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Protein tyrosine phosphatase 1B inhibitors from *Morus* root bark

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Abstract—An organic layer prepared from the Chinese crude drug 'Sang-Bai-Pi' (*Morus* root bark) was studied in order to identify the inhibitory compounds for protein tyrosine phosphatase 1B (PTP1B). Bioassay-guided fractionation resulted in the isolation of sanggenon C (**1**), sanggenon G (**2**), mulberrofuran C (**3**) and kuwanon L (**4**) as PTP1B inhibitors, along with moracin O (**5**) and moracin P (**6**). Compounds **1**–**4** inhibited PTP1B with IC_{50} values ranging from 1.6 ± 0.3 μM to 16.9 ± 1.1 μM.

Protein tyrosine phosphatases (PTPs), which dephosphorylate the insulin receptor (IR) and other proteins, have an important role in insulin signaling and metabolism. Although several PTPs such as PTP-α, leukocyte antigen-related tyrosine phosphatase (LAR), and SH2-domain-containing phosphotyrosine phosphatase (SHP2) have been implicated in the regulation of insulin signaling, there is substantial evidence that PTP1B is the critical point among various PTPs in the insulin signaling pathway.[1] Its overexpression has been shown to inhibit the IR signaling cascade and increased expression of PTP1B occurs in insulin-resistant states.[2] Furthermore, recent evidence has shown that the leptin signaling pathway can be attenuated by PTPs and there is compelling evidence that PTP1B is also involved in this process.[3] Therefore, it has been suggested that the PTP1B inhibitor could not only be used for treating Type 2 diabetes but also obesity. Although there have been a number of reports on the designing and development of synthetic PTP1B inhibitors, only a few studies have been reported as PTP1B inhibitors derived from plants.[4]

In an effort to search for PTP1B inhibitors from medicinal plants, an ethyl acetate-soluble extract of a Chinese crude drug, 'Sang-Bai-Pi,' the root bark of *Morus* sp., was found to inhibit PTP1B activity (63% inhibition at 30 μg/mL). The root bark of *Morus* sp. (*Morus* root bark) was purchased from the herbal medicine association of Daejon, Korea, and was identified by Prof. KiHwan Bae, College of Pharmacy, Chungnam National University (Voucher specimen, KRIBB 1132, has been deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea). The leaf of *Morus* sp. has been used in traditional medicine to treat diabetes mellitus, and recently many reports suggest that N-containing sugars from the leaf of *Morus alba* and *Morus bombysis* are a potent glucosidase inhibitor.[5] In our present work, we report the isolation of sanggenon C (**1**), sanggenon G (**2**), mulberrofuran C (**3**), and kuwanon L (**4**) as new PTP1B inhibitors, and also moracin O (**5**) and moracin P (**6**).

The dried *Morus* root bark (3 kg) was extracted with MeOH (6 L) for 24 h. After solvent fractionation, the EtOAc-soluble fraction (5 g) was chromatographed over silica gel (70–230 mesh) using a gradient of CH_2Cl_2–MeOH (from 10/1 to 3/1) to yield seven fractions (Fr. 1–Fr. 7). For further fractionation of active compounds, Fr. 5 (202.5 mg) and Fr. 6 (195.7 mg) having the PTP1B inhibitory activity (76% and 87% inhibition at 20 μg/mL) were re-chromatographed on a C18 reversed-phase column with a gradient of MeOH–H_2O (60/40–90/10). The active fraction that had eluted with MeOH–H_2O (60/40) was applied to C18 reversed-phase HPLC column (10 μM, 250 × 10 mm) and eluted with a gradient

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of CH_3CN–H_2O (40/60–73/27) at a flow rate of 2.0 mL/min. This resulted in the isolation of three compounds, sanggenon C (**1**, 12.9 mg), sanggenon G (**2**, 10.2 mg), and mulberrofuran C (**3**, 7.5 mg). Further purification of the fraction eluted from MeOH–H_2O (90/10) led to the isolation of kuwanon L (**4**, 31.3 mg), moracin O (**5**, 3.2 mg), and moracin P (**6**, 5.6 mg).

Compound **1** was obtained as a pale yellow amorphous powder and showed a pair of quasimolecular ion peaks at *m*/*z* 707 $[M-H]^+$ and *m*/*z* 731 $[M+Na]^+$, respectively, in the ESI mass spectrum. Signals typical of a trisubstituted methylcyclohexene were observed in the ^1H NMR spectrum at δ 1.51, 2.20, 2.50, 4.23, 3.79, and 4.06. The ^{13}C NMR and HMQC spectra showed that methine signals of the methylcyclohexene ring at δ 4.23 were attached to the carbon at δ 95.6. The data from ^1H, ^{13}C NMR (acetone-d_6), and $[\alpha]_D$, in comparison of previously reported literatures confirmed the structure of **1** as sanggenon C.[6]

Compound **2** was obtained as a pale yellow amorphous powder and displayed a pair of base peaks at *m*/*z* 693 $[M-H]^+$ and *m*/*z* 717 $[M+Na]^+$, respectively, in the ESI mass spectrum. ^1H and ^{13}C NMR spectra (acetone-d_6) indicated the presence of trisubstituted methylcyclohexene ring, prenyl and hydroxyl groups. The structure of **2** was identified as sanggenon G by analyses of MS, $[\alpha]_D$, ^1H, ^{13}C NMR data and comparison of a previously reported literature.[7]

The ESI-MS exhibited each pair of quasimolecular ion peaks in compounds **3**, **4**, **5**, and **6** indicating molecular weight of 580, 626, 326, and 326, respectively. The structures of **3**, **4**, **5**, and **6** were identified readily as mulberrofuran C (**3**), kuwanon L (**4**), moracin O (**5**), and moracin P (**6**) by their spectroscopic properties (MS, $[\alpha]_D$, ^1H, and ^{13}C NMR (acetone-d_6)) and comparison with the data of a previously reported literature.[8,9]

The ability of isolated compounds to inhibit PTP1B, dual-specificity protein tyrosine phosphatase (DS-PTP), and protein serine/threonine phosphatase (PP1) was studied by a previously described method.[10] PTP1B (human, recombinant) was purchased from BIOMOL® International LP (USA). The enzyme activity was measured by adding 2 mM *p*NPP and PTP1B (0.05–0.1 μg) in a buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) with or without test compounds. Following incubation at 37 °C for 30 min, the reaction was terminated with 1 M NaOH. The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 405 nm. The nonenzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.[11] DS-PTP was assayed with the VH1-related human protein (VHR) expressed in *Escherichia coli*. The reaction mixture containing VHR enzyme, 2 mM *p*NPP, and assay buffer (50 mM succinate, 1 mM EDTA, 140 mM NaCl, and 0.05% Tween 20, pH 6.0) was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1 M NaOH, and the dephosphorylation activity was measured.[12] For protein serine/threonine phosphatase 1 (PP1) assay, PP1 (Sigma Chemical Co., St. Louis, MO, USA) was added in the assay buffer (50 mM Tris–HCl, 0.1% β-mercaptoethanol, 1 mM EDTA, 1 mM $MnCl_2$, and 20 mM $MgCl_2$, pH 7.6) and incubated for 30 min. After the reaction was stopped by the addition of 1 M NaOH, the amount of *p*-nitrophenol was measured by absorbance at 405 nm.[11,12] As shown in Table 1, compounds **1**–**4** inhibited PTP1B activity in a dose-dependent manner with IC_{50} values ranging from 1.6 ± 0.3 μM to 16.9 ± 1.1 μM. A known phosphatase inhibitor, RK-682 ($IC_{50} = 4.5 \pm 0.5$ μM), was used as a positive control in this assay.[13] In addition, when we tested for the inhibitory effects on other types of protein phosphatases with the isolated compounds, it was shown that the compounds, except **2**, have no inhibitory effects toward VHR and PP1 at levels up to 50 μM. This suggests that compounds **1**–**4** have strong inhibitory activity against PTP1B.

To elucidate the inhibition modes of compounds **1**–**3** with strong inhibitory activity against PTP1B, kinetic analyses were performed. The reaction mixture consisting of four or five different concentrations of *p*NPP (1.0, 2.0, 4.0, 8.0, and 16.0 mM) was used as a PTP1B substrate in the absence or presence of compounds. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of PTP1B were determined by Lineweaver–Burk plots using a GraphPad Prism® 4 program (GraphPad Software Inc., USA).[4b,12] As shown in Figure 1, when *p*NPP is used as the substrate, reciprocal plots of the isolated compounds are intersected to the left of $1/v$ axis. From these results, we found that **1**–**3** inhibited PTP1B in the mixed-type manner, indicating that they may bind both at the active site and an additional binding site of the PTP1B enzyme.

Although the exact molecular mechanism of type 2 diabetes has not been fully elucidated, a variety of evidences suggest that the disease is a result of a defect in the insulin signaling pathway.[14] Insulin exerts its effects by binding to the insulin receptor (IR) on insulin target tissues (adipose, liver, and muscle). When insulin binds to

Table 1. Comparison of the inhibitory activity of the compounds isolated from *Morus* root bark against PTP1B, VHR, and PP1

Compounds	IC_{50} (μM)[a]		
	PTP1B	VHR DS-PTP	PP1
1	2.6 ± 0.3	>80	>80
2	1.6 ± 0.3	>80	17.8 ± 2.5
3	4.9 ± 0.2	>80	>50
4	16.9 ± 1.1	>80	>80
5	>80	>80	>80
6	>80	>80	>80
RK-682[b]	4.5 ± 0.5	10.2 ± 1.2	NT[c]

[a] IC_{50} values were determined by regression analyses and expressed as means ± SD of three replicates.
[b] Positive control.
[c] Not tested.

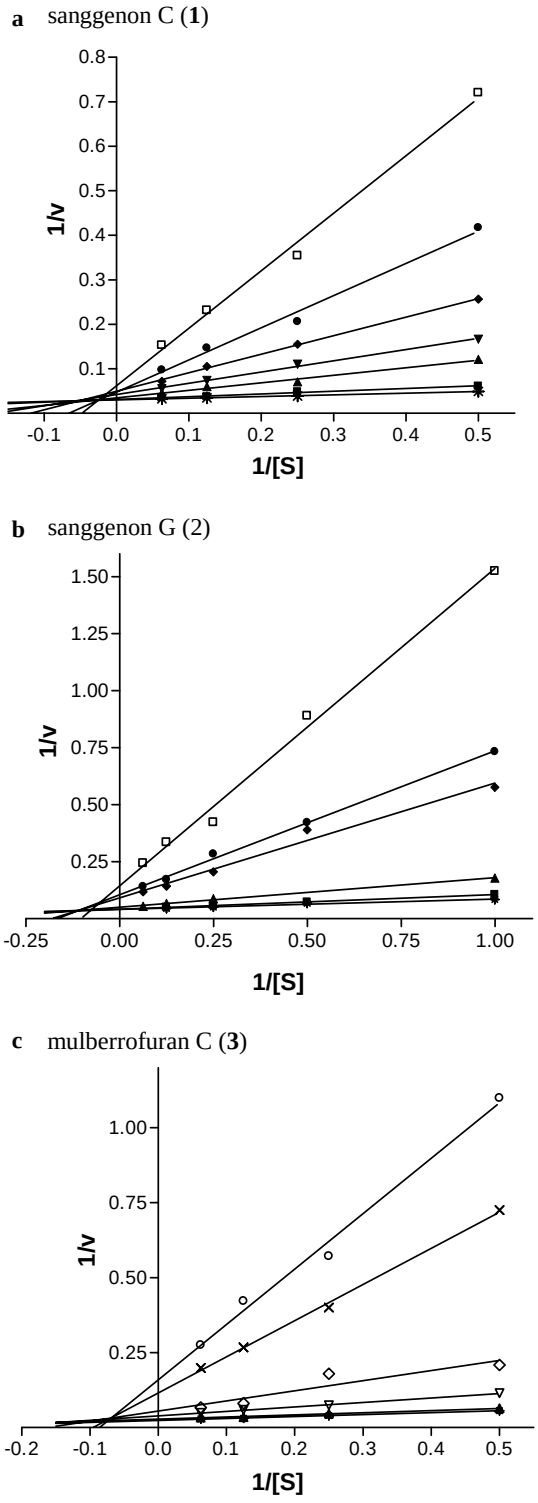

Figure 1. Inhibition kinetics of sanggenon C (**1**), sanggenon G (**2**), and mulberrofuran C (**3**). (a–c) The Lineweaver–Burk plot of the inhibitory effect of compounds **1**–**3** on PTP1B-catalyzed hydrolysis of *p*NPP, respectively. Data are expressed as mean initial velocity (μg *p*-nitrophenol/min) for $n = 3$ replicates at each substrate concentration. Symbols: (a)—(∗) 0 μM, (■) 2.8 μM, (▲) 3.5 μM, (▼) 4.5 μM, (♦) 5.6 μM, (●) 7.1 μM, (□) 8.5 μM sanggenon C (**1**). (b)—(∗) 0 μM, (■) 2.8 μM, (▲) 3.5 μM, (♦) 5.6 μM, (●) 7.1 μM, (□) 8.5 μM sanggenon G (**2**). (c)—(*) 0 μM, (△) 4.3 μM, (▽) 5.5 μM, (◊) 6.9 μM, (×) 10.3 μM, (○) 17.2 μM mulberrofuran C (**3**).

extracellular α-subunits of insulin-binding site, IR triggers a conformational change that activates the intrinsic tyrosine kinase activity in the intracellular β-subunit within the activation loop of IRβ.[15] The insulin action on IR is attenuated by PTPs, PTP1B emerging as a key player that negatively regulates insulin signaling by dephosphorylating the IR as well as IRS proteins.[16] As part of our ongoing study to search for natural PTP1B inhibitors from plants, *Morus* root bark was chosen for detailed investigation, since the MeOH extract was found to inhibit PTP1B at 30 μg/mL. Bioassay-guided fractionation of the MeOH extract of the plant resulted in the isolation of six compounds. Four compounds among isolated materials exhibited strong inhibitory activity against PTP1B. When we determined the inhibition mode on the activity of PTP1B, it showed that tested compounds were mixed-type inhibitors of PTP1B. To our knowledge, this is the first time that purified flavonoids of *Morus* root bark are described as PTP1B inhibitors. It was well known that N-containing sugars from leafs and root of *Morus alba*, 1-deoxynojirimycin and *N*-methyl-1-deoxynojirimycin, had hypoglycemic activity due to their inhibitory activity on glycosidase.[5,17] Another recent study, conducted by Singab et al. (2005), also has demonstrated that flavonoid-rich fraction of the Egyptian *Morus alba* root bark (MRBF-3) has hypoglycemic activity on streptozotocin-induced diabetic rats. When they administered MRBF-3 for 10 days (600 mg/kg/day), the level of glucose in blood was significantly reduced from control level (379 ± 9 mg/dl) to a lower level (155 ± 8 mg/dl).[18] Although the hypoglycemic activity of isolated compounds on diabetes-related animal models is not directly evaluated yet, this report could be supported by the in vivo anti-diabetic activity of isolated compounds because the PTP1B enzyme plays an important role in insulin signaling.

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