NOTES

Inhibition of PTP1B by Metabolites from *Micromucor ramannianus* var. *angulisporus* CRM000232

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Several protein tyrosine phosphatases (PTPases) play a critical role in the regulations of a variety of cellular processes, such as growth, proliferation and differentiation, metabolism, immune response, cell-cell adhesion, and cell-matrix contacts.[1,2] Particularly, protein tyrosine phosphatase1B (PTP1B) is a major nontransmembrane phosphotyrosine phosphatase in human tissues and a negative regulator of the insulin-stimulated signal transduction pathway.[3] A number of evidences accumulated from the various biochemical studies are now suggesting that PTP1B is a major negative regulator of insulin receptor signaling.[4,5] Therefore, PTP1B is considered as an attractive target for the treatment of type 2 diabetes and related metabolic syndromes.[6~8] Although there have been a number of reports on the designing and development of synthetic PTP1B inhibitors,[6,9] little has been studied for PTP1B inhibitors derived from natural resources such as plant or microbial resources.[8] Et-3,4-dephostatin is one of few PTP1B inhibitors derived from microbial source. As a stable analogue of dephostatin isolated from *Streptomyces*, Et-3,4-dephostatin has been shown to strongly inhibit PTP1B activity *in vitro*, and to potentiate insulin-related signal transduction in cultured mouse adipocytes.[10] Considering the track record of success in the development of a number of useful therapeutics, it seems reasonable to search for PTP1B inhibitors from natural resources.

In the course of the screening program for PTP1B inhibitors from microbial resources, a fungal isolate CRM000232 was selected on the basis of its potent inhibitory effect against PTP1B. The fungal isolate CRM000232 was identified as a Zygomycete fungus, *Micromucor ramannianus* var. *angulisporus* belonging to the Mucorales based on the morphological and cultural characteristics.

To identify PTP1B inhibitor(s) from CRM000232, the organic extract from the fungal culture was fractionated and purified by utilizing the solvent extraction, reversed-phase (C_{18}) column chromatography, and HPLC. The PTP1B inhibitory compounds isolated in this study were identical to KS-506a and KS-506m, which were previously reported from *Mortierella vinacea*.[11] Details of isolation of the active compound and its PTP1B inhibitory activity are described here.

Micromucor ramannianus var. *angulisporus* was cultured on 24 1-liter Elenmeyer flasks, each containing 200 ml of the medium contained glucose 20 g, yeast extract 2 g, peptone 5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and KH_2PO_4 1 g in 1 liter of distilled water, pH 5.6~5.8. Flasks were individually inoculated with 2 ml seed cultures of *M. ramannianus* var. *angulisporus*. Flask cultures were incubated at 28°C and aerated by agitation on a rotary shaker at 150 rpm for a period of 7 days. Extraction of the filtered fermentation broth with EtOAc (5×1 liter) provided an organic phase, which was then concentrated using a rotary evaporator to yield 1.17 g of crude extract. Further purification of the extract as guided by an *in vitro* PTP1B inhibitory assay afforded three related compounds **1**~**3**.

1 (white powder, 32.5 mg): ESI-MS *m/z* 801 $(M+Na)^+$; ^1H NMR (500 MHz, $CDCl_3$) δ 11.35 (1H, s, 2, 2′-O*H*), 2.47 (3H, s, H-10, 10′), 2.31 (3H, s, H-18, 18′), 2.18 (3H, s, H-19, 19′), 2.13 (3H, s, H-9, 9′), 2.12 (3H, s, H-8, 8′), 2.00 (3H, s, H-20, 20′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 188.5 (C-17, 17′), 170.5 (C-7, 7′), 161.1 (C-2, 2′), 157.8 (C-4, 4′), 154.8 (C-15, 15′), 143.7 (C-11, 11′), 138.8 (C-6, 6′), 133.6 (C-13, 13′), 124.1 (C-12, 12′), 121.1 (C-14, 14′), 115.4 (C-5, 5′), 114.8 (C-16, 16′), 107.5 (C-3, 3′), 104.6

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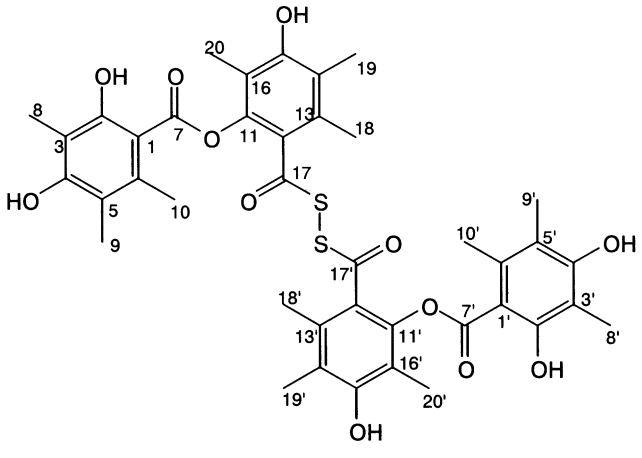
(C-1, 1′), 19.1 (C-10, 10′), 16.8 (C-18, 18′), 12.0 (C-9, 9′), 11.9 (C-19, 19′), 9.9 (C-20, 20′), 8.1 (C-8, 8′).

2 (white powder, 72.9 mg): FAB-MS (NBA matrix) *m/z* 483 $(M+Na)^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.43 (1H, s, 2-O*H*), 5.34 (1H, s, 4-O*H*), 5.05 (1H, s, 15-O*H*), 3.06 (2H, m, H-21), 2.54 (3H, s, H-10), 2.51 (2H, m, H-22), 2.21 (3H, s, H-18), 2.18 (3H, s, H-9), 2.16 (3H, s, H-9), 2.14 (3H, s, H-8), 2.04 (3H, s, H-20), 1.93 (3H, s, H-24); ^{13}C NMR (125 MHz, $CDCl_3$) δ 206.2 (C-23), 194.7 (C-17), 170.5 (C-7), 161.1 (C-2), 157.8 (C-4), 153.8 (C-15), 143.4 (C-11), 138.5 (C-6), 132.4 (C-13), 126.4 (C-12), 120.9 (C-14), 115.5 (C-5), 114.8 (C-16), 107.6 (C-3), 104.8 (C-1), 43.0 (C-22), 29.5 (C-24), 23.5 (C-21), 19.1 (C-10), 16.8 (C-18), 12.0 (C-9), 11.9 (C-19), 9.9 (C-20), 8.1 (C-8).

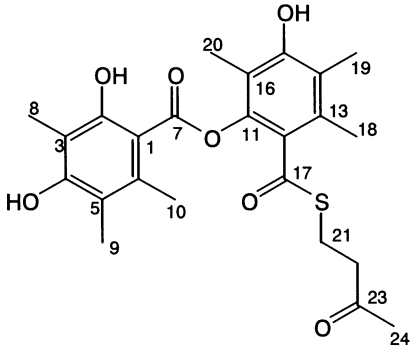
3 (pale yellowish solid, 16.3 mg): FAB-MS (NBA matrix) *m/z* 233 $(M+Na)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.42 (1H, s, 2-O*H*), 5.12 (1H, s, 4-O*H*), 3.91 (3H, s, H-11), 2.41 (3H, s, H-10), 2.124 (3H, s, H-9), 2.116 (3H, s, H-8); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.7 (C-7), 159.6 (C-2), 156.7 (C-2), 137.5 (C-6), 114.8 (C-5), 107.2 (C-1), 106.2 (C-3), 51.8 (C-11), 18.8 (C-10), 11.8 (C-9), 8.0 (C-8).

PTP1B (human, recombinant) was purchased from BIOMOL Research Laboratories, Inc. The enzyme activity was measured in a reaction mixture containing 20 mM *p*-nitro-phenyl phosphate (pNPP) in 50 mM citrate, pH 6.0, 0.1 M NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT). The reaction mixture was placed in a 30°C incubator for 30 minutes, and the reaction was terminated with 1 N NaOH. The amount of produced *p*-nitro-phenol was estimated by measuring the increase in absorbance at 405 nm. The nonenzymatic hydrolysis of 20 mM pNPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.[12] In the kinetic analysis, the reaction mixture consisting of different concentrations of pNPP used as a PTP1B substrate in the absence or presence of compound **1**. Michaelis constant (*Km*) and maximum velocity (Vmax) of PTP1B were determined by Lineweaver-Burk's plot. Dual-specificity protein tyrosine phosphatases (DS-PTPases) was assayed with the glutathione *S*-transferase-VH1-related human protein (GST-VHR) fusion enzyme, over-expressed in *Escherichia coli*.[13] The reaction mixture containing GST-VHR fusion enzyme,

Fig. 1. Structures of compounds **1**～**3**.



1



2

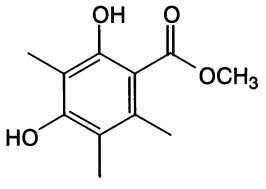


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Table 1. Comparison of the inhibitory activity of the compounds **1**~**3** isolated from *M. ramannianus* var. *angulisporus* against PTP1B, VHR, and PPase1.

Compound	IC_{50} (μM)		
	PTP-1B	VHR DS-PTPase	PPase1
1	4.9	–[b]	–
2	69.9	–	–
3	–	–	–
(Sodium orthovanadate)[a]	2.0	NT[c]	NT

[a]Known positive control.

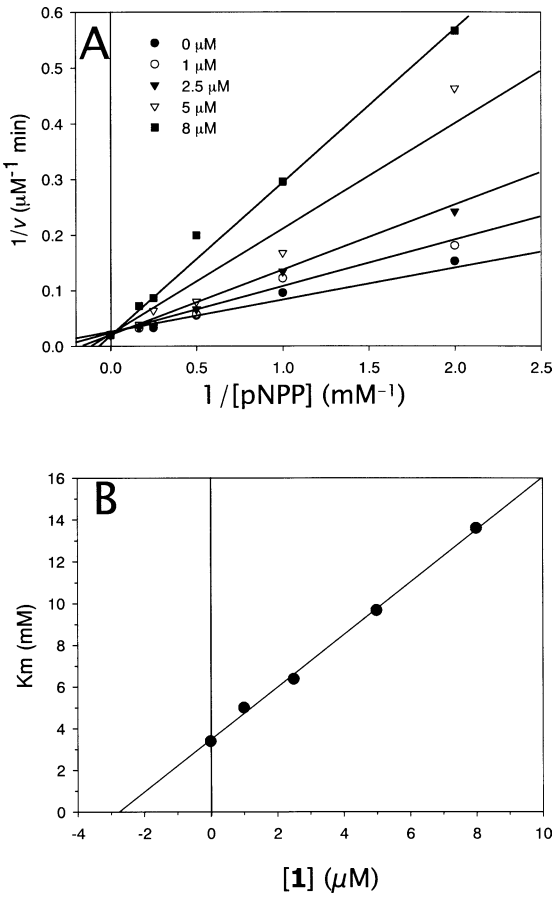
[b]Inactive (IC_{50} values above 200 μM).

[c]Not tested.

10 mM pNPP and assay buffer (50 mM succinate, 1 mM EDTA, 140 mM NaCl, 0.05% Tween 20, pH 6.0) was incubated at 30°C for 1 hour. The reaction was terminated by the addition of 1 N NaOH, and the dephosphorylation activity measured at 405 nm.[14] The catalytic activity of protein phosphatase1 (PPase1), from Sigma Co., was measured at 37°C using pNPP as a substrate. Reactions were performed for 30 minutes in the assay buffer (50 mM Tris-HCl, 0.1% β-mercaptoethanol, 1 mM Na_2EDTA, 1 mM $MnCl_2$, 20 mM $MgCl_2$, pH 7.6). The reaction was stopped by the addition of 1 N NaOH, and the amount of *p*-nitrophenol was measured by absorbance at 405 nm.[15]

Bioassay guided fractionation on the EtOAc extract of *M. ramannianus* var. *angulisporus* has led to the isolation of active compound **1**, along with two related compounds **2** and **3** (Fig. 1). The structures of the isolated compounds were identified by analysis of MS and NMR data, and by comparison with those in the literature.[11,16] Compound **1** inhibited PTP1B activity in a dose-dependent manner with an IC_{50} value of 4.9 μM. On the other hand, related compounds **2** and **3** showed moderate or no inhibitory activity (Table 1). To elucidate the inhibition mode of compound **1** on the activity of PTP1B, kinetic analysis was conducted with different concentrations of a substrate (Fig. 2). When pNPP was used as a substrate, compound **1** increased the *Km* value, but did not alter the Vmax value of PTP1B (Fig. 2). Accordingly, compound **1** was determined as a competitive inhibitor with the *Ki* value of 2.7 μM. In addition, the isolated compounds were tested for the inhibitory effects on other types of protein phosphatases, and it was shown that the compounds have no inhibitory effects toward dual-specificity protein tyrosine phosphatase (VHR) and protein serine/threonine phosphatase (PPase1).

Fig. 2. Effect of compound **1** on PTP1B-catalyzed hydrolysis of pNPP.



(A) Lineweaver-Burk plot of the effect of compound **1** on PTP1B activity, where various concentrations of **1** was or was not treated.

(B) Second-order plot of (A).

Therefore, it was suggested that compound **1** has specific inhibitory effect on protein tyrosine phosphatase such as PTP1B.

Compounds **1** and **2**, named KS-506a and KS-506m, respectively, have been isolated from culture broth of *Mortierella vinacea*.[11] KS-506a has been reported to have inhibitory effect on cyclic nucleotide phosphodiesterase purified from bovine cerebral cortex with IC_{50} of 1.22 μg/ml.[11] KS-506a was reported to inhibit histamine release (IC_{50}=1.2 μg/ml) from rat peritoneal exudates cells.[11] Nevertheless, it is noteworthy that these sulfur-containing depsides have been previously described only from the genus *Mortierella*.

In summary, dimeric depside-type fungal metabolite **1**, along with biogenetically related compounds **2** and **3**, was isolated as a PTP1B inhibitor by bioassay guided fractionation from the EtOAc extract of *M. ramannianus* var. *angulisporus*. To our best knowledge, compound **1** is the first PTP1B inhibitor reported from fungal sources,[9] and it could serve as a lead compound in design and development of potent PTP-1B inhibitor.

Acknowledgements

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