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ORIGINAL ARTICLE

Penicyrones A and B, an epimeric pair of α-pyrone-type polyketides produced by the marine-derived *Penicillium* sp.

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Two polyketides containing an α-pyrone unit, named penicyrones A (1) and B (2), were isolated from a culture broth of the marine-derived *Penicillium* sp. TPU1271 together with nine known compounds: verrucosidin (3), fructigenine A (4), verrucofortine (5), cyclo-(L-Trp-L-Phe) (6), cyclopenol (7), cyclopenin (8), penipratynolene (9), aspterric acid (10) and viridicatol (11). The structures of 1 and 2 were elucidated by analyzing the spectroscopic data of 1, 2 and their *O*-acetyl derivatives (1a and 2a). Compounds 1 and 2 were epimers of each other at the C-9 position. The absolute configurations of 1 and 2 were assigned on the basis of NOESY data for 1, 2, 1a and 2a, a conformational analysis and the identity of the biogenetic pathway with verrucosidin (3). The planar structure of penicyrones was found in the SciFinder as a compound in the commercial chemical libraries; however, the stereostructure and spectroscopic data were not available. Therefore, this is the first study on the isolation and structure elucidation, including the absolute configurations, of penicyrones A (1) and B (2) as fungal metabolites. Compound 3 exhibited growth inhibitory activity against *Mycobacterium smegmatis* at 40 μg per disc (inhibition zone of 11 mm). This is the first study to demonstrate that verrucosidin (3) exhibited anti-mycobacterial activity.
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INTRODUCTION

Marine natural resources have continued to provide structurally and biologically novel compounds, some of which are promising leads for the development of new drugs.[1–3] Marine microorganisms, especially marine-derived fungi, have been attractive and important sources of pharmacologically active secondary metabolites.[4–6] Marine fungal metabolites, possessing unique structures and biological properties, are expected to become candidates for advanced medicines and biochemical reagents.[4–6]

In the course of our search for useful bioactive substances from marine microorganisms, the marine-derived *Penicillium* sp. TPU1271 was found to produce various types of secondary metabolites. HPLC separation from a culture broth of strain TPU1271 led to the isolation of an epimeric pair of polyketides containing α-pyrone and the tetrahydrofuran rings, penicyrones A (**1**) and B (**2**) (Figure 1), as well as nine known compounds: verrucosidin (**3**),[7–11] fructigenine A (**4**),[12,13] verrucofortine (**5**),[9,12] cyclo-(L-Trp-L-Phe) (**6**),[14,15] cyclopenol (**7**),[9,16] cyclopenin (**8**),[9,17] penipratynolene (**9**),[18,19] aspterric acid (**10**)[20] and viridicatol (**11**)[16,21] (Figure 1). Although the planar structure of penicyrones was found in the SciFinder as a compound in commercial chemical libraries, the stereostructure, spectroscopic data and origin were not available. We described herein the isolation of penicyrones A (**1**) and B (**2**) as secondary metabolites from the marine-derived *Penicillium* sp., structures including the absolute configurations of **1** and **2** and the anti-mycobacterial activity of verrucosidin (**3**). This is the first study to demonstrate that verrucosidin (**3**) exhibited inhibitory activity against *Mycobacterium smegmatis*.

RESULTS AND DISCUSSION

Structure elucidation

Compounds **3–11** were identified as verrucosidin, fructigenine A, verrucofortine, cyclo-(L-Trp-L-Phe), cyclopenol, cyclopenin, penipratynolene, aspterric acid and viridicatol, respectively (Figure 1), by comparing their spectroscopic data with the reported values.[7–21]

Penicyrones A (**1**) and B (**2**) were first obtained as a mixture by HPLC (octadecylsilyl (ODS)) with CH_3OH-H_2O (1:1). The mixture showed a clear ^1H NMR spectrum; however, two sets of signals were detected for several carbons in the ^{13}C NMR spectrum. The isolation of **1** and **2** was achieved by repeated HPLC (ODS) with CH_3OH-H_2O (2:3).

Compound **1** was isolated as a colorless oil. The MW and formula of **1**, 434 and $C_{24}H_{34}O_7$, respectively, were determined from the HRFABMS (high-resolution fast atom bombardment mass spectra) and NMR data. ^1H and ^{13}C NMR spectra showed 32 protons and 24 carbons (Table 1), which were classified into eight methyl, one oxygenated methyl, three sp^3 oxygenated methine, three sp^3 oxygenated quaternary, two sp^2 methine, four sp^2 quaternary, two sp^2 oxygenated quaternary and one carbonyl carbons by an analysis of HMQC and DEPT spectra. The ^1H NMR spectrum of **1** resembled

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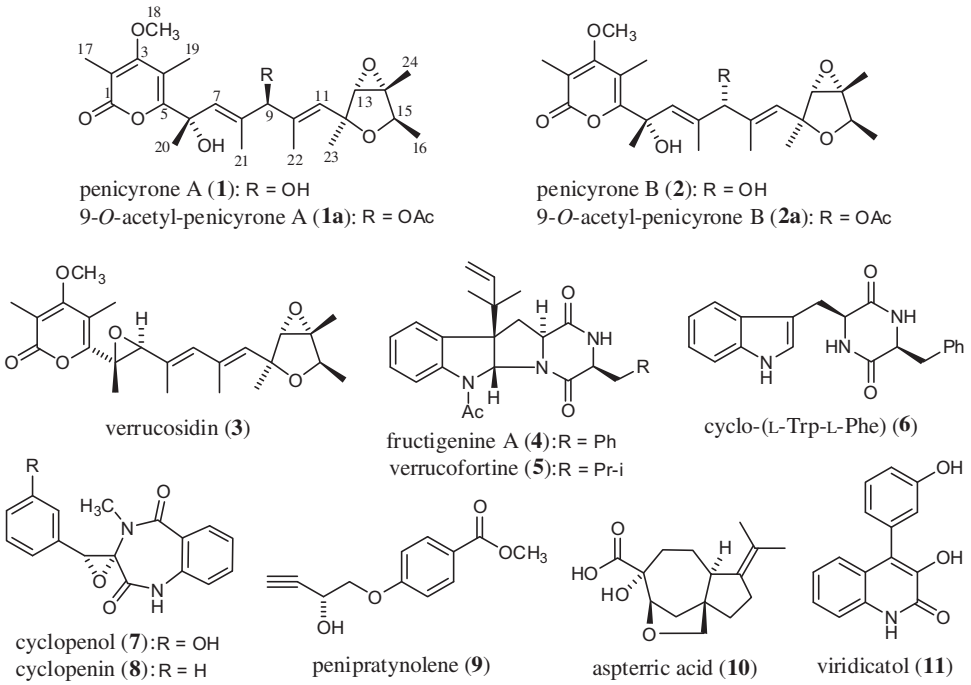

Figure 1 Structures of compounds **1**–**11** produced by the marine-derived fungus *Penicillium* sp. TPU1271.

Table 1 ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR data for penicyrones A (1) and B (2) in CD_3OD

	1		*2*	
C no.	δ_C	δ_H, *mult. (J in Hz)*	δ_C	δ_H, *mult. (J in Hz)*
1	167.7		167.7	
2	110.9		110.9	
3	171.9		171.9	
4	113.4		113.3	
5	162.8		162.8	
6	75.3		75.3	
7	133.8	5.88, t (1.2)	134.1	5.86, t (1.2)
8	137.7		137.8	
9	82.6	4.23, s	82.6	4.24, s
10	139.7		139.8	
11	130.3	5.70, t (1.5)	129.9	5.69, t (1.2)
12	81.7		81.7	
13	68.9	3.55, s	68.89	3.54, s
14	68.8		68.86	
15	78.4	4.03, q (6.8)	78.4	4.03, q (6.9)
16	19.4	1.14, d (6.8)	19.4	1.13, d (6.9)
17	10.3	2.02, s	10.3	2.02, s
18	61.3	3.85, s	61.3	3.85, s
19	11.1	2.10, s	11.1	2.09, s
20	27.9	1.63, s	28.0	1.64, s
21	13.1	1.33, s (overlapped)	13.1	1.33, br d (1.0)
22	14.4	1.70, br d (1.2)	14.7	1.71, d (1.2)
23	22.1	1.33, s (overlapped)	22.1	1.32, s
24	13.9	1.43, s	13.8	1.42, s

that of verrucosidin (**3**), which suggested the presence of α-pyrone and tetrahydrofuran rings in **1**. ^{1}H-^{1}H COSY data for **1** revealed two partial structures of C-15–C-16 and C-7–C-11 with C-21 and C-22. The connections of the two partial structures with α-pyrone and tetrahydrofuran rings were assigned by HMBC data for **1** (Figure 2). HMBC correlations were detected from H_3-20 (δ 1.63) to C-5 (162.8), C-6 (75.3) and C-7 (133.8), from H-11 (5.70) to C-12 (81.7) and C-13 (68.9) and from H_3-23 (1.33) to C-11 (130.3), C-12 and C-13. The NOESY correlations between H-7 (δ 5.88)/H-9 (δ 4.23), H-7/H_3-20 (1.63), H-11 (5.70)/H-13 (3.55), H_3-21 (1.33)/H_3-22 (1.70) and H_3-22/H_3-23 (1.33) established the *E*-orientation of two double bonds at C-7 and C-10 (Figure 2). Thus, the planar structure of **1** was determined as shown in Figure 2.

HRFABMS and NMR data for compound **2** revealed that the MW and formula (434, $C_{24}H_{34}O_7$) of **2** were the same as those of **1**. ^{1}H and ^{13}C NMR data for **2** were very similar to those for **1** (Table 1). Moreover, the 2D NMR spectra (^{1}H-^{1}H COSY, HMQC, HMBC and NOESY) of **2** showed the same correlations as those of **1**. Therefore, the planar structure of **2** was elucidated to be the same as that of **1** (Figure 2).

This planar structure was found in the SciFinder as a commercially available reagent in chemical libraries for the screening of drug discovery. However, the origin, spectroscopic data and stereochemistry were not described. Consequently, this is the first study to isolate **1** and **2** as natural products from a fungal culture broth.

Absolute configurations of penicyrones A (1) and B (2)

NOE correlations between H-11 (**1**, δ 5.70; **2**, δ 5.69)/H-13 (**1**, 3.55; **2**, 5.54), H-13/H-24 (**1**, 1.43; **2**, 1.42) and H-16 (**1**, 1.14; **2**, 1.13)/H-24 observed in the NOESY spectra of **1** and **2** suggested that the relative configurations of **1** and **2** were the same as that of **3**. As compounds **1** (+110.0), **2** (+96.0) and **3** (+70.0) showed similar specific rotations and were produced by the same fungal strain TPU1271 through the identical biogenetic pathway, the absolute configurations at the tetrahydrofuran ring (C-12–C-16) in **1** and **2** were assigned the same as those in **3**. The absolute configuration of **3** was previously established by organic synthetic studies.[22–24]

Slight differences in ^{13}C chemical shifts were detected for the signals due to C-7, C-8 and C-22, which indicated that **1** and **2** were the

epimers of each other at the C-9 position. As NOESY data for **1** and **2** could not distinguish the configuration at C-9, 9-*O*-acetyl derivatives (**1a** and **2a**) were prepared. The ^{1}H NMR spectra of **1a** and **2a** showed marked differences in the chemical shifts at H-9 (**1a**, δ 5.32 and **2a**, 5.30) and the acetyl methyl (**1a**, 2.05 and **2a**, 2.02). NOESY data for **1a** showed the correlations between H_3-26 (δ 2.05)/H_3-21 (1.39) and H_3-26/H_3-22 (1.76), and a correlation between H_3-26 (2.02)/H_3-20 (1.62) was observed in the NOESY spectrum of **2a**. Based on the NOESY correlations, the most stable conformers of 9*R* and 9*S* isomers for *O*-acetyl derivatives were calculated by a Monte Carlo conformational analysis with an MMFF94 force field using Spartan'14. The stereostructures of the 9*R* isomer (Figure 3a) and 9*S* isomer (Figure 3b) agreed with the configurations of **1a** and **2a**, respectively. The configurations at the C-6 positions in **1** and **2** were assigned by the correlations between H-7 and H_3-20 in the NOESY spectra of **1**, **2**, **1a** and **2a**.

Thus, the absolute configurations of **1** and **2** were elucidated as (6*S*, 9*R*, 12*S*, 13*S*, 14*R*, 15*R*) and (6*S*, 9*S*, 12*S*, 13*S*, 14*R*, 15*R*), respectively, as shown in Figure 1.

Biological activity

The anti-bacterial activities of compounds **1**–**11** against *M. smegmatis* NBRC 3207 were evaluated by the paper disc method.[25,26] Verrucosidin (**3**) showed an inhibition zone of 11 mm at 40 μg per disc. Compound **3** was initially isolated from *Penicillium verrucosum* var. *cyclopium* as a potent neurotoxin,[7] and its inhibitory effects on GRP78 promoter expression has recently been reported.[11] Consequently, this is the first study to demonstrate that verrucosidin (**3**) exhibited anti-mycobacterial activity. In spite of the structural similarities of **1**–**3**, the two new compounds did not inhibit the growth of *M. smegmatis*.

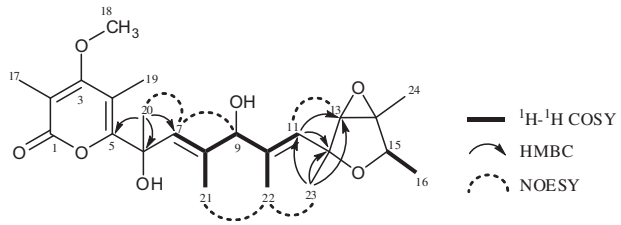

Figure 2 ^{1}H-^{1}H COSY, key HMBC and key NOESY correlations for penicyrones A (**1**) and B (**2**).

MATERIALS AND METHODS

General experimental procedures

FABMS was performed using a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were recorded on a JNM-AL-400 NMR spectrometer (JEOL) at 400 MHz for ^{1}H and 100 MHz for ^{13}C in CD_3OD (δ_H 3.31, δ_C 49.15). Optical rotations were measured with a JASCO P-2300 digital polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on a Hitachi U-3310 UV-Visible spectrophotometer (Hitachi, Tokyo, Japan) and IR spectra on a Perkin-Elmer Spectrum One Fourier transform infrared spectrometer (Perkin-Elmer, Waltham, MA, USA). CD spectra were measured with a spectrometer (J-720; JASCO). Secondary metabolites were analyzed by a LaChrom Elite HTA system (Hitachi). Preparative HPLC was conducted using a Toyosoda CCPU dual pump (Toyosoda Kogyo, Tokyo, Japan) with a Tosoh UV-8010 detector (Tosoh, Tokyo, Japan).

Materials

Potato dextrose agar, Middlebook 7H9 broth, polysorbate 80 and Middlebook OADC were purchased from BD (Franklin Lakes, NJ, USA). All other chemicals including organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Isolation and identification of strain TPU1271

The fungal strain TPU1271 was isolated from organic debris attached to a cultured oyster shell collected at a depth of 10 m in the Oshika Peninsula, Miyagi Prefecture, Japan, in June 2012. The debris was suspended in 25 ml of sterilized seawater, and 200 μl of the mixture was spread on an agar plate (potato dextrose agar, 90% seawater) and incubated at 25 °C. Strain TPU1271 was identified by the sequence of its ITS1 rDNA (231 nucleotides). Twenty-nine known *Penicillium* species including *P. polonicum*, *P. cordubense* and *P. crustosum* showed 100% identity, and, therefore, strain TPU1271 was identified as a *Penicillium* sp. The following sequence was used in a BLAST search: 5′-AGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTTT GGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGG CCCGCCTTTACTGGCCGCCGGGGGGCTCACGCCCCCGGGCCCGCGCC CGCCGAAGACACCCCCGAACTCTGTCTGAAGATTGAAGTCTGAGTGAA AATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGG-3′.

Fermentation

Strain TPU1271, which was grown on the potato dextrose agar plate, was inoculated into a 100-ml Erlenmeyer flask containing 40 ml of the seed medium (2.0% glucose, 0.5% polypeptone, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% yeast extract, 0.1% KH_2PO_4, 0.1% agar in natural seawater; adjusted to pH 6.0 before sterilization). The flask was shaken reciprocally for 3 days at 25 °C to obtain the seed culture, which was then transferred to the production medium (3.0% sucrose, 3.0% soluble starch, 1.0% malt extract, 0.30% Ebios (Asahi Food and Healthcare, Tokyo, Japan), 0.50% KH_2PO_4 and 0.050% $MgSO_4{\cdot}7H_2O$ in

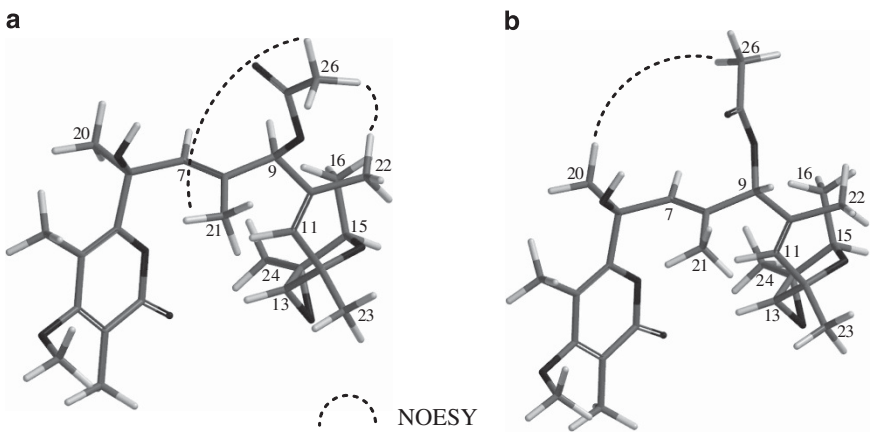

Figure 3 The calculated most stable conformers with key NOESY correlations for (**a**) **1a** and (**b**) **2a**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

natural seawater; adjusted to pH 6.0 before sterilization). The production culture was carried out at room temperature for 21 days under static conditions.

Extraction and isolation of compounds 1–11

Acetone (3.0 l) was added to the culture broth (3.0 l) after 21 days and filtered. The filtrate was concentrated to remove acetone, and the aqueous residue was adsorbed on an ODS column. The column was eluted stepwise with 10, 30, 40, 50, 60, 70, 80, 90 and 100% CH_3OH in H_2O for separation into nine fractions. Fr. 5 (171.4 mg, 60% CH_3OH eluate) was separated by HPLC (column; PEGASIL ODS SP100 (Senshu Scientific, Tokyo, Japan), 10×250 mm^2; mobile phase, 50% CH_3OH; detection, UV at 210 nm; flow rate, 2.0 ml min^{-1}) to give compounds **6** (2.1 mg), **8** (11.2 mg), **9** (2.4 mg) and **11** (5.5 mg), and a mixture of **1** and **2** (54.7 mg). Compounds **1** (5.5 mg) and **2** (4.1 mg) were purified from a portion of the mixture by repeated HPLC (PEGASIL ODS SP100, 40% CH_3OH, UV 215 nm, 2.0 ml min^{-1}, $t_R = \sim 180$ min). Compounds **4** (19.0 mg), **5** (2.6 mg) and **10** (80.5 mg) were isolated from Fr. 7 (161.0 mg, 80% CH_3OH eluate) by HPLC (PEGASIL ODS SP100, 70% CH_3OH, UV at 210 nm, 2.0 ml min^{-1}). Compound **3** (17.5 mg) was obtained by HPLC (80% CH_3OH) from Fr. 8 (70.5 mg, 90% CH_3OH eluate). Fr. 3 (200.2 mg, 40% CH_3OH eluate) was separated by a Sephadex LH-20 column (GE Healthcare UK Ltd., Buckinghamshire, UK) with 30% CH_3OH to yield compound **7** (90.0 mg).

*Penicyrone A (**1**)*: A colorless oil; $[\alpha]_D^{20}$+110 (*c* 0.10, CH_3OH); IR (KBr) ν_{max} 3426, 2978, 2929, 2857, 1688, 1558, 1451, 1377, 1095, 1043 and 1012 cm^{-1}; UV (CH_3OH) λ_{max} (nm) ($\log \varepsilon$) 306 (3.40); CD (CH_3OH) $\lambda_{extermum}$ (nm) ($\Delta\varepsilon$) 298 (+12.9), 213 (−40.9); HRFABMS *m/z* 435.2389 ([M+H]$^+$; calcd for $C_{24}H_{35}O_7$, 435.2383); ^{1}H and ^{13}C NMR (CD_3OD) (see Table 1).

*Penicyrone B (**2**)*: A colorless oil; $[\alpha]_D^{20}$+96.0 (*c* 0.10, CH_3OH); IR (KBr) ν_{max} 3436, 2978, 2934, 2863, 1683, 1558, 1451, 1380, 1092, 1048 and 1026 cm^{-1}; UV (CH_3OH) λ_{max} (nm) ($\log \varepsilon$) 304 (3.63); CD (CH_3OH) $\lambda_{extermum}$ (nm) ($\Delta\varepsilon$) 298 (+12.0), 213 (−38.8); HRFABMS *m/z* 435.2391 ([M+H]$^+$; calcd for $C_{24}H_{35}O_7$, 435.2383); ^{1}H and ^{13}C NMR (CD_3OD) (see Table 1).

*Verrucosidin (**3**)*: A pale yellow oil; $[\alpha]_D^{22}$ +70.0 (*c* 0.10, CH_3OH); CD (CH_3OH) $\lambda_{extermum}$ (nm) ($\Delta\varepsilon$) 291 (+1.48), 241 (+8.78); EIMS *m/z* 416 [M]$^+$; ^{1}H NMR (CD_3OD): δ 5.83 (1H, s), 5.44 (1H, ts), 4.10 (1H, q, $J = 6.8$ Hz), 3.80 (3H, s), 3.46 (1H, s), 3.40 (1H, s), 2.02 (3H, s), 2.01 (3H, s), 1.93 (3H, brs), 1.87 (3H, brs), 1.44 (3H, s), 1.40 (3H, s), 1.39 (3H, s) and 1.16 (3H, d, $J = 6.8$ Hz).

Preparation of 9-*O*-acetyl penicyrones A (1a) and B (2a)

Acetic anhydride (2.0 μl, 21 nmol), pyridine (2.0 μl, 25 nmol) and 4-(dimethylamino)pyridine (0.1 mg, 0.83 nmol) were added to a solution of compound **1** (2.0 mg, 4.6 nmol) in CH_2Cl_2 (100 μl), and the solution was stirred at room temperature for 10 h. The reaction mixture was evaporated *in vacuo*, dissolved in water and extracted with EtOAc. The organic layer was concentrated *in vacuo* to give 9-*O*-acetyl-penicyrone A (**1a**, 1.66 mg, 3.5 mmol, 76.1%). The purity of the product was sufficient for NMR experiments.

9-*O*-Acetyl-penicyrone B (**2a**, 1.73 mg, 3.6 mmol, 78.3%) was prepared by similar procedures as described above.

*9-O-Acetyl-penicyrone A (**1a**)*: A colorless oil; $[\alpha]_D^{21}$+122 (*c* 0.10, CH_3OH); IR (KBr) ν_{max} 3434, 2973, 2929, 1739, 1714, 1690, 1561, 1454, 1372, 1355, 1237, 1089, 1043 and 1029 cm^{-1}; UV (CH_3OH) λ_{max} (nm) ($\log \varepsilon$) 300 (3.97); CD (CH_3OH) $\lambda_{extermum}$ (nm) ($\Delta\varepsilon$) 297 (+10.9), 213 (−33.5); HRFABMS *m/z* 477.2492 ([M+H]$^+$; calcd for $C_{26}H_{37}O_8$, 477.2488); ^{1}H NMR (CD_3OD): δ 5.84 (1H, t, $J = 0.9$ Hz, H-7), 5.65 (1H, t, $J = 0.9$ Hz, H-11), 5.32 (1H, s, H-9), 4.02 (1H, q, $J = 6.8$ Hz, H-15), 3.85 (3H, s, H-18), 3.54 (1H, s, H-13), 2.08 (3H, s, H-19), 2.05 (3H, s, H-26), 2.02 (3H, s, H-17), 1.76 (3H, br d, $J = 0.7$ Hz, H-22), 1.61 (3H, s, H-20), 1.41 (3H, s, H-24), 1.39 (3H, br d, $J = 1.0$ Hz, H-21), 1.30 (3H, s, H-23) and 1.11 (3H, d, $J = 6.8$ Hz, H-16).

*9-O-Acetyl-penicyrone B (**2a**)*: A colorless oil; $[\alpha]_D^{21}$+118 (*c* 0.10, CH_3OH); IR (KBr) ν_{max} 3432, 2978, 2934, 1742, 1714, 1690, 1561, 1451, 1372, 1353, 1235, 1089, 1078, 1040 and 1026 cm^{-1}; UV (CH_3OH) λ_{max} (nm) ($\log \varepsilon$) 297 (3.85); CD (CH_3OH) $\lambda_{extermum}$ (nm) ($\Delta\varepsilon$) 297 (+13.8), 213 (−41.2); HRFABMS *m/z* 477.2498 ([M+H]$^+$; calcd for $C_{26}H_{37}O_8$, 477.2488); ^{1}H NMR (CD_3OD): 5.82 (1H, t, $J = 0.9$ Hz, H-7), 5.67 (1H, t, $J = 0.9$ Hz, H-11), 5.30 (1H, s, H-9), 4.02 (1H, q, $J = 6.8$ Hz, H-15), 3.85 (3H, s, H-18), 3.55 (1H, s, H-13), 2.08 (3H, s, H-19), 2.024 (3H, s, H-26), 2.020 (3H, s, H-17), 1.76 (3H, br d, $J = 0.7$ Hz, H-22), 1.62 (3H, s, H-20), 1.42 (3H, s, H-24), 1.38 (3H, br d, $J = 1.0$ Hz, H-21), 1.30 (3H, s, H-23) and 1.11 (3H, d, $J = 6.8$ Hz, H-16).

Conformational analysis

The most stable conformers of **1a** and **2a** were predicted using Spartan' 14 (Wavefunction, Irvine, CA, USA) by a preliminary conformational analysis with the MMFF94 force field followed by geometry optimization using the density functional theory with the B3LYP functional and 6-31G* basis set.

Anti-microbial assay

An anti-bacterial assay was carried out using *M. smegmatis* NBRC 3207 by the paper disc method.[25,26] Strain NBRC 3207 was obtained from the Biological Resource Center (NBRC) and NITE (Chiba, Japan), and maintained in 20% glycerol at − 80 °C.

The test microorganism was cultured in Middlebook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol and 10% Middlebook OADC at 37 °C for 2 days and adjusted to 1.0×10^6 CFU ml^{-1}. The inoculum was spread on the above medium containing 1.5% agar in a square plate. Each sample in CH_3OH was adsorbed to a sterile filter disc (6 mm; Advantec, Tokyo, Japan), and, after the evaporation of CH_3OH, the disc was placed on an agar plate and incubated for 2 days at 37 °C. Streptomycin sulfate and CH_3OH were used as positive and negative controls, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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