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NOTE

Biphenyl ether derivatives with protein tyrosine phosphatase 1B inhibitory activity from the freshwater fungus *Phoma* sp.

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Type 2 diabetes mellitus (T2DM) and obesity are serious public health issues in developed countries.[1,2] One of the potential strategies for the prevention and treatment of T2DM and obesity is to inhibit protein tyrosine phosphatase 1B (PTP1B) activity because this enzyme has been shown to play a significant role as a negative regulator of signal transduction from insulin and leptin receptors.[3,4] Therefore, PTP1B inhibitors are expected to become new therapeutic agents for T2DM and obesity.[5,6]

During the course of our screening program on new types of PTP1B inhibitors from microorganisms, we have reported tricoketides,[7] verruculides[8] and asperdichrome.[9] Continuous efforts revealed that a culture broth of the freshwater-derived fungus *Phoma* sp. TPU1222 inhibited PTP1B activity. Bioassay-guided separation of the EtOAc extract from the culture broth led to the isolation of a new biphenyl ether derivative, 1-methoxy-3,5′-dimethyl-2,3′-oxybiphenyl-5,1′,2′-triol (**1**), together with three known phenolic compounds, 5-methoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,1′,2′-triol (**2**),[10] cyperine (**3**)[11] and 6-methylsalicylic acid (**4**)[12,13] (Figure 1). We herein describe the isolation, structural elucidation and biological properties of compounds **1–4**.

Fungal strain TPU1222 was isolated from a freshwater sample collected at Hayakake lake, Aomori, Japan, in November 2012. Namely, ~1 ml of the freshwater sample was spread on a potato dextrose agar plate (BD, Franklin Lakes, NJ, USA) containing 0.005% rose bengal (Wako, Osaka, Japan) and 0.01% kanamycin (Wako), and was then incubated at 25 °C for 7 days. Strain TPU1222 grown on the plate was isolated and then maintained on a Miura's medium (LCA) slant (0.1% glucose (Wako), 0.08% KH_2PO_4 (Wako), 0.02% K_2HPO_4 (Wako), 0.02%, $MgSO_4{\cdot}7H_2O$ (Wako), 0.02% KCl (Wako), 0.2% $NaNO_3$ (Wako), 0.02% yeast extract (BD) and 1.5% agar (Wako) in freshwater and adjusted to pH 6.0 before sterilization). The 196 bp ITS1 rDNA sequence of strain TPU1222 was identical to 11 known species of the genus *Phoma* including *P. herbarum*, and, thus, the strain was identified as *Phoma* sp.

The mycelia grown on Miura's medium (LCA) were inoculated into a 100-ml Erlenmeyer flask containing 50 ml of seed medium (2.0% glucose, 0.50% polypeptone (Wako), 0.050% $MgSO_4{\cdot}7H_2O$, 0.20% yeast extract, 0.10% KH_2PO_4 and 0.10% agar in freshwater and 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 production medium (3.0% sucrose (Wako), 3.0% soluble starch (Wako), 1.0% malt extract (BD), 0.30% Ebios (Asahi Food & Healthcare Co. Ltd., Tokyo, Japan), 0.50% KH_2PO_4 and 0.050% $MgSO_4{\cdot}7H_2O$ in freshwater and adjusted to pH 6.0 before sterilization). The production culture was performed at 25 °C for 7 days under agitation.

Acetone (2.4 l) was added to the culture broth (2.4 l) after 7 days and filtered. The filtrate was concentrated to remove acetone and extracted with EtOAc. The EtOAc extract was concentrated *in vacuo* to dryness, and the brown residue (0.34 g) was then suspended in 30% CH_3OH and adsorbed on an ODS column (100 g). The ODS column was eluted stepwise with 30, 50, 70, 85 and 100% CH_3OH in H_2O (200 ml each × 2) and separated into 10 fractions (Fr. 1–Fr. 10). Fr. 5 (the first 200 ml of the 70% CH_3OH eluate) exhibited inhibitory activity against PTP1B and was concentrated *in vacuo* to dryness in order to give a dark black oil (38.9 mg), which was purified by preparative HPLC (column; PEGASIL ODS (Senshu Scientific Co., Ltd., Tokyo, Japan), 10 × 250 mm; mobile phase, 45% CH_3CN containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 ml min^{-1}) to give compounds **1** (5.7 mg), **2** (15.3 mg) and **3** (4.8 mg). Active Fr. 4 (20.9 mg, second 200 ml of the 50% CH_3OH eluate) was separated by preparative HPLC (column; PEGASIL ODS, 10 × 250 mm; solvent, 40% CH_3CN containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 ml min^{-1}) to obtain compounds **2** (0.8 mg) and **4** (7.9 mg).

Compounds **2–4** were identified by comparing their spectroscopic data with those for 5-methoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,1′,2′-triol,[10] cyperine[11] and 6-methylsalicylic acid[12,13] (Figure 1a).

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Compound **1** was obtained as a brown oil, and its molecular formula was deduced to be $C_{15}H_{16}O_5$ from HREIMS data (*m/z* 276.0999 $[M]^+$, Δ +0.1 m.m.u.). ^1H and ^{13}C NMR spectra (in CD_3OD) showed 13 proton and 15 carbon signals (Supplementary Figures S1 and S2 and Supplementary Table S1), which were classified into two methyl, one oxygenated methyl, four sp^2 methine, two sp^2 quaternary and six sp^2 oxygenated quaternary carbons by an analysis of the HMQC and DEPT spectra of **1**. UV absorption at 277 nm (log ε 3.6) and an IR band at 3400 cm^{-1} revealed the presence of phenyl and OH groups, respectively. These spectral properties of **1** were very similar to those of **2**.[10] Therefore, compound **1** was presumed to have a biphenyl ether skeleton, and its structure was elucidated as 1-methoxy-3,5′-dimethyl-2,3′-oxybiphenyl-5,1′,2′-triol by an analysis of its ^1H–^1H COSY and HMBC spectra (Figure 1b). In order to confirm the positions of OMe groups in **1** and **2**, the NOESY spectra of **1** and **2** were examined and compared with each other. Compound **1** showed NOE correlations between 1-OCH_3 (δ_H 3.63)/H-6 (6.22), 1-OCH_3/H-4′ (6.43) and 3-CH_3 (2.23)/H-4 (6.44) (Figure 1c). On the other hand, NOE correlations were observed between 3-CH_3 (2.03)/H-4 (6.31), H-4/5-OCH_3 (3.74) and 5-OCH_3/H-6 (6.37) in the NOESY spectrum of **2** (Figure 1c). Consequently, the structure of **1** was elucidated as shown in Figure 1a.

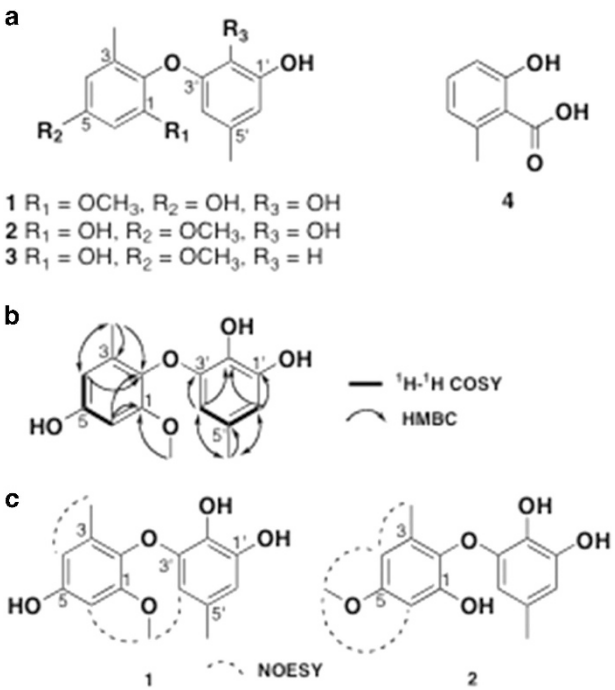

Figure 1 (**a**) Structures of compounds **1**–**4** produced by the freshwater fungus *Phoma* sp. TPU1222. (**b**) ^1H-^1H COSY and key HMBC correlations for compound **1**. (**c**) Key NOESY correlations for compounds **1** and **2**.

Compounds **1–4** were evaluated for their PTP1B inhibitory activities using an enzyme assay method.[14] As shown in Figure 2 and Supplementary Table S2, compound **2** showed 32% inhibition at 36 μM, and **4** was inactive at 66 μM. Compounds **1** and **3** exhibited PTP1B inhibitory activities in a dose-dependent manner with IC_{50} values of 13 μM and 17 μM, respectively (Figure 2 and Supplementary Table S2). Oleanolic acid (Tokyo Chemical Industry, Tokyo, Japan),[15] a positive control, had an IC_{50} value of 1.3 μM in the same experiment (Supplementary Table S2).

Protein tyrosine phosphatases (PTPs) constitute a major family of more than 100 members including PTP1B and regulate various cellular functions. Therefore, selective activity against PTP1B over the other PTPs is one of the important properties for the development of PTP1B inhibitors. The inhibitory effects of compounds **1–3** on T-cell protein tyrosine phosphatase (TCPTP) as one of the non-transmembrane PTPs, CD45 tyrosine phosphatase (CD45) as one of the receptor-like PTPs and *vaccinia* H-1-related phosphatase (VHR) as one of the dual-specificity phosphatases were evaluated. Although compounds **1** and **3** exhibited similar inhibitory activities against PTP1B and VHR, their activities against TCPTP and CD45 were weaker than those against PTP1B (Figure 2 and Supplementary Table S2).

PTP1B is mainly located in insulin-targeted tissues such as liver, muscle and fat cells,[3,4] and, thus, Huh-7 cells (human hepatoma cell line) are used in cell-based experiments in order to investigate the mechanisms of action of PTP1B inhibitors.[16] Thus, the growth inhibitory effects of compounds **1–4** on Huh-7 cells were examined using the WST-1 assay.[17] After a treatment for 72 h, compound **3** exhibited modest cytotoxicity (26% growth inhibition at 50 μM), whereas compounds **1**, **2** and **4** did not affect cell proliferation at 50 μM. Consequently, compound **1** as a PTP1B inhibitor has potential as a drug candidate.

The polybromobiphenyl ether, 2-(3′,5′-dibromo-2′-methoxyphenoxy)-3,5-dibromophenol, was obtained from the Indonesian marine sponge *Lamellodysidea herbacea* as a potent PTP1B inhibitor (IC_{50} = 0.85 μM),[14] and exhibits greater inhibitory activity than those of **1–3**. Therefore, Br atom(s) play an important role in the inhibition of PTP1B activity. Cell-based experiments on compound **1** and bromobiphenyl ethers in Huh-7 cells are now underway.

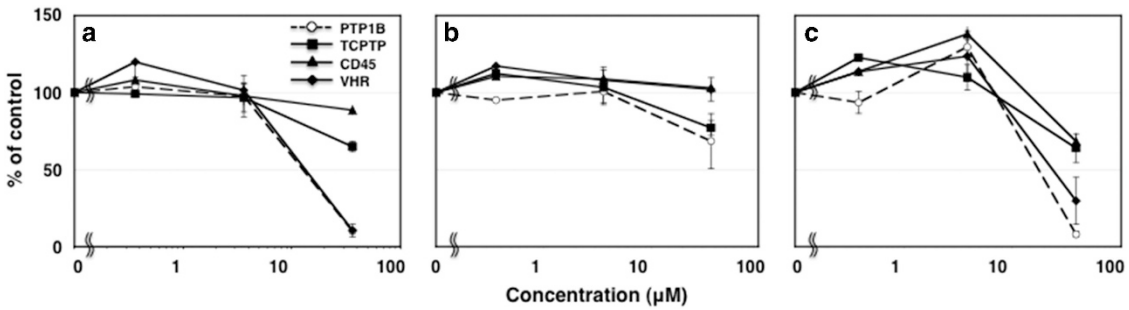

Figure 2 Inhibitory activities of compounds **1** (**a**), **2** (**b**) and **3** (**c**) against PTP1B, TCPTP, CD45 and VHR.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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