NOTE

A new benzoxepin metabolite isolated from endophytic fungus *Phomopsis* sp.

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Endophytes live symptomlessly and intracellularly inside host plants. The interaction of endophytes with host plants has, in particular, received considerable attention. Studies carried out in the last two decades have shown that endophytes are rich sources of structurally diverse natural products with interesting biological activities.¹ These studies emphasize that chemical compounds help control the equilibrium between the endophytes and the host plants.

In our previous research on novel bioactive compounds isolated from endophytic fungi, we reported eremoxylarins A and B isolated from endophytic fungus xylariaceous YUA-026 as novel calcineurin inhibitors. These compounds were obtained as potential inhibitors of Ca²⁺-signaling by the use of a yeast-based screening system for the activity that restores the growth of a Ca²⁺-sensitive, drug-sensitive strain of *Saccharomyces cerevisiae* (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) on solid medium containing CaCl₂.^{2,3} Among the microbial compounds similarly screened, we found that benzophomopsin A (**1**) inhibited the Ca²⁺-signal transduction more strongly than did a known compound, xylarinol A (**2**) (Figure 1). Herein, we report the isolation and structure elucidation of a new benzoxepin derivative **1** together with **2** and their effects on Ca²⁺-signal transduction.

The producing strain, *Phomopsis* sp. KS-37-2, was isolated from the stem of a cherry tree in Yamagata, Japan.

Phomopsis sp. KS-37-2 was cultivated on sterilized, unpolished rice (20 g/Petri dish × 50) at 25 °C for 3 weeks. The moldy, unpolished rice was extracted with MeOH, and the MeOH extract was concentrated. The resulting aqueous concentrate was extracted with *n*-hexane at first, and the water layer was subsequently partitioned with EtOAc. The purification of the EtOAc layer was guided by the intense blue characteristic coloration with vanillin–sulfuric acid solution on TLC plates. The EtOAc residue was purified on a silica gel column using a stepwise gradient of *n*-hexane–EtOAc (100:0–0:100). The 50–60% EtOAc fractions (85.0 mg) were combined and further purified by

octa decyl silyl (ODS, Fuji Silysia Chemical Ltd., Aichi, Japan) column chromatography using 80% aqueous MeOH as the eluent to afford fractions 1–15 (100 ml each). Fraction 5 (35.0 mg) was rechromatographed on a silica gel column using CHCl₃–MeOH (80:20) as the eluent to yield benzophomopsin A (**1**, 5.5 mg) and xylarinol A (**2**, 11.0 mg).

The metabolite xylarinol A (**2**) was isolated as a white powder. Xylarinol A has recently been isolated from the fruiting bodies of *Xylaria polymorpha* as a radical scavenger.⁴ A comparison of our spectroscopic data with the literature values confirmed that **2** was xylarinol A.

The molecular formula of **1** was C₁₂H₁₂O₃, which required seven degrees of unsaturation, as revealed by HR–FAB–MS. The IR spectrum of **1** showed absorption bands at 3384, 1584 and 1465 cm^{−1}, which are characteristic of the hydroxyl and aromatic groups. The formation of a monomethoxyl derivative (**1a**) [C₁₃H₁₄O₃ (FAB–MS: *m/z* 241 [M+Na]⁺); δ_H 3.83 (3H, s, OMe)] after treating **1** with trimethylsilyldiazomethane confirmed the presence of a phenolic hydroxyl group. The UV spectrum of **1** revealed the presence of aromatic rings. The ¹³C NMR and DEPT spectra of **1** showed peaks corresponding to five sp² methine (δ 103.5, 114.8, 123.0, 128.0, 131.3), three sp² quaternary carbons (δ 126.6, 136.9, 152.3), one sp³ quaternary carbon (δ 104.1), one sp³ methylene (δ 56.8), one sp³ methine (δ 66.9) and one methyl group (δ 16.0). The seven unsaturation equivalents implied by the molecular formula indicated that **1** has three rings.

The ¹H-NMR spectrum showed signals attributable to a vicinal sp² spin network [δ 6.82 (1H, d, *J*=7.6 Hz), 6.89 (1H, d, *J*=7.6 Hz), 7.09 (1H, t, *J*=7.6 Hz)] and two protons of a *cis* double bond [δ 5.95 (1H, d, *J*=12.7 Hz) and 6.82 (1H, d, *J*=12.7 Hz)]. In addition, the ¹H NMR spectrum revealed signals that were due to an isolated oxy-methylene [δ 4.63 (1H, d, *J*=13.9 Hz), 5.12 (1H, d, *J*=13.9 Hz)], a doublet methyl group [δ 1.05 (3H, d, *J*=6.5 Hz)] and an oxymethine

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Figure 1 Structures of compounds **1** and **2**.

Figure 2 Important HMBC correlations for **1**.

group [δ 4.15 (1H, q, $J=6.5$ Hz)]. For the connectivity of partial structures, we conducted heteronuclear multiple bond correlation (HMBC) experiments (Figure 2 and Table 1). The aromatic moiety in **1** was confirmed on the basis of HMBC correlations from H-6 and H-8 to C-9a and from H-7 to C-5a and C-9. HMBC correlations from H-1 to C-3, C-5a and C-9, from H-4 to C-10, and from H-5 to C-3, C-6 and C-9a were also observed. These results indicated the presence of a 1,3-dihydro-benzo[*c*]oxepine moiety in **1**. The presence of an epoxy group at C-3 and C-10 was confirmed from the molecular formula, the chemical shifts of the ¹H and ¹³C NMR signals at these positions, and the large ¹*J*_{CH} values for C-10 ($J_{C-10,H-10}=153$ Hz).⁵ The residual OH group was hence thought to be a phenolic OH located at C-9. The relative stereochemistry of **1** was deduced from NOE experiments. An NOE was observed between H-4 and Me-11. From this result, the relative configuration at the position of C-10 was determined, as shown in Figure 1.

Benzophomopsin A (**1**), which possesses benzo-fused oxacycloalkenes moiety, was closely related to heptacyclosordariolone and cladoacetal A that were isolated earlier from cultures of *Sordaria macrospora* and *Cladosporium* sp., respectively.^{6,7}

Intracellular Ca²⁺ is thought to have an important function in various biological signaling processes. Ca²⁺ has also been found to affect the progression of the G2/M cell cycle multilaterally in the yeast *S. cerevisiae*.⁸ The mutant yeast strain (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) used in this study is unable to grow at high CaCl₂ concentrations because of the hyperactivation of the Ca²⁺-signal pathway. Two parallel pathways of Ca²⁺-signaling, calcineurin and Mpk1 MAP kinase cascade, coordinately activate Swe1 in response to CaCl₂. Swe1, in turn, activates the inhibitory phosphorylation of the Cdc28 cyclin-dependent protein kinase in the G2 phase, resulting in cell cycle arrest in G2/M.⁹ Thus, the calcineurin inhibitor, FK506, and the heat-shock protein 90 inhibitor, radicicol, by inhibiting the Ca²⁺-induced Swe1 activation, promote the growth of the *zds1Δ* strain yeast on Ca²⁺-agar plate.^{9,10} The growth-promoting activities of compounds **1** and **2** were

Table 1 ¹H and ¹³C-NMR data of **1**

No	δ _C	δ _H	HMBC
1	56.8 t	4.63 (1H, d, 13.9) 5.12 (1H, d, 13.9)	3, 5a, 9 3, 5a, 9
3	104.1 s		
4	103.5 d	5.95 (1H, d, 12.7)	5a, 10
5	131.3 d	6.82 (1H, d, 12.7)	3, 6, 9a
5a	136.9 s		
6	123.0 d	6.89 (1H, d, 7.6)	8, 9a
7	128.0 d	7.09 (1H, t, 7.6)	5a, 9
8	114.8 d	6.82 (1H, d, 7.6)	6, 9a
9	152.3 s		
9a	126.6 s		
10	66.9 d	4.15 (1H, q, 6.5)	3, 4, 11
11	16.0 q	1.05 (3H, d, 6.5)	3, 10

¹³C- (100 MHz) and ¹H- (400 MHz) NMR spectra were taken on JEOL NMR system EX-400 in CDCl₃, the solvent peak were used as internal standard (δ_C 77.0, δ_H 7.26), and values in parentheses were coupling constants in Hz.

a Benzophomopsin A (**1**)

b Xylarinol A (**2**)

Figure 3 Restored growth activity of **1** (**a**) and **2** (**b**) against the mutant strain of *Saccharomyces cerevisiae* (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) with 0.3 M CaCl₂. Assays were carried out as described earlier.³ 1: 10 μg per disc, 2: 5 μg per disc, 3: 2.5 μg per disc, 4: 1.25 μg per disc, 5: 0.625 μg per disc, 6: 0.31 μg per disc, 7: 0.02 μg per disc (FK506).

determined semi-quantitatively by similar procedures used for the screening assay.³ Paper discs containing different amounts of compounds **1** and **2** were placed on a YPD agar plate containing CaCl₂ and the cell-growth zones formed around the discs were observed after 3 days of incubation at 28 °C. Compound **1** gave rise to a growth zone at 1.25 μg per disc and it restored the growth inhibition caused by a high Ca²⁺ dose dependently (Figure 3). In contrast, the activity of compound **2** was much weaker, giving rise to only a faint growth zone at 10 μg per disc of this compound. The activity of **1** observed by this assay was apparently similar to those observed with eremoxylarins A and B, which were found to inhibit calcineurin.³ However, **1** did not inhibit calcineurin even at 100 μM (data not shown). Though YCM1008A is already known as a Ca²⁺-signaling inhibitor from *Fusarium* sp.,¹¹ **1** has different structures and producing strain from it. Thus, further studies are required for an in-depth understanding of the inhibitory activity of **1**.

PHYSICO-CHEMICAL PROPERTIES

1: White powder amorphous; [α]_D²⁰ –46° (*c* 0.11, MeOH), UV $\lambda_{\max}$ MeOH nm (ϵ) 253 (27,000), 261 (sh, 22,000), IR (KBr) $\nu_{\max}$ cm^{–1} 3384, 1584, 1465, 1014, FAB–MS: *m/z* 205 (M+H)⁺, HR–FAB–MS *m/z* 205.0872 (calcd for C₁₂H₁₂O₃+H, 205.0865), for ¹H and ¹³C NMR see Table 1.

1a: Yellow oil, $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 1.05 (1H, d, $J=6.5$ Hz, 11-Me), 3.83 (3H, s, 9-OMe), 4.58 (1H, d, $J=13.8$ Hz, 1-H), 5.18 (1H, d, $J=13.8$ Hz, 1-H), 5.97 (1H, d, $J=12.5$ Hz, 4-H), 6.76 (1H, d, $J=12.5$ Hz, 5-H), 6.82 (1H, d, $J=7.6$ Hz, 8-H), 6.94 (1H, d, $J=7.6$ Hz, 6-H), 7.24 (1H, t, $J=7.6$ Hz, 7-H). FAB-MS: m/z 241 ($\text{M}+\text{Na}$) $^+$.

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- 1 Gunatilaka, A. A. L. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J. Nat. Prod.* **69**, 509–526 (2006).
- 2 Shiono, Y. & Murayama, T. New eremophilane-type sesquiterpenoids, eremoxylarins A and B from xylariaceous endophytic fungus YUA-026. *Z. Naturforsch.* **60b**, 885–890 (2005).
- 3 Ogasawara, Y., Yoshida, J., Shiono, Y., Miyakawa, T. & Kimura, K. New eremophilane sesquiterpenoid compounds, eremoxylarins A and B directly inhibit calcineurin in a manner independent of immunophilin. *J. Antibiot.* **61**, 496–502 (2008).
- 4 Lee, I. *et al.* Xylarinols A and B, two new 2-benzoxepin derivatives from the fruiting bodies of *Xylaria polymorpha*. *J. Antibiot.* **62**, 163–165 (2009).
- 5 Someno, T. *et al.* ICM0301s, new angiogenesis inhibitors from *Aspergillus* sp. F-1491. II. Physico-chemical properties and structure elucidation. *J. Antibiot.* **57**, 104–109 (2004).
- 6 Bouillant, M. L., Bernillon, J., Favre-Bonvin, J. & Salin, N. New hexaketides related to sordariol in *Sordaria macrospora*. *Z. Naturforsch.* **44c**, 719–723 (1989).
- 7 Höller, U., Gloer, J. B. & Wicklow, D. T. Biologically active polyketide metabolites from an undetermined fungicolous hyphomycete resembling *Cladosporium*. *J. Nat. Prod.* **65**, 876–882 (2002).
- 8 Mizunuma, M., Hirata, D., Miyahara, K., Tsuchiya, E. & Miyakawa, T. Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. *Nature* **392**, 303–306 (1998).
- 9 Shitamukai, A., Mizunuma, M., Hirata, D., Takahashi, H. & Miyakawa, T. A positive screening for drugs that specifically inhibit the Ca^{2+} -signaling activity on the basis of the growth promoting effect on a yeast mutant with a peculiar phenotype. *Biosci. Biotechnol. Biochem.* **64**, 1942–1946 (2000).
- 10 Chanklan, R. *et al.* Inhibition of Ca^{2+} -signal-dependent growth regulation by radicicol in budding yeast. *Biosci. Biotechnol. Biochem.* **72**, 132–138 (2008).
- 11 Koizumi, F. *et al.* YCM1008A, a novel Ca^{2+} -signaling inhibitor, produced by *Fusarium* sp. YCM1008. *J. Antibiot.* **60**, 455–458 (2007).