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Protein tyrosine phosphatase 1B inhibitory activity of triterpenes isolated from *Astilbe koreana*

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Abstract—Bioassay-guided fractionation of a MeOH extract of the rhizomes of *Astilbe koreana* (Saxifragaceae), using an in vitro protein tyrosine phosphatase 1B (PTP1B) inhibitory assay, resulted in the isolation of a new triterpene, 3α,24-dihydroxyolean-12-en-27-oic acid (**4**), along with four triterpenes, 3-oxoolean-12-en-27-oic acid (**1**), 3β-hydroxyolean-12-en-27-oic acid (β-peltoboykinolic acid; **2**), 3β-hydroxyurs-12-en-27-oic acid (**3**), and 3β,6β-dihydroxyolean-12-en-27-oic acid (astilbic acid; **5**). Compounds **1**–**5** inhibited PTP1B with IC_{50} values of 6.8 ± 0.5, 5.2 ± 0.5, 4.9 ± 0.4, 11.7 ± 0.9, and 12.8 ± 1.1 μM, respectively. Our results indicate that 3-hydroxyl group and a carboxyl group in this type of triterpenes may be required for the activity, while addition of one more hydroxyl group at C-6 or C-24 may be responsible for a loss of activity. Thus, compounds **2** and **3** which possess only one hydroxyl group at C-3 and a carboxyl group at C-27 could be potential PTP1B inhibitors.

Protein tyrosine phosphatases (PTPs), which dephosphorylate the tyrosine residues of proteins, have an important role in intracellular 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 are substantial evidences supporting PTP1B as the critical PTP controlling insulin signaling pathway.[1,2] PTP1B can interact with and dephosphorylate the activated insulin receptor (IR) as well as insulin receptor substrate (IRS) proteins.[1,2] Its overexpression has been shown to inhibit the IR signaling cascade and increased expression of PTP1B occurs in insulin-resistant states.[3] Furthermore, recent genetic evidence has shown that PTP1B gene variants are associated with changes in insulin sensitivity.[4] As with the insulin signaling pathway, the leptin signaling pathway can be attenuated by PTPs and there is compelling evidence that PTP1B is also involved in this process.[1,2] Therefore, it has been suggested that compounds that reduce PTP1B activity or expression levels 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,[1,5] only a few studies have been reported as PTP1B inhibitors derived from plants.[6]

In our screening program to search for PTP1B inhibitors from plants, a MeOH extract of the rhizomes of *Astilbe koreana* exhibited PTP1B inhibitory activity (>70% inhibition at 30 μg/mL), which led us to investigate the PTP1B inhibitory compounds from this plant. *A. koreana* belongs to Saxifragaceae and is a perennial herb growing in the moist fields and mountains, usually to a height of 60 cm. The plants of this genus have been used to treat pain, headache, arthralgia, chronic bronchitis, and inflammation.[7] Bergenin and its galloyl ester, flavonoids, triterpenes, and phytosterols have been reported as constituents of the genus *Astilbe*, and have been found to possess analgesic and cytotoxic activities.[8,9] Our previous study has demonstrated that the EtOH extract of *A. koreana* inhibited UVB-induced generation of proinflammatory cytokines.[10] Despite a number of studies on the genus *Astilbe*, there have been no investigations regarding the chemical constituents and

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the PTP1B inhibitory activity of *A. koreana*. Therefore, we have investigated the PTP1B inhibitory compounds of this plant. Bioassay-guided fractionation of a MeOH extract of the rhizomes of this plant led to the isolation of a new triterpene, 3α,24-dihydroxyolean-12-en-27-oic acid (**4**), along with four triterpenes, 3-oxoolean-12-en-27-oic acid (**1**), 3β-hydroxyolean-12-en-27-oic acid (β-peltoboykinolic acid; **2**), 3β-hydroxyurs-12-en-27-oic acid (**3**), and 3β,6β-dihydroxyolean-12-en-27-oic acid (astilbic acid; **5**), as the active principles. In this report, we describe the isolation and structure determination of these compounds, and the evaluation of their PTP1B inhibitory activity.

The rhizomes of *A. koreana* (3 kg), collected at Mt. Sulak, Korea, in July 2001 (voucher No. CNU 0880), were extracted with MeOH at room temperature for two months. The MeOH extract (IC_{50} = 22.7 μg/mL, 460 g) was suspended in H_2O (3 L) and sequentially partitioned with hexane (3× 3 L), EtOAc (3× 3 L), and BuOH (3× 3 L), to obtain hexane-soluble fraction (IC_{50} = 18.5 μg/mL), EtOAc-soluble fraction (IC_{50} = 9.5 μg/mL), BuOH-soluble fraction (IC_{50} = 47.3 μg/mL), and H_2O-soluble fraction (IC_{50} > 100.0 μg/mL), respectively. Since the EtOAc-soluble fraction showed the strongest PTP1B inhibitory activity, this fraction (102 g) was further separated by silica gel column chromatography (10 × 30 cm; 63–200 μm particle size) using a stepwise gradient of CH_2Cl_2/MeOH (from 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 3:1, 1:1 to 0:1; 5 L for each step), to yield seven fractions (Fr. 1–Fr. 7). Of these, Fr. 1, Fr. 2 and Fr. 3 showed the most potent PTP1B inhibitory activity (80%, 76%, and 74% inhibition at 10 μg/mL). Fr. 1 [eluted with CH_2Cl_2/MeOH (10:1), 10.4 g] was chromatographed over silica gel (6.5 × 35 cm; 63–200 μm particle size) using a mixture of $CHCl_3$/MeOH (20:1), to yield four subfractions (Fr. 1-1–Fr. 1-4). Further purification of Fr. 1-1 (2.3 g) by preparative MPLC [LiChroprep® Si 60 column (25 × 310 mm; 40–63 μm particle size); mobile phase $CHCl_3$/MeOH (20:1); flow rate 5 mL/min] resulted in the isolation of compounds **1** (6.2 mg), **2** (7.5 mg), and **3** (3.9 mg). Another active fraction, Fr. 3 [eluted with CH_2Cl_2/MeOH (8:1), 9.8 g], was subjected to silica gel column chromatography using a stepwise gradient of $CHCl_3$/MeOH (from 20:1, 15:1, 10:1, 9:1, 8:2, 7:3, 6:4 to 1:1; 2 L for each step) to yield six subfractions (Fr. 3-1–Fr. 3-6). Fr. 3-2 [eluted with $CHCl_3$/MeOH (from 15:1 to 10:1), 1.5 g] was purified by preparative MPLC [LiChroprep® Si 60 column (25 × 310 mm; 40–63 μm particle size); mobile phase $CHCl_3$/MeOH (15:1); flow rate 5 mL/min], to afford compound **4** (12 mg). Additional silica gel column chromatography (10 × 30 cm; 40–63 μm particle size) of Fr. 3-3 [eluted with $CHCl_3$/MeOH (from 10:1 to 9:1), 2.0 g] with hexane/acetone (2:1) resulted in the isolation of compound **5** (37 mg). The structures of the isolated compounds **1**, **2**, **3**, and **5** were identified as 3-oxoolean-12-en-27-oic acid {compound **1**; mp: 210 °C; $[\alpha]_D^{25}$ +140° (*c* 1.3, $CHCl_3$); ESI-MS *m/z*: 453 $[M-H]^+$, 477 $[M+Na]^+$}, 3β-hydroxyolean-12-en-27-oic acid {compound **2**; mp: 227 °C; $[\alpha]_D^{25}$ +105° (*c* 1.3, $CHCl_3$); ESI-MS *m/z*: 455 $[M-H]^+$, 479 $[M+Na]^+$}, 3β-hydroxyurs-12-en-27-oic acid {compound **3**; mp: 240–241 °C; $[\alpha]_D^{25}$ +102° (*c* 0.9, $CHCl_3$); ESI-MS *m/z*: 455 $[M-H]^+$, 479 $[M+Na]^+$}, and 3β,6β-dihydroxyolean-12-en-27-oic acid {compound **5**; mp: 220–223 °C; $[\alpha]_D^{25}$ +100° (*c* 1.0, $CHCl_3$); ESI-MS *m/z*: 471 $[M-H]^+$, 495 $[M+Na]^+$} by analyses of MS and NMR data, and comparison with those in the literature (Fig. 1).[9,11]

Compound **4** was obtained as a white amorphous powder, mp 245–247 °C, $[\alpha]_D^{25}$ +110° (*c* 1.1, $CHCl_3$). A molecular formula of $C_{30}H_{48}O_4$ was determined for this compound from the molecular ion peak at *m/z* 472.3553 $[M]^+$ (calcd for $C_{30}H_{48}O_4$: 472.3554) obtained by HREIMS. The ^1H NMR spectrum of **4** displayed the characteristic signals of an olefinic proton (δ_H 5.61), an oxygenated methine (δ_H 3.74), a hydroxymethylene group (δ_H 3.63 and 3.41), and six tertiary methyl groups (δ_H 1.10, 1.05, 0.92× 2, 0.91, 0.73). Thirty carbon signals appeared in ^{13}C and DEPT NMR spectra including a carbonyl carbon signal at δ_C 179.6 (C-27), olefinic carbon signals at δ_C 137.8 (C-13) and 126.0 (C-12), an oxygenated methine at δ_C 70.9 (C-3), an oxygenated methylene at δ_C 66.5, and six methyls at δ_C 33.1, 28.5, 25.1, 23.9, 18.4, and 16.7 indicated that compound **4** had a skeleton similar to those of β-peltoboykinolic acid (**2**) and astilbic acid (**5**).[9,11] When compared to compound **2** or **5**, C-3 of **4** showed a upfield chemical shift (6–8 ppm), and the signal of H-3 in **4** was displayed as a broad singlet. This suggested that compound **4** should have a 3α-OH rather than 3β-OH.[12,13] The proton signals of hydroxymethylene showed HMBC correlation to a hydroxymethine (δ_C 70.9, C-3), a quaternary carbon (δ_C 44.2, C-4), and an aliphatic methine (δ_C 49.7, C-5), indicating that the hydroxymethylene is located at

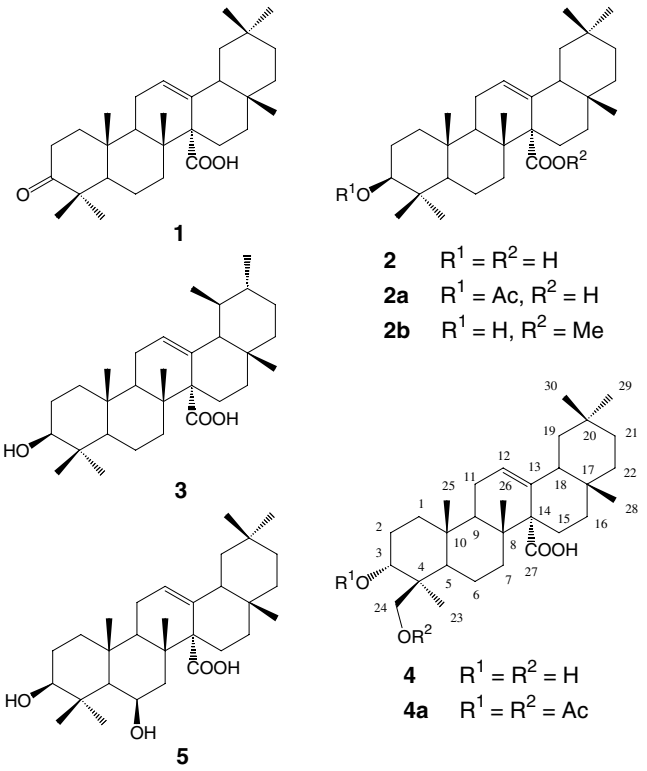

Figure 1. Structures of compounds **1**–**5** isolated from *A. koreana*.

C-23 or 24. The upfield shift of the oxygenated methylene (δ_C 66.5) in **4** suggested that this group could be assigned to the axial C-24.[12–17] In order to confirm the position of hydroxymethylene, **4** was acetylated using pyridine and acetic anhydride at room temperature for 12 h, to yield the diacetate (**4a**).[18] Comparison of the average chemical shift value for the acetoxymethylene protons of **4a** with those of reported values in analogous triterpenoids indicated an axial orientation for the hydroxymethylene group, which is β (C-24) in configuration.[12–17] This was further supported by the NOE correlations from Me-25 to both the H-24a and H-24b. Thus, the structure of this new compound (**4**) was determined as 3α,24-dihydroxyolean-12-en-27-oic acid (Fig. 1).[19]

PTP1B (human, recombinant) was purchased from BIOMOL® International LP (USA) and the enzyme activity was measured using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate.[20] To each 96-well (final volume: 200 μL) were added 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 10 M NaOH. The amount of produced *p*-nitro phenol 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. All the isolates were assayed for their inhibitory activity against PTP1B, and the results are presented in Table 1. Of the compounds tested, 3β-hydroxyurs-12-en-27-oic acid (**3**) and 3β-hydroxyolean-12-en-27-oic acid (**2**) exhibited the strongest inhibitory activity with IC_{50} values of 4.9 ± 0.4 and 5.2 ± 0.5 μM, respectively, which were comparable to that of RK-682 (IC_{50} = 4.5 ± 0.5 μM) used as a positive control.[21] This result indicates that only a positional change of methyl groups at C-29 and C-30 may not affect the PTP1B inhibitory activity. Compound **1** (IC_{50} = 6.8 ± 0.5 μM) converted a hydroxyl group at C-3 to ketone was somewhat less active than **2**. Moreover, addition of an acetyl group to C-3 (**2a**,[22] IC_{50} = 8.5 ± 0.7 μM) resulted in loss of in vitro inhibitory activity. These results suggest that 3-hydroxyl group in this type of triterpenes may be required for the activity. Compound **5** (IC_{50} = 12.8 ± 1.1 μM) substituted a hydroxyl group at C-6 of **2** exhibited significantly lower activity than **2**. Compound **4**, which possesses one more hydroxyl group at C-24, was also found to be less effective than **2**. Although the structure–activity relationships of these triterpenes bearing a carboxyl group at C-27 were not thoroughly investigated, our results indicate that introduction of one more hydroxyl group at C-6 or C-24 may be responsible for a loss of activity. To evaluate the role of a carboxyl group at C-27, we assayed a group of related triterpenes that are commercially available or can be easily prepared by standard method.[22] Oleanolic acid with the same substitution pattern as that of compound **2** except for a position of carboxyl group at C-28 retained the activity, whereas addition of methyl ester to C-28 carboxylic acid resulted in a loss in its PTP1B inhibitory activity (Table 1). A similar loss of activity was observed in the 27-methyl ester, **2b**,[22] as compared to that in compound **2** (Table 1). These results indicate that a carboxyl group in oleanane triterpenes is important for the PTP1B inhibitory activity, however, the position of carboxyl group, whether it is at C-27 or C-28, seems not to be critical for the activity. Additionally, we tested the ability of a group of related natural triterpenes to inhibit in vitro PTP1B activity. As a result, 18β-glycyrrhetic acid (3β-hydroxy-11-oxo-18β,20β-olean-12-en-30-oic acid) and the 18α-isomer displayed IC_{50} values of 13.8 ± 1.0 and 15.6 ± 1.1 μM, respectively, whereas glycyrrhizin did not show any activity in our assay (Table 1). PTP1B is known to have several binding sites such as electrostatic, hydrophobic, and hydrogen-bonding sites, and to have several N-terminal favorable for binding to acidic site.[1,23] Although the inhibition mode of action with regard to this type of triterpenes has not been elucidated yet, considering the molecular features of active triterpenes, pentacyclic ring might facilitate the hydrophobic interaction, and the hydroxyl group at C-3 may be presumed to form hydrogen bond. Besides, a free carboxyl group at C-27 or C-28 might play a role in binding to N-terminal residues. Of the isolates, thus, compounds **2** and **3** which possess only one hydroxyl group at C-3 and a carboxyl group at C-27 can be potential PTP1B inhibitors.

Table 1. The inhibitory activity of the isolated compounds **1**–**5** and related compounds against PTP1B

Compound	PTP1B inhibitory activity IC_{50}[a] (μM)
3-Oxoolean-12-en-27-oic acid (**1**)	6.8 ± 0.5
3β-Hydroxyolean-12-en-27-oic acid (**2**)	5.2 ± 0.5
3β-Acetoxyolean-12-en-27-oic acid (**2a**)	8.5 ± 0.7
Methyl 3β-hydroxyolean-12-en-27-oate (**2b**)	16.0 ± 1.3
3β-Hydroxyurs-12-en-27-oic acid (**3**)	4.9 ± 0.4
3α,24-Dihydroxyolean-12-en-27-oic acid (**4**)	11.7 ± 0.9
3β,6β-Dihydroxyolean-12-en-27-oic acid (**5**)	12.8 ± 1.1
Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid)	3.9 ± 0.5
Methyl 3β-hydroxyolean-12-en-28-oate	14.2 ± 1.1
Glycyrrhizin	>100
18β-Glycyrrhetic acid	13.8 ± 1.0
18α-Glycyrrhetic acid	15.6 ± 1.1
RK-682[b]	4.5 ± 0.5

[a] IC_{50} values were determined by regression analyses and expressed as means ± SD of three replicates.
[b] Positive control.[21]

Pentacyclic triterpenes with a carboxyl group at C-27 have been isolated rarely from the genera *Astilbe*, *Peltoboykinia*, *Boykinia*, *Chrysosplenium*, and *Aceriphyllum* of the family Saxifragaceae,[9,24–26] the genus *Cordia* of Boraginaceae,[11] and the genus *Cornulaca* of Chenopodiaceae.[27] This type of triterpenes has been reported to possess ant-repellent,[11] cytotoxic, and antitumor activities,[9,25] and to inhibit acyl-CoA cholesterol acyltransferase (ACAT) related to atherosclerosis.[26] However, to our knowledge, PTP1B inhibitory activity of these triterpenes is now being reported for the first time in this study. Our results indicate that the PTP1B inhibitory

activity for this unique type of triterpenes warrants further investigation and optimization.

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18. Compound **4** (8 mg) was treated with pyridine (0.5 mL) and acetic anhydride (0.5 mL) at room temperature for 12 h. To the mixture 5 mL of H_2O was added, which was then extracted with CH_2Cl_2 (3× 5 mL). The combined organic extract was purified over a silica gel column (1.6 × 15 cm) using a mixture of hexane/$CHCl_3$/MeOH (5:1:1), to afford the diacetate (**4a**): white amorphous powder; R_f: 0.5 [hexane/$CHCl_3$/MeOH (4:1:1)]; FABMS *m/z*: 579 $[M+Na]^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 5.71 (1 H, br s, H-12), 4.82 (1H, br s, H-3), 4.11 (1H, d, J = 10.0 Hz, H-24a), 3.86 (1H, d, J = 10.0 Hz, H-24b), 2.29 (1H, dd, J = 5.2, 11.6 Hz, H-9), 2.10 (1H, m, H-18), 2.00 (3H, s, OAc), 1.99 (3H, s, OAc), 1.07 (3H, s, Me), 1.03 (3H, s, Me), 1.01 (3H, s, Me), 0.86 (3H, s, Me), 0.84 (3H, s, Me), 0.80 (3H, s, Me).
19. 3α,24-Dihydroxyolean-12-en-27-oic acid (**4**): white amorphous powder, mp 245–247 °C; $[\alpha]_D^{25}$ +110° (*c* 1.1, $CHCl_3$); ESI-MS *m/z*: 471 $[M-H]^+$, 495 $[M+Na]^+$; HREIMS *m/z*: 472.3553 $[M]^+$ (calcd for $C_{30}H_{48}O_4$: 472.3554); ^1H NMR (400 MHz, $CDCl_3$): δ 5.61 (1H, br s, H-12), 3.74 (1H, br s, H-3), 3.63 (1H, d, J = 11.6 Hz, H-24a), 3.41 (1H, d, J = 11.6 Hz, H-24b), 2.34 (1H, dd, J = 5.2, 12.0 Hz, H-9), 2.13 (1H, m, H-18), 1.10 (3H, s, H-26), 1.05 (3H, s, H-25), 0.92 (6H, s, H-28 and H-29), 0.91 (3H, s, H-30), 0.73 (3H, s, H-23); ^{13}C NMR (100 MHz, $CDCl_3$): δ 36.8 (C-1), 27.9 (C-2), 70.9 (C-3), 44.2 (C-4), 49.7 (C-5),18.0 (C-6), 32.9 (C-7), 35.9 (C-8), 47.0 (C-9), 37.0 (C-10), 22.9 (C-11), 126.0 (C-12), 137.8 (C-13), 56.1 (C-14), 22.3 (C-15), 27.9 (C-16), 31.3 (C-17), 49.4 (C-18), 42.2 (C-19), 33.5 (C-20), 34.6 (C-21), 37.0 (C-22), 25.1 (C-23), 66.5 (C-24), 16.7 (C-25), 18.4 (C-26), 179.6 (C-27), 28.5 (C-28), 33.1 (C-29), 23.9 (C-30).
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22. (a) Compound **2a** was prepared by stirring **2** with acetic anhydride in pyridine in the presence of 4-dimethylaminopyridine (DMAP): colorless needles; mp 228–230 °C; $[\alpha]_D^{25}$ +101° (*c* 0.6, $CHCl_3$), ESI-MS *m/z*: 497 $[M-H]^+$, 521 $[M+Na]^+$; (b) Reaction of compound **2** with diazomethane in ether yielded the methyl ester, **2b**: colorless needles; mp 210–213 °C; $[\alpha]_D^{25}$ +115° (*c* 0.5, $CHCl_3$), ESI-MS *m/z*: 469 $[M-H]^+$, 493 $[M+Na]^+$; (3) reaction of oleanolic acid with diazomethane in ether afforded the methyl ester: colorless needles; mp 202–205 °C; $[\alpha]_D^{25}$ +70° (*c* 1.0, $CHCl_3$), ESI-MS *m/z*: 469 $[M-H]^+$, 493 $[M+Na]^+$.
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