

PTP1B inhibitory activity of kaurane diterpenes isolated from *Siegesbeckia glabrescens*

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(Received 8 February 2006; in final form 10 March 2006)

Abstract

Protein tyrosine phosphatase 1B (PTP1B) is considered as a therapeutic target for the treatment of diabetes and obesity. In our preliminary screening study, a MeOH extract of the aerial part of *Siegesbeckia glabrescens* was found to inhibit PTP1B activity at 30 µg/mL. Bioassay-guided fractionation led to the isolation of two active diterpenes, *ent*-16βH,17-isobutyryloxy-kauran-19-oic acid (**1**) and *ent*-16βH,17-acetoxy-18-isobutyryloxy-kauran-19-oic acid (**2**), along with *ent*-16βH,17-hydroxy-kauran-19-oic acid (**3**). Compounds **1** and **2** inhibited the PTP1B activity with IC₅₀ values of 8.7 ± 0.9 and 30.6 ± 2.1 µM, respectively. Kinetic studies suggest that both **1** and **2** are non-competitive inhibitors of PTP1B. However, compound **3** substituted with a hydroxyl group at C-17 in kaurane-type showed no inhibitory effects towards PTP1B.

Keywords: Protein tyrosine phosphatase 1B, *Siegesbeckia glabrescens*, kaurane diterpenes, *ent*-16βH,17-isobutyryloxy-kauran-19-oic acid, *ent*-16βH,17-acetoxy-18-isobutyryloxy-kauran-19-oic acid, non-competitive inhibitors, inhibition

Introduction

Binding of insulin to the extracellular α-subunit of the insulin receptor (IR) triggers a conformational change that activates the intrinsic tyrosine kinase activity of the β-subunit via autophosphorylation of specific tyrosine residues. This results in the phosphorylation of IR substrates (IRS) 1–4, which then activates several signaling cascades that give a biological response, such as glucose transport into the cell and glycogen synthesis [1,2]. Protein tyrosine phosphatases (PTPs) are responsible for the dephosphorylation of tyrosine residues, and are considered as negative regulators of insulin signaling. Although several PTPs such as PTP-α, leukocyte antigen-related tyrosine phosphatase (LAR), SH2-domain-containing phosphotyrosine phosphatase (SHP2) have been implicated in the regulation of insulin signaling, there is substantial evidence supporting protein tyrosine phosphatase 1B

(PTP1B) as the critical PTP controlling insulin signaling pathway [1,2]. The overexpression of PTP1B has been shown to inhibit the IR signaling cascade and occurs in insulin-resistant states [3,4]. Furthermore, recent genetic evidence has demonstrated that PTP1B gene variants are associated with changes in insulin sensitivity [5,6]. 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 [1,2,7]. 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 [1,2]. Although there have been a number of reports on the design and development of synthetic PTP1B inhibitors [1,8], only a few studies have been reported on PTP1B inhibitors derived from plants [9,10].

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In our preliminary screening study, a methanol extract of the aerial part of *Siegesbeckia glabrescens* (Compositae) was found to inhibit PTP1B activity at 30 $\mu\text{g/ml}$ [11]. The genus *Siegesbeckia* has been used as a traditional medicine, 'Hi-Chum' in Korea (common name: *Siegesbeckia* herb), to treat inflammatory diseases [12]. Previous phytochemical investigations on this genus have resulted in the isolation of sesquiterpenes and diterpenes [13,15]. The aqueous extract of *S. glabrescens* has been reported to reduce the mast cell-mediated anaphylactic reaction and inhibit the immunoglobulin E (IgE) production [16,17]. Since there has been no study with regard to its inhibitory effect on PTP1B, we searched for the PTP1B-inhibitory compounds from this plant. Bioassay-guided fractionation of the methanol extract resulted in the isolation of two active diterpenes, *ent*-16 β H,17-isobutyryloxy-kauran-19-oic acid (**1**) and *ent*-16 β H,17-acetoxy-18-isobutyryloxy-kauran-19-oic acid (**2**), along with *ent*-16 β H,17-hydroxy-kauran-19-oic acid (**3**) (Figure 1). Although these diterpenes have been isolated from some other species, they have not previously been reported in this plant.

Materials and methods

Plant material

The aerial parts of *S. glabrescens* was purchased from Il-sin Pharmaceutical Co., Ltd. in Daejeon, Korea. A voucher specimen (KRIBB No. 1144) has been deposited in the authors' laboratory.

Extraction and isolation

The dried aerial parts of *S. glabrescens* (2 kg) was extracted with MeOH (10 L \times 3) for 6 h at room temperature. The dried MeOH extract (680 g) was suspended in water and partitioned with hexane, EtOAc, and BuOH, sequentially. Since the hexane-soluble fraction exhibited the strongest activity (80% inhibition at 30 $\mu\text{g/ml}$), a portion of this fraction (1.45 g) was further separated by silica gel column chromatography (5.5 \times 45 cm, 0.063–0.200 mm particle size) eluting with a stepwise gradient of hexane-EtOAc (from 100:0 to 0:100), to afford 22 subfractions (Fr.1 – Fr. 22). Of the subfractions that showed PTP1B inhibitory activity, Fr. 9 (eluted with 85% hexane in EtOAc, 63.4 mg) was further purified by preparative reverse phase HPLC using a gradient from 80% – 100% AcCN in 0.1% formic acid (*aq.*) over 40 min [RS tech Optima Pak C₁₈ column (Korea); 250 \times 10 mm; 10 μm particle size; 2 mL/min; UV detection at 210 nm], to yield compound **1** (9.3 mg, t_R 29.5 min). The other active subfraction, Fr. 15 (eluted with 70% hexane in EtOAc, 68.4 mg), was also subjected to preparative reverse phase HPLC on Shiseido Capcell Pak C₁₈

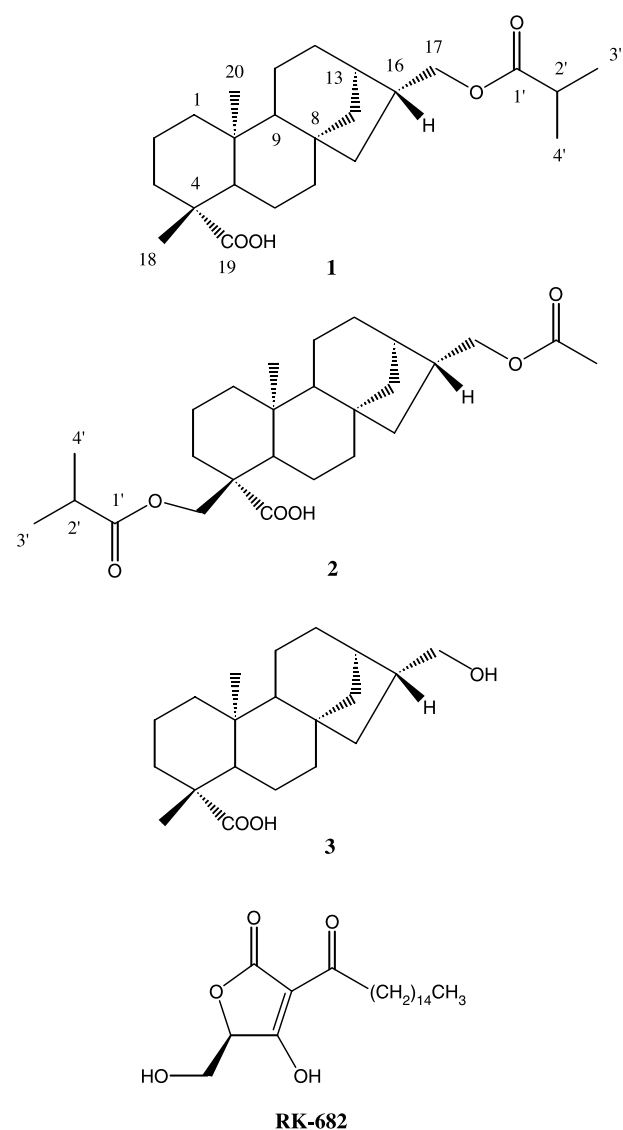


Figure 1. Structures of isolated compounds **1** – **3** and RK-682.

column (250 \times 10 mm; 5 μm particle size; Japan), eluted with a gradient from 80%–92% AcCN in 0.1% formic acid (*aq.*) over 30 min (2 mL/min; UV detection at 210 nm), to afford compounds **2** (9.7 mg, t_R 21.3 min) and **3** (11.8 mg, t_R 10.3 min).

Ent-16 β H,17-isobutyryloxy-kauran-19-oic acid (**1**). white amorphous powder; ESI-MS m/z : 389 [M – H]⁺; ¹H-NMR (600 MHz, pyridine-*d*₅) δ : 4.01 (2H, br d, J = 8.4 Hz, H-17), 2.61 (1H, sept, J = 6.6 Hz, H-2'), 2.50 (1H, d, J = 12.6 Hz, H-3), 2.14 (1H, m, H-16), 2.06 (1H, br s, H-13), 1.88 (2H, d, J = 12.0 Hz, H-1 and H-14), 1.37 (3H, s, H-18), 1.19 (6H, d, J = 6.6 Hz, H-3' and H-4'), 1.16 (3H, s, H-20), 1.08 (1H, m, H-3), 1.05 (1H, br d, J = 11.4 Hz, H-14), 0.84 (1H, m, H-1); ¹³C-NMR (150 MHz, pyridine-*d*₅) δ : 41.59 (C-1), 20.34 (C-2), 39.18 (C-3), 44.36 (C-4), 57.47 (C-5), 23.64 (C-6),

42.34 (C-7), 45.60 (C-8), 55.88 (C-9), 40.39 (C-10), 19.42 (C-11), 32.01 (C-12), 39.42 (C-13), 37.84 (C-14), 45.74 (C-15), 40.48 (C-16), 68.98 (C-17), 29.86 (C-18), 180.52 (C-19), 16.45 (C-20), 177.24 (C-1'), 34.73 (C-2'), 19.65 (C-3'), 19.64 (C-4').

Ent-16 β H,17-acetoxy-18-isobutyryloxy-kauran-19-oic acid (2). white amorphous powder; ESI-MS m/z : 447 $[M - H]^+$, 471 $[M + Na]^+$; 1H -NMR (300 MHz, $CDCl_3$) δ : 4.40 (1H, d, $J = 10.5$ Hz, H-18a), 3.97 (1H, d, $J = 10.5$ Hz, H-18b), 3.84 (2H, d, $J = 7.2$ Hz, H-17), 2.53 (1H, sept, $J = 7.2$ Hz, H-2'), 2.05 (3H, s, $OCOCH_3$), 1.15 (6H, d, $J = 7.2$ Hz, H-3' and H-4'), 0.96 (3H, s, H-20); ^{13}C -NMR (75 MHz, $CDCl_3$) δ : 40.32 (C-1), 18.94 (C-2), 32.53 (C-3), 47.64 (C-4), 52.46 (C-5), 22.54 (C-6), 41.26 (C-7), 44.82 (C-8), 55.51 (C-9), 39.63 (C-10), 18.51 (C-11), 31.39 (C-12), 38.70 (C-13), 37.34 (C-14), 45.03 (C-15), 39.76 (C-16), 68.50 (C-17), 72.38 (C-18), 180.70 (C-19), 15.64 (C-20), 177.61 (C-1'), 34.30 (C-2'), 19.23 (C-3'), 19.23 (C-4'), 170.90 ($OCOCH_3$), 21.01 ($OCOCH_3$).

Ent-16 β H,17-hydroxy-kauran-19-oic acid (3). white amorphous powder; ESI-MS m/z : 319 $[M - H]^+$; 1H -NMR (400 MHz, pyridine- d_5) δ : 3.64 (2H, m, H-17), 2.50 (1H, br d, $J = 12.6$ Hz, H-3), 2.35 (2H, m, H-2), 2.21 (2H, m, H-5 and H-16), 1.92 (1H, m, H-1), 1.37 (3H, s, H-18), 1.18 (3H, s, H-20), 0.75 (1H, td, $J = 12.4, 4.8$ Hz, H-1); ^{13}C -NMR (100 MHz, pyridine- d_5) δ : 42.16 (C-1), 19.90 (C-2), 37.43 (C-3), 45.07 (C-4), 57.10 (C-5), 23.25 (C-6), 41.20 (C-7), 43.90 (C-8), 55.68 (C-9), 39.97 (C-10), 19.23 (C-11), 31.92 (C-12), 38.74 (C-13), 38.76 (C-14), 45.80 (C-15), 44.23 (C-16), 67.03 (C-17), 29.41 (C-18), 180.19 (C-19), 16.05 (C-20).

PTP1B assay

PTP1B (human, recombinant) was purchased from BIOMOL[®] International LP (USA). The enzyme activity was measured using *p*-nitrophenylphosphate (*p*NPP) as described previously [18]. To each 96 well (final volume: 200 μ l) was added 2 mM *p*NPP and PTP1B (0.05(0.1 μ g) 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 *p*-nitro phenol produced was estimated by measuring the absorbance at 405 nm. The non-enzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

Dual-specificity protein tyrosine phosphatase (DS-PTP) assay

DS-PTP was assayed with VHR-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, 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 measured at 405 nm [18,19].

Protein serine/threonine phosphatase 1 (PP1) assay

The PP1 (Sigma Chemical Co., St. Louis, MO, USA) activity was measured at 37°C using *p*NPP as a substrate. Reactions were performed for 30 min in the assay buffer (50 mM Tris-HCl, 0.1% β -mercaptoethanol, 1 mM EDTA, 1 mM $MnCl_2$, 20 mM $MgCl_2$, pH 7.6). The reaction was stopped by the addition of 1 M NaOH, and the amount of *p*-nitro phenol was measured by absorbance at 405 nm [18,19].

Inhibition kinetics

In the kinetic analysis, the reaction mixture consisting of four or five different concentrations of *p*NPP (0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mM) used as a PTP1B substrate in the absence or presence of compounds **1** and **2**. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of PTP1B were determined by Eadie-Hofstee plots using a GraphPad Prism[®] 4 program (GraphPad Software Inc., USA).

Results and discussion

Recent evidence suggests a key role for PTPs in the control of insulin signaling and metabolism [1,2]. Of the PTPs, PTP1B appears to be an attractive therapeutic target because the PTP1B levels in muscles and adipose tissues correlate with the degree of insulin resistance in subjects with diabetes and obesity [1,2]. Furthermore, recent genetic evidence has supported the view that PTP1B inhibitors could be potential agents for the treatment of type 2 diabetes and obesity [5,7].

As part of our ongoing study to search for natural PTP1B inhibitors from plants, the aerial part of *S. glabrescens* were chosen for detailed investigation, since the methanol extract was found to inhibit PTP1B at 30 μ g/mL [11]. Bioassay-guided fractionation on the MeOH extract led to the isolation of three diterpenes **1**, **2** and **3** (Figure 1). The structures of the isolated compounds were identified as *ent*-16 β H,17-isobutyryloxy-kauran-19-oic acid (**1**), *ent*-16 β H,17-acetoxy-18-isobutyryloxy-kauran-19-oic acid (**2**) and *ent*-16 β H,17-hydroxy-kauran-19-oic acid (**3**) by analyses of MS and NMR data, and comparison with those reported in the literature [14,20–23]. Although compound **1** was previously reported by Murakami

et al. for the first time [20], only the chemical structure of its alkaline hydrolysate was confirmed by the analysis of $^1\text{H-NMR}$ data. In this study, the structure was more precisely elucidated by $^{13}\text{C-NMR}$, DEPT and 2D-NMR as indicated in Materials and Methods.

The three isolates were tested for their inhibitory activity against PTP1B, and the results are shown in Table I. Of the isolates, *ent*-16 β H,17-isobutyryloxy-kauran-19-oic acid (**1**) which has an isobutyryloxy moiety at C-17 of the kaurane-type structure exhibited the strongest inhibitory activity ($\text{IC}_{50} = 8.7 \pm 0.9 \mu\text{M}$). However, compound **3** ($\text{IC}_{50} > 200 \mu\text{M}$) with a hydrolyzed isobutyryloxy group at C-17 of **1** exhibited significantly lower activity than **1**. This suggests that an isobutyryloxy moiety at C-17 of the kaurane diterpene is essential for activity. Compound **2** substituted with an acetoxy and an isobutyryloxy group at C-17 and C-18, respectively, inhibited PTP1B activity in a dose-dependent manner with an IC_{50} value of $30.6 \pm 2.1 \mu\text{M}$, which was less effective than **1**. A known phosphatase inhibitor, RK-682 ($\text{IC}_{50} = 3.5 \pm 0.4 \mu\text{M}$) [24], was used as a positive control in this assay. In addition, the isolates were assayed for their inhibitory effects on other types of protein phosphatases, and it was shown that the compounds had no inhibitory effects toward VHR and PP1 at levels up to $200 \mu\text{M}$. To elucidate the inhibition mode of **1** and **2** on the activity of PTP1B, kinetic analyses were performed with different concentrations of substrate. As shown in Figure 2, the mechanisms of inhibition by the two compounds were determined using an Eadie-Hofstee plot. When *p*NPP was used as substrate, both **1** and **2** decreased the V_{max} values, but did not alter the K_m values of PTP1B (Figure 2). Accordingly, **1** and **2** were found to be non-competitive inhibitors with K_i values of 9.1 and $31.8 \mu\text{M}$, respectively. This indicates that they may bind to the enzyme-substrate complex or interact with an allosteric site distinct from the active site of the enzyme [25]. Kaurane diterpenes have been reported to inhibit TNF- α secretion in trypsin-stimulated HMC-1 cells [22], to reduce nitric oxide and prostaglandin E_2 production in LPS-induced

Table I. Comparison of the inhibitory activity of compounds **1**–**3** isolated from *S. glabrescens* against PTP1B, VHR and PP1.

| Compounds | IC_{50} (μM) ^a | | |
|---------------------|---|----------------|-----------------|
| | PTP1B | VHR DS-PTP | PP1 |
| 1 | 8.7 ± 0.9 | >200 | >200 |
| 2 | 30.6 ± 2.1 | >200 | >200 |
| 3 | >200 | >200 | >200 |
| RK-682 ^b | 3.5 ± 0.4 | 20.0 ± 1.7 | NT ^c |

^a IC_{50} values were determined by regression analyses and expressed as mean \pm SD of three replicates. ^bPositive control [24]. ^cNot tested.

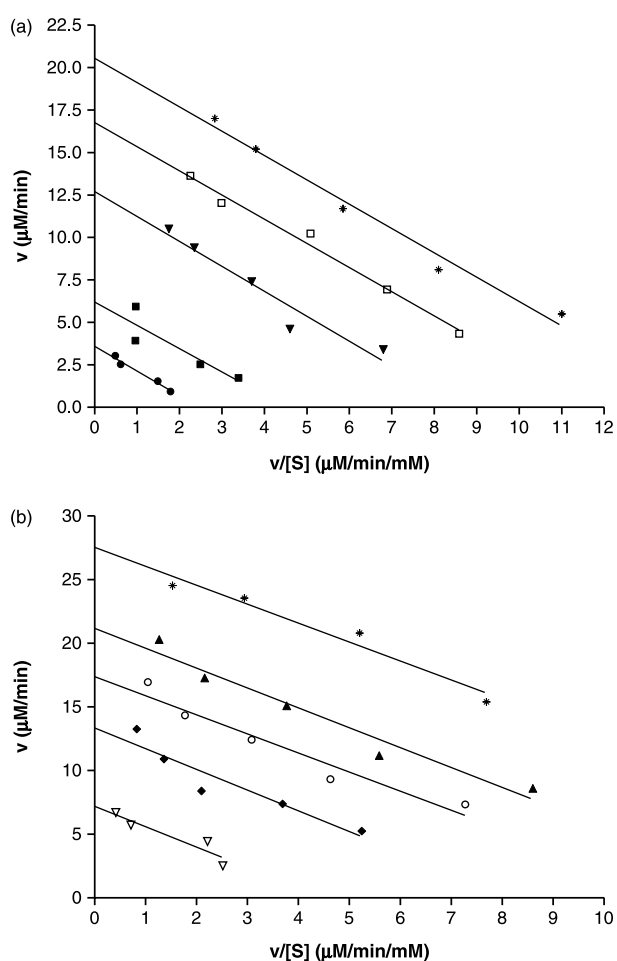


Figure 2. Inhibition kinetics of compounds **1** and **2**. Panel **a** shows an Eadie-Hofstee plot of the inhibitory effect of compound **1** on PTP1B-catalyzed hydrolysis of *p*NPP. Data are expressed as mean initial velocity for $n = 3$ replicates at each substrate concentration. Symbols: (*) $0 \mu\text{M}$, (\square) $3 \mu\text{M}$, (\blacktriangledown) $7 \mu\text{M}$, (\blacksquare) $15 \mu\text{M}$, (\bullet) $30 \mu\text{M}$ compound **1**. Panel **b** shows an Eadie-Hofstee plot of the inhibitory effect of compound **2** on PTP1B. Data are expressed as mean initial velocity for $n = 3$ replicates at each substrate concentration. Symbols: (*) $0 \mu\text{M}$, (\blacktriangle) $12 \mu\text{M}$, (\circ) $21 \mu\text{M}$, (\blacklozenge) $35 \mu\text{M}$, (∇) $60 \mu\text{M}$ compound **2**.

RAW264.7 cells [26], and to induce caspase-dependent apoptotic or necrotic cell death [27]. Besides, this type diterpenes have been described to have anti-HIV, anti-platelet aggregation and cytotoxic activities [28–30]. However, to our knowledge, PTP1B inhibitory activity of these diterpenes is being reported here for the first time in this study.

In conclusion, bioassay-guided investigation of the methanol extract of the aerial part of *S. glabrescens* afforded two active kaurane diterpenes, *ent*-16 β H, 17-isobutyryloxy-kauran-19-oic acid (**1**) and *ent*-16 β H, 17-acetoxy-18-isobutyryloxy-kauran-19-oic acid (**2**), along with *ent*-16 β H,17-hydroxy-kauran-19-oic acid (**3**). The present study indicates that the isolates **1** and **2** are non-competitive inhibitors of PTP1B. Because PTP1B is an intracellular enzyme, a further study to confirm their cellular effects is under progress.

Acknowledgements

This research was supported in part by the grants from the Plant Diversity Research Center of 21st Frontier Research Program (PF0320903-00), the Molecular and Cellular BioDiscovery Research Program (M1-0311-00-0023) of the Ministry of Science and Technology of Korea, and from KRIBB Research Initiative Program.

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