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NOTE

Xylaropyrone, a new γ-pyrone from the endophytic fungus *Xylaria feejeensis* MU18

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Novel bioactive compounds have been intensively screened to identify their potential benefit in many fields of human life, including medicine, agriculture and industry. Natural compounds have been a continuous and important source of bioactive compounds, and have led to the discovery of not less than 200 000 bioactive compounds so far,[1]accounting for more than 50% of new medicines registered as anticancer, antibacterial, antifungal and antiviral agents during the period of 1981–2006.[2]

Fungal endophytes are defined as filamentous fungi that reside in the tissues of living plants without exerting any pathogenic effects. Judging from the fact more than one endophyte often inhabit a single plant, new and interesting endophytic microorganisms are likely to be found from the nearly 300 000 plant species, which inhabit the diverse environments and ecosystems of the earth.[3] Moreover, considering that a great number of secondary metabolites with diverse chemical structures and various biological activities[6–8] have been discovered from endophytes, endophytes can be regarded as a rich source of bioactive natural products.[4,5]

Thailand is located in a tropical zone with abundant biodiversity and bioresources, suggesting that Thai endophytic fungi may be a rich source of bioactive compounds. A series of recent discoveries of novel bioactive substances, such as xylariaquinone A, scopararanes A and B, 11-hydroxymonocerin, phomoenamide, phomonitroester and deacetylphomoxanthone B, have confirmed the usefulness of fungal endophytes from Thai medicinal plants as promising bioresources.[9–12]

In this study, novel compounds were screened from the endophytic fungus *Xylaria feejeensis* MU18, isolated from *Eryngium foetidum* Linn., a medicinal plant in Thailand. From the crude extract, one novel compound was isolated and its chemical structure was determined. The compound possesses a novel chemical structure comprising a γ-pyrone with a hydroxymethyl group and a methylpentyl group at C-2 and C-5, respectively. This is the first report of a natural or even a synthetic compound possessing a γ-pyrone moiety having these two side chains.

MATERIALS AND METHODS

General experimental procedures

The UV spectrum was recorded on a Hitachi U-3200 spectrophotometer (Hitachi Ltd., Tokyo, Japan). NMR spectra were recorded on a on a JEOL JNM-ECS400 (JEOL, Tokyo, Japan) at 400 MHz. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent signal (δH 7.26 and δC 77.0 in $CDCl_3$). HRFABMS was recorded on a JEOL JMS-700 spectrometer. Optical rotation was measured on a JASCO P-1020 polarimeter (Jasco, Tokyo, Japan). IR spectra were recorded on a FTIR-8400S (Shimadzu, Kyoto, Japan).

Microorganism

The endophytic fungus MU18 was isolated from leaves of *Eryngium foetidum* Linn., obtained from Mahidol University, Bangkok, Thailand. The fungus was identified as *X. feejeensis* based on the DNA sequences of the internal transcribed spacer (ITS) ribosomal RNA region (DNA data bank of Japan (DDBJ) accession number AB569622). A Genbank search for similar ITS sequences confirmed that the fungus was *X. feejeensis*, with 99% sequence identity.[13] The fungus was deposited as *X. feejeensis* MU18 at the culture collection of International Center for Biotechnology (ICBiotech; Osaka University, Osaka, Japan).

Fermentation and isolation

All chemicals, media and reagents were purchased from Wako (Osaka, Japan) unless stated otherwise. For seed culture preparation, the mycelia of *X. feejeensis* MU18 grown on a potato dextrose agar slant was inoculated into 5 ml of medium two (soluble starch 5%, Pharmamedia 2%, oatmeal 0.5%, KH_2PO_4 0.35%, Na_2HPO_4 0.25% and $(NH_4)_2SO_4$ 0.6%) in test tubes (Ø12.5 mm× 10.5 cm), and incubated for 3 days at 28 °C on a reciprocal shaker at 120 r.p.m. The seed culture (2 ml) was inoculated into 100 ml of medium two in 500-ml baffled flasks and cultivated for 21 days at 28 °C under a static condition.

After cultivation, culture broth (100 ml×10 flasks) was mixed with an equal amount of EtOAc and left to stir for 1 h at room temperature. Mycelia were removed by filtration with Miracloth (Calbiochem, La Jolla, CA, USA), and the EtOAc layer was recovered from a separation funnel, dried over anhydrous Na_2SO_4 and evaporated to afford a crude extract (brown gum, 650 mg).

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The crude extract (200 mg each) was at first separated on a C_{18} cartridge (Sep-Pak Vac 35 cc; Waters, Milford, MA, USA) by stepwise elution with increasing MeOH concentrations (MeOH/H_2O=2:8, 4:6, 6:4, 8:2 and 1:0 v/v). The 60% MeOH fractions containing compound **1** were combined and evaporated (50 mg from two repeats). The compound **1** was further purified by preparative reversed-phase C_{18} HPLC (PU-1570; Jasco, equipped with a UVIDEC-100-V detector) using a CAPCELL PAK C_{18} column (UG80S5; Shiseido, Tokyo, Japan) with a shallow MeOH gradient in 0.1% TFA (a 60–75% MeOH gradient over a period of 20 min) to yield 10 mg of pure compound **1**.

Antimicrobial assay

The minimum inhibitory concentration of xylaropyrone (**1**) was determined by a twofold broth microdilution method in three individual experiments according to the procedures of the Clinical and Laboratory Standards Institute for antimicrobial, anti-yeast and antifungal activity.[14] Kojic acid was used as a reference for the γ-pyrone compound. The minimum inhibitory concentration is defined as the lowest concentration of the compound at which there is no visible growth of the indicator strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29923, *Enterococcus faecalis* ATCC 29212, *Saccharomyces cerevisiae* ATCC 6275, *Candida albicans* OUT 6266, *Aspergillus niger* ATCC 6275, *Rhizopus oryzae* ATCC10404 and *Candida candidum* IFO4598.

Xylaropyrone (**1**) was obtained as a yellow oil. The molecular formula was determined to be $C_{12}H_{18}O_3$ on the basis of HRFABMS (obs. *m/z* 211.1335 $[M+H]^+$, calcd. 211.1334 for $C_{12}H_{19}O_3$), ^1H and ^{13}C NMR spectra data (Table 1). The IR spectrum showed a broadened OH absorption band at 3390 cm^{-1}.

^1H NMR data of **1** showed two methyl signals at δ 0.82 (t, *J*=6.9 Hz, 3H) and 0.85 (d, *J*=6.9 Hz, 3H), eight methylene protons at δ 1.14 (ddq, *J*=7.6, 15.0, 11.0 Hz, 1H), 1.31 (m, 1H), 1.41 (m, 1H), 1.61(dddd, J=6.7, 7.6, 13.4, 16.6 Hz, 1H), 2.48 (m, 2H) and 4.42 (s, 2H), one methine proton at δ 1.33 (m, 1H) and two aromatic protons at δ 7.74 (s, 1H) and 6.20 (s, 1H) (Supplementary Figure S1). The ^{13}C NMR spectrum of **1** indicated one carbonyl carbon at δ 180.4, two quaternary carbons at δ 126.8 and 171.4, two methyl carbons at δ 11.2 and 18.3, four sp^3 methylene carbons at δ 29.1, 31.3, 33.4 and 58.3, one sp^3 methine carbon at δ 33.8 and two sp^2 methine carbons at δ 113.4 and 152.4 (Supplementary Figure S2).

Three partial structures of **1**, namely, a hydroxymethyl group, a methylpentyl group and a 2,5-disubstituted γ-pyrone, were deduced by comprehensive interpretation of its ^1H, ^{13}C NMR, COSY, heteronuclear single quantum correlation (HSQC) and HMBC spectra and other spectroscopic data (Figure 1). The observed carbon signals at δ 113.4, 126.8, 152.3, 171.4 and 180.4 in the ^{13}C NMR spectrum suggested the presence of a γ-pyrone moiety.[15] This was further supported by the maximum UV absorption at 252 nm and strong absorption band at 1660 cm^{-1} in the IR spectrum.[15]

The key long range connections of H-8 (δ 2.48) with C-2 (δ 171.4) and C-3 (δ 113.4) and of H-7 (δ 4.42) with C-4 (δ 180.4), C-5 (δ 126.8) and C-6 (δ 152.4) indicated that the hydroxylmethyl and methylpentyl groups were connected to C-5 and C-2 of the γ-pyrone nucleus, respectively. Regarding the absolute configuration at C-10, it was deduced to be *R*, from the comparison of optical rotations on compounds having similar aliphatic chain with 3-methyl or 3-hydroxymethyl group: those of R-configuration were all minus ((*R*)-2-(3-(hydroxymethyl)pentyl)-4H-*pyran*-4-one ($[\alpha]_D^{20}$ −0.567), (*R*)-(3-methylpentyl)benzene ($[\alpha]_D^{25}$ −5.52), (*R*)-4-methylhexan-1-ol ($[\alpha]_D^{20}$ −8.1),[16–18] whereas those of S-configuration were all plus.[19–21] Thus, the structure of **1** was elucidated as (*R*)-5-(hydroxymethyl)-2-(3-methylpentyl)-4*H*-pyran-4-one (Figure 2).

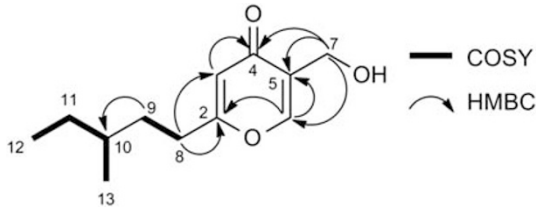

Figure 1 COSY and HMBC correlation of xylaropyrone (**1**).

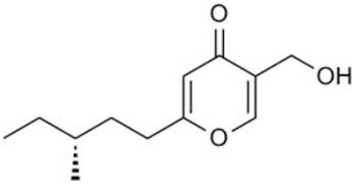

Figure 2 Structure of xylaropyrone (**1**).

Table 1 NMR spectroscopic data of xylaropyrone (1) in $CDCl_3$

Position	δ_C	δ_H	*H–H COSY*	*HMBC*
2	171.4	—		H-3, H-6, H-8
3	113.4 CH	6.20 (s)		H-8
4	180.4	—		H-3, H-6, H-7
5	126.8	—		H-3, H-6, H-7
6	152.4 CH	7.74 (s)		H-7
7	58.3 CH_2	4.42 (s)		H-6
8	31.3 (CH_2)	2.48 (m)	H-9	H-3, H-9
9	33.4 (CH_2)	1.41 (m)	H-8,H-9	H-8, H-10, H-12, H-13
		1.61 (dddd, J=6.7, 7.6, 13.4, 16.6 Hz)	H-8, H-9	
10	33.8 (CH)	1.33 (m)	H-11, H-13	H-9, H-11, H-12, H-13
11	29.1 (CH_2)	1.14 (ddq, *J*=7.6, 15.0, 11.0 Hz)	H-10, H-12	H-9, H-10, H-12, H-13
		1.31 (m)	H-10, H-12	
12	11.2 (CH_3)	0.82 (t, *J*=6.9 Hz)	H-11	H-11
13	18.8 (CH_3)	0.85 (d, *J*=6.9 Hz)	H-10	H-9, H-10, H-11

^1H, ^{13}C NMR and 2D NMR spectra were obtained on JOEL JNM-ECS400 NMR spectrometers, in $CDCl_3$ at room temperature, and the solvent peak was used as an internal standard (δ_H 7.26 and δ_C 77.0 in $CDCl_3$).

To the best of our knowledge, xylaropyrone, which consists of a γ-pyrone moiety, a hydroxymethyl group and a methylpentyl group, is a novel compound that has not previously been identified in natural resources or derived from chemical synthesis.

Xylaropyrone (**1**): a yellow oil; $[\alpha]_D^{26}$ −4.1 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 215 (3.48), 252 (3.85); HRFABMS *m/z* $[M+H]^+$ 211.1335 (calcd. for $C_{12}H_{19}O_3$, 211.1334). IR ν_{max} (film) 3161–3502 (OH), 2958, 2523, 2858, 1654 (C=O), 1596, 1419, 1338, 1184, 1128 and 1029 cm^{-1}. ^1H ($CDCl_3$, 400 MHz), ^{13}C ($CDCl_3$, 100 MHz), H–H COSY and HMBC see Table 1.

As no antimicrobial data is available on a γ-pyrone compound possessing two side chains, especially to evaluate the effect of two side chains on a γ-pyrone on biological activities, the antimicrobial activities against typical prokaryotes and eukaryotes were measured using kojic acid as a reference. Xylaropyrone showed moderate activity against *S. cerevisiae* (minimum inhibitory concentration=32 μg ml^{-1}), whereas kojic acid did not show any inhibition even at a concentration of 128 μg ml^{-1}. Neither xylaropyrone nor kojic acid showed any inhibitory activity against *E. coli, P. aeruginosa, S. aureus* or *E. faecalis* when used at a concentration of 512 μg ml^{-1}, or against *A. niger, R. oryzae* or *C. candidum* when administered at 128 μg ml^{-1}.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)