324 (calcd. for $C_{25}H_{33}O_5$, 413.2328, [M–H]$^-$). – MS (negative mode, FAB): m/z = 413 [M–H]$^-$.

*Preparation of acetonide **1a** of **1***

To compound **1** (1.0 mg) in 2,2-dimethoxylpropane (0.3 mL) and CH_2Cl_2 (0.3 mL), were added catalytic amounts of *p*-toluenesulfonic acid, and the mixture was left to stir at r. t. for 4 h. After evaporation of the solvents the residue was subjected to silica gel column chromatography ($CHCl_3$: MeOH = 20 : 1, v/v) to yield the 16,17-*O*-isopropylidene-compound (**1a**, 0.6 mg) as an amorphous powder.

*Acetonide derivative (**1a**)*

MS (positive mode, FAB): *m/z* = 471 $[M+H]^+$. – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.08 (d, *J* = 6.6 Hz, 3H, 23-H_3), 1.09 (d, *J* = 6.6 Hz, 3H, 22-H_3), 1.10 (s, 3H, 25-H_3), 1.38 (s, 3H, 26-H_3), 1.49 (s, 3H, 27-H_3), 1.69 (s, 3H, 24-H_3), 1.83 (s, 3H, 21-H_3), 1.69 – 1.72 (m, 1H, 12-H), 2.05 (t, *J* = 12.5 Hz, 1H, 8-H), 2.15 (s, 3H, 1-H_3), 2.18 (d, *J* = 15.6 Hz, 1H, 3-H), 2.35 – 2.45 (2H, m, 9-H and 11-H), 2.65 (dd, *J* = 12.5, 6.0 Hz, 1H, 8-H), 3.44 (d, *J* = 15.6 Hz, 1H, 3-H), 5.74 (s, 1H, 6-H), 3.15 (d, *J* = 10.3 Hz, 1H, 13-H), 5.48 (d, *J* =10.3 Hz, 1H, 14-H), 4.22 (s, 1H, 16-H), 6.97 (d, *J* = 15.5 Hz, 1H, 18-H), 6.10 (d, *J* = 15.5 Hz, 1H, H-19).

*Preparation of MTPA ester derivatives (**1b** and **1c**) from **1***

To **1** (1.0 mg) in CH_2Cl_2 (0.5 mL) were added 2.0 mg of (*S*)-(–)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA,), 2.0 mg of dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (2.0 mg). The mixture was stirred at r. t. for 12 h. EtOAc was added to the reaction mixture, and the resulting solution was washed with a saturated solution of aqueous $NaHCO_3$ and brine, and concentrated *in vacuo*. Purification by column chromatography on silica gel (*n*-hexane-EtOAc) gave the (*S*)-(–)-MTPA ester (**1b**) of **1**. Compound **1** (1.0 mg) was treated with (*R*)-(+)-MTPA (2.0 mg) in the same manner to afford the (*R*)-(+)-MTPA ester (**1c**, 0.9 mg).

*Compound **1b***

MS (positive mode, FAB): *m/z* = 647 $[M+H]^+$. – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.09 (d, *J* = 6.6 Hz, 3H, 22-H_3), 1.05 (d, *J* = 6.6 Hz, 3H, 23-H_3), 1.38 (s, 3H, 25-H_3), 1.56 – 1.63 (m, 1H, 12-H), 1.65 (s, 3H, 24-H_3), 1.81 (s, 3H, 21-H_3), 1.94 (t, *J* = 11.8 Hz, 1H, 8-H), 2.14 (s, 3H, 1-H_3), 2.33 – 2.44 (2H, m, 9-H and 11-H), 2.63 (dd, *J* = 11.8, 6.0 Hz, 1H, 8-H), 2.76 (d, *J* = 15.6 Hz, 1H, 3-H), 3.08 (d, *J* = 10.3 Hz, 1H, 13-H), 3.38 (d, *J* = 15.6 Hz, 1H, 3-H), 3.49 (3H, s, MTPA-OCH_3), 5.28 (s, 1H, 16-H), 5.35 (d, *J* =10.3 Hz, 1H, 14-H), 5.72 (s, 1H, 6-H), 5.94 (d, *J* = 15.5 Hz, 1H, H-19), 7.20 (d, *J* = 15.5 Hz, 1H, 18-H), 7.43 – 7.52 (m, 5H, MTPA-Ar*H*).

*Compound **1c***

MS (positive mode, FAB): *m/z* = 647 $[M+H]^+$. – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.06 (d, *J* = 6.3 Hz, 3H, 23-H_3), 1.09 (d, *J* = 6.4 Hz, 3H, 22-H_3), 1.33 (s, 3H, 25-H_3), 1.56 – 1.60 (m, 1H, 12-H), 1.71 (s, 3H, 24-H_3), 1.83 (s, 3H, 21-H_3), 1.94 (t, *J* = 11.8 Hz, 1H, 8-H), 2.13 (s, 3H, 1-H_3), 2.33 – 2.44 (2H, m, 9-H and 11-H), 2.64 (dd, *J* = 11.8, 6.5 Hz, 1H, 8-H), 2.79 (d, 1H, *J* = 15.3 Hz, 3-H), 3.11 (d, *J* = 10.6 Hz, 1H, 13-H), 3.36 (d, 1H, *J* = 15.3 Hz, 3-H), 3.49 (3H, s, MTPA-OCH_3), 5.36 (s, 1H, 16-H), 5.40 (d, *J* = 10.6 Hz, 1H, 14-H), 5.73 (s, 1H, 6-H), 5.86 (d, *J* = 15.7 Hz, 1H, 19-H), 7.15 (d, *J* = 15.7 Hz, 1H, 18-H), 7.41 – 7.52 (m, 5H, MTPA-Ar*H*).

*Antimicrobial activity of compounds **1** and **2***

Test organisms were *Staphylococcus aureus* NBRC 13276, *Pseudomonas aeruginosa* ATCC 15442, *Aspergillus clavatus* F 318a, *Pyricularia oryzae* NBRC 31178 and *Candida albicans* ATCC 2019. Antimicrobial assays were carried out by the paper disk diffusion method using a published protocol [8].

Cell cytotoxicity assay

Human chronic myelogenous leukemia K562 (ATCC CCL-243, 5×10^4 cells mL^{-1}), human promyelocytic leukemia HL-60 (RCB0041, 4×10^4 cells mL^{-1}), human prostate carcinoma LNCaP (ATCC CRL-1740, 1×10^5 cells mL^{-1}), and human cervical cancer HeLa (ATCC CCL-2, 1×10^5 cells mL^{-1}) cells were treated with compounds **1** and **2** at various concentrations using a 96-well microplate at 37 °C under a humidified, 5 % CO_2 atmosphere for 4 days in RPMI 1640 medium (for K562, HL-60 and LNCaP cells, GIBCO) and DMEM (for HeLa cell, GIBCO) supplemented with 10 % fetal bovine serum (BIOWEST), 50 units mL^{-1} of penicillin and 50 μg mL^{-1} of streptomycin (GIBCO). Cytotoxicity was determined by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, Dojin) assay. Percentages of viable cells were calculated as the ratio of the A_{570} values of treated and control cells (treated with 5 % MeOH vehicle for 100 μL of K562, LNCaP and HeLa cells, and 2 % MeOH vehicle for 100 μL of HL-60). Values are the averages of two independent experiments.

Brine shrimp lethality assay

The screening for brine shrimp toxicity was performed using a slight modification of the original method [9]. The egg of brine shrimp, *Artemia salina*, was hatched in a beaker filled with artificial sea water. Approximately twenty hatched brine shrimp larvae (*A. salina*) in 3.0 mL artificial seawater were added to each well containing different concentrations of sample in 20 μL of MeOH. Samples and controls were run in duplicate. After 24 h at 25 °C, the number of alive, immobile and dead brine shrimp larvae were counted with a magnifying glass.

Acknowledgements

We thank Ms. Teiko Yamada of the Faculty of Agriculture at Tohoku University for HRMS measurements. Finally we wish to thank Professor Dr. Hartmut Laatsch of the Institute for Organic and Biomolecular Chemistry, University of Göttingen, for his advise in structure assignments.

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