Bioactivity-Guided Isolation of 1,2,3,4,6-Penta-*O*-galloyl-D-glucopyranose from *Paeonia lactiflora* Roots As a PTP1B Inhibitor

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The inhibition of protein tyrosine phosphatase 1B (PTP1B) is of substantial interest for the treatment of type-2 diabetes mellitus. Using an in vitro enzyme assay with human recombinant PTP1B 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (**1**) was isolated from the roots of *Paeonia lactiflora* as an inhibitor of PTP1B, with an IC_{50} value of 4.8 μM. Additionally, **1** was shown to act as an insulin sensitizer in human hepatoma cells (HCC-1.2) at a concentration of 10 μM. Thus, a potential new mechanism of action is provided explaining the antidiabetic properties of *P. lactiflora*.

Treatment and prevention of type-2 diabetes mellitus (T2DM) are major challenges of the present age. According to the International Diabetes Federation, 285 million people worldwide will suffer from diabetes mellitus in 2010. This number will increase to 439 million by 2030.[1] The majority of these patients will be affected by T2DM. Insulin resistance due to inadequate insulin action generally precedes the onset of T2DM and can be linked causally to aberrantly high levels of visceral fat tissue. Notably, abdominal obesity and insulin resistance are also characteristics of this metabolic syndrome.[2] Currently used insulin sensitizers still have many side effects including weight gain.[3] An ideal treatment for T2DM would result in the alleviation of the insulin-resistant state combined with weight loss. A drug target offering both of these approaches is protein tyrosine phosphatase 1B (PTP1B).[4] PTP1B is a negative regulator of insulin and leptin signaling, and its inhibition results in increased insulin sensitivity as well as higher energy expenditure, less food intake, and less weight gain.[5−7]

The roots of *Paeonia lactiflora* Pall. or *Paeonia suffruticosa* Andrews (Ranunculaceae) are used in Traditional Chinese Medicine (TCM) as "Paeoniae radix rubra" (as deduced from the reddish color of the dried root bark) or "Cortex Moutan". This is used commonly in TCM for amenorrhea and dysmenorrhea,[8] inflammation, spasms,[9] and high blood pressure.[10] Moreover, this is found in formulations for the treatment of symptoms related to metabolic syndrome and T2DM.[11] An aqueous extract of the root bark of *P. suffruticosa* was reported to inhibit intestinal glucose absorption and to promote glucose uptake in cell models including the intestinal brush border membrane vesicles, hepatocytes, and adipocytes.[12] However, knowledge of the active components in these *Paeonia* species and their respective targets is limited. In particular, there is no report investigating the potential insulin- sensitizing action of *P. lactiflora* by means of PTP1B inhibition. Therefore, the first goal of the present study was to investigate if a methanol extract of *P. lactiflora* roots inhibits PTP1B in an enzyme-based in vitro assay. In a second step, the active compound was isolated by bioassay-guided fractionation and its activity confirmed in a cell-based model.

Figure 1 shows that 10 μg/mL of the crude methanol extract of *P. lactiflora* roots reduced human recombinant PTP1B residual activity to 30%, compared to 100% enzyme activity in the DMSO-treated control group (Co). A known natural product-derived PTP1B inhibitor, ursolic acid (UA),[13] and a pan-protein tyrosine phosphatase (PTP) inhibitor, sodium orthovanadate (SOV),[14] were used as positive controls. Having proven activity of the crude methanol extract against PTP1B, the extract was separated by bioguided fractionation.

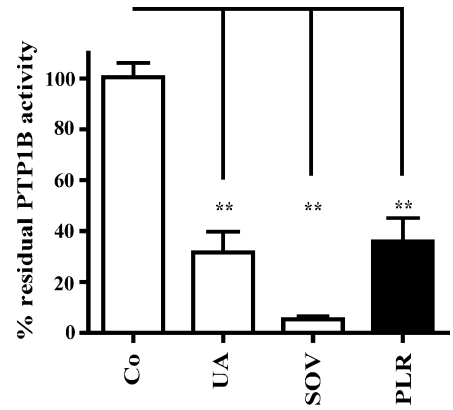
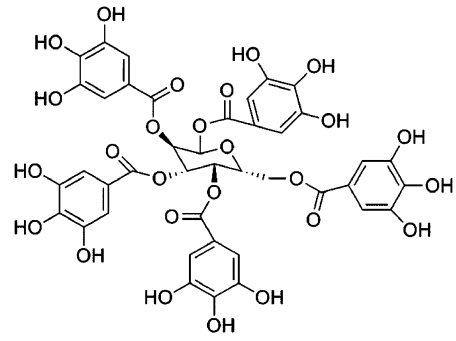


Figure 1. The methanol extract of *P. lactiflora* roots (PLR) inhibits PTP1B in vitro. The crude PLR extract (10 μg/mL) was subjected to testing in a PTP1B enzymatic assay. Ursolic acid (UA, 30 μM) and sodium orthovanadate (SOV, 3 μM) were used as positive controls. All compounds were dissolved in DMSO at a final concentration of 1%. The DMSO control (Co) was set to 100% enzyme activity [$^{**}p < 0.01$ ($n = 3$)].



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The crude extract was fractionated using column chromatography with methanol as mobile phase and Sephadex LH-20 as stationary phase. The resulting fractions were tested for their inhibitory activity on PTP1B. The most potent inhibited PTP1B to 5.86% residual

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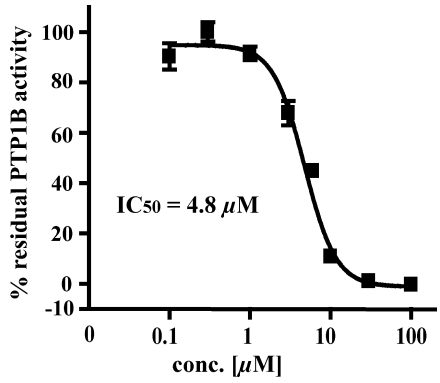

Figure 2. Compound **1** inhibits PTP1B in a dose-dependent manner. Different concentrations of **1** (0.1−100 μM) were evaluated in the in vitro PTP1B enzyme assay to establish a sigmoidal dose−response curve by nonlinear regression ($n = 3$).

activity, which was 5 times more potent than the crude extract tested at the same concentration (10 μg/mL) (Supporting Information, Figure S1). Thus, this fraction was selected for further Lobar purification on C_{18} material, leading to the isolation of a pure compound, which was identified by HPLC-DAD, MS, and 1D and 2D NMR experiments and subsequent comparison with published data[15−18] as 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (**1**). Quantification of **1** in the roots of *P. lactiflora* by HPLC-DAD showed a content of 0.88% w/w in the dried plant material.

Among other compounds isolated in a similar fashion (e.g., paeonol, paeoniflorin; Supporting Information, Figure S2), **1** was identified as the most active compound from *P. lactiflora* roots with regard to PTP1B inhibition. The enzyme-based in vitro assay showed a dose-dependent inhibition of PTP1B by **1** (Figure 2), with an apparent IC_{50} value of 4.8 μM.

To verify the PTP1B-inhibitory effect in a cell-based system, the human hepatoma cell line HCC-1.2 was used. Usually, upon insulin stimulation the insulin receptor (IR) is autophosphorylated and thereby triggers signal transduction. The major inhibitor of this initial phosphorylation is PTP1B, which dephosphorylates the IR at the tyrosine residues 1146, 1150, and 1151, thereby stopping the insulin signaling cascade.[5] Therefore, the phosphorylation status of the IR was examined at the above-mentioned sites in the presence and absence of **1** and known PTP inhibitors after stimulation with insulin. As shown in Figure 3, stimulation of starved HCC-1.2 cells with 10 nM insulin for 5 min resulted in an increase in IR phosphorylation in DMSO-treated control cells. In the presence of 10 μM **1**, this increase was augmented significantly. This demonstrated clearly a marked insulin-sensitizing effect of **1** in living cells at a concentration range similar to its IC_{50} value in the enzymatic in vitro assay. Moreover, the insulin-sensitizing effect of **1** was more potent than that of SOV (9 μM) and the crude methanol extract of *P. lactiflora* roots (10 μg/mL), respectively.

Inhibiting a protein tyrosine phosphatase, such as PTP1B, poses the question of specificity due to the high structural similarity of the catalytic center throughout the family of tyrosine phosphatases.[19] Insulin receptor signaling is regulated negatively not only by PTP1B but also by other phosphatases such as T-cell protein tyrosine phosphatase (TCPTP), leukocyte antigen-related protein tyrosine phosphatase (LAR), and SH2-domain-containing phosphatase (SHP)-2. Although their inhibition can influence positively insulin signaling, these phosphatases play important roles in immunity, neuronal development, and growth-factor signaling, and optimally should remain unaffected by an identified PTP1B inhibitor.[20] Therefore, compound **1** was tested against TCPTP, LAR, and SHP-2. In respective enzyme-based in vitro assays, **1** inhibited TCPTP, which shares 74% structural similarity with PTP1B,[21] with an IC_{50} of 0.07 μM. However, **1** did not inhibit LAR and SHP-2 at concentrations below 50 μM. Although the levels of TCPTP in HCC-1.2 cells

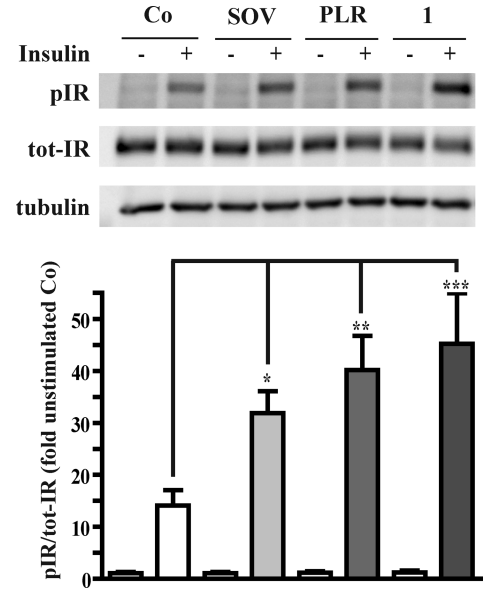

Figure 3. Effect of the methanol extract of the roots of *P. lactiflora* (PLR) and **1** on IR phosphorylation. Human hepatocytes were starved and pretreated for 2 h with the extract (PLR, 10 μg/mL), **1** (10 μM), or the controls, DMSO (0.1%) or SOV (9 μM), prior to insulin stimulation (10 nM) for 5 min. Total cell lysates were subjected to western blot analysis for phospho-IR, tot-IR, and tubulin. The bar graph depicts compiled data of densitometric analysis of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; one way ANOVA and Bonferroni post-test).

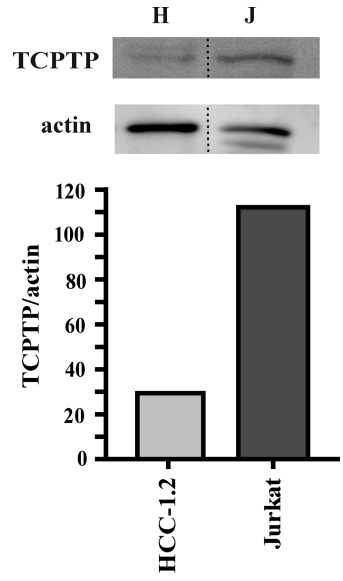

Figure 4. Cell lysates of HCC-1.2 display low levels of TCPTP. HCC-1.2 hepatocytes (H) and Jurkat T-lymphocytes (J) were analyzed by western blot for their levels of TCPTP. Actin was taken as loading control.

(H) are rather low when compared, for example, to Jurkat T-lymphocytes (J) (Figure 4), and 5 times lower than PTP1B levels (data not shown), the present data cannot rule out that the effect of **1** on IR phosphorylation in HCC-1.2 cells is due to the simultaneous inhibition of PTP1B and TCPTP. The problem of inseparable inhibition of both enzymes has been encountered by most research groups aiming to find selective PTP1B inhibitors.[22] Although concerns were raised against TCPTP inhibition due to a massive inflammatory phenotype observed in TCPTP knockout mice,[23] a recent study found no abnormalities in mice having only one copy

of PTP1B and TCPTP. Therefore, incomplete inhibition of both PTP1B and TCPTP by small-molecule inhibitors may still be a promising approach against diseases such as obesity and T2DM.[24]

Overall, in this study an important new finding has been made that explains the antidiabetic activity of *P. lactiflora* roots at the molecular level. Its PTP inhibitory activity in crude root extracts has been revealed, with **1** identified as a bioactive compound by bioassay-guided isolation. PTP inhibition and insulin sensitization by **1** were confirmed in an enzyme-based in vitro assay as well as in a cell-based system using HCC-1.2 cells. To the best of our knowledge, this is the first report of the PTP-inhibitory activity of **1**.

Experimental Section

General Experimental Procedures. HPLC-DAD analyses were performed on a Shimadzu UFLC-XR instrument (Kyoto, Japan), using water (A) and acetonitrile (CH_3CN) (B), both containing 0.3% formic acid (FA), as mobile phase, and a Synergi MAX-RP 100A (2.5 μm, 50 × 3.0 mm; Phenomenex, Torrance, CA) column as stationary phase.

For screening purposes a steep solvent gradient was used (from 5% B in 8 min to 40% B, then in another 4 min to 100% B, and held at this composition for 3 min). For quantification of **1** the following gradient was employed: from 1% B in 7 min to 25% B, in 2 min to 100% B, which was held for 3 additional min. In all experiments, the flow rate, column temperature, and sample volume were kept at 1 mL/min, 25 °C, and 5 μL, respectively. The detection wavelength was 210 nm.

Plant Material. "Paeoniae radix rubra" (purchased December 2008; lot: 810117, 500 g) was obtained from Plantasia (Oberndorf, Austria). It was identified as the roots of *Paeonia lactiflora* Pall. by one of the authors (D.S.) using macroscopic and microscopic procedures described in the respective monograph in the German translation of the Pharmacopoeia of the People's Republic of China (2005).[25] Voucher specimens (08-12-PRR-PL-01) are deposited at the herbarium of the Institute of Pharmacy, University of Innsbruck, Austria.

Extraction and Isolation. About 300 g of the ground material was extracted three times with 500 mL of MeOH for 1 h each in an ultrasonic bath. The supernatant was filtered through a filter paper, combined, and evaporated to dryness. The initial fractionation of the extract was performed on Sephadex LH-20 using MeOH as mobile phase. The collected fractions were assayed by TLC (silica gel 60 F_{254} plates; Merck, Darmstadt, Germany), using a mixture of toluene, ethyl acetate, formic acid, ethanol, and MeOH (4:3:1:2:1) as mobile phase. Results were evaluated at 254 and 360 nm and by spraying with vanillin/sulfuric acid reagent.

Further purification of the most active fraction was conducted using a preparative Lobar system (LiChropep RP-18, 240 × 10 mm, 40−63 μm; Merck, Darmstadt, Germany). Water (A) and CH_3CN (B), with 0.3% formic acid each, were used as mobile phase (gradient conditions: 5% B for 4 h, to 100% B, flow rate: 1 mL/min). Monitoring the separation at 210 nm with an Agilent HP UV detector series 1100 (Böblingen, Germany) led to the isolation of **1**.

Compound **1** was obtained as a pale brown, amorphous powder with UV maxima at 211, 231, and 278 nm. For MS analysis, **1** was diluted in MeOH and infused into a Bruker Esquire 3000plus mass spectrometer (Bremen, Germany) by syringe pump; spectra were recorded in the negative ESI mode and showed an *m/z* value of 939.6. NMR spectra of the isolated compound in deuterated methanol (CD_3OD) were recorded on a Bruker Avance 300 NMR spectrometer (Rheinstetten, Germany). Identification was achieved by the comparison of the spectroscopic data obtained with those in the literature.[15−18]

For quantitation purposes, 0.5 g of the ground plant material was extracted three times with 3 mL of MeOH by sonication (10 min each). After centrifugation, the extracts were combined in a 10 mL volumetric flask, and the flask was filled with extraction solvent. As a control for exhaustive extraction, the sample was extracted a fourth time and the resulting extract was assayed by HPLC. No remains of **1** were found.

Reagents. PTP1B enzyme was purchased from R&D Systems (Minneapolis, MN). TCPTP, LAR, and SHP-2 were from Enzo Life Science (Farmingdale, NY). DMSO, MOPS, DTT, sodium orthovanadate, and ursolic acid were from Sigma (St. Louis, MO). Human hepatoma cells (HCC-1.2) were a gift from M. Eisenbauer of the Institute of Cancer Research at the Medical University, Vienna, Austria.[26] RPMI 1640 was purchased from Lonza (Basel, Switzerland), and FBS from Gibco/Invitrogen (Carlsbad, CA). Complete protease inhibitor was from Roche Diagnostics (Penzberg, Germany), and PVDF membranes were from Bio-Rad Laboratories (Hercules, CA). The following antibodies were used: against the phosphorylated insulin receptor (Y1158, 1162, 1163) from Sigma (St. Louis, MO), anti-total insulin receptor β from New England Biolabs (Beverly, MA), anti-TCPTP from R&D Systems (Minneapolis, MN), and anti-tubulin and anti-actin from Santa Cruz (Santa Cruz, CA). Secondary antibodies: goat anti-rabbit IgG was from New England Biolabs (Beverly, MA) and goat anti-mouse IgG from Upstate (Billerica, MA).

All solvents and additives used for analysis and isolation (CH_3CN, formic acid) were of HPLC grade and purchased from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius Arium 611 UV water purification system (Göttingen, Germany). TLC solvents and reagents (toluene, ethanol, MeOH, DCM, ethyl acetate, sulfuric acid, vanillin) came from VWR (Vienna, Austria).

In Vitro Enzyme Assay. PTP1B, TCPTP, LAR, and SHP-2 activity was determined using 2 mM pNPP in 50 mM MOPS, pH 6.5, as a substrate. The known PTP inhibitors, sodium orthovanadate (SOV, 10−1000 μM) and ursolic acid (UA, 30 μM), were used as positive controls. Fractions and compounds were first dissolved in 100% DMSO and diluted to 1% DMSO in 100 μL overall assay volume. The inhibitory action toward the phosphatases was measured in 96-well format in the presence of either 1 mM (PTP1B, LAR, SHP-2) or 10 mM DTT (TCPTP). The reactions were subjected to kinetic absorbance readings at 405 nm for 30 min in a Tecan/Sunrise photometer. Subsequently, the reaction was stopped with 10 M NaOH, and the absorbance was again measured at 405 nm.

Cell Culture. Human hepatoma cells (HCC-1.2) were maintained in RPMI 1640 medium supplemented with 10% FBS, glutamine (584 mg/mL), and penicillin (100 U/mL)/streptomycin (100 μg/mL), under 5% CO_2 environment at 37 °C.

SDS-PAGE and Western Blot Analysis. HCC-1.2 cells were grown in six-well plates, serum-starved, and preincubated with plant extracts or compound **1** dissolved in DMSO for 2 h. For cell-based assays, DMSO did not exceed a final concentration of 0.1%. Then, cells were stimulated for 5 min with 10 nM insulin. The plates were then put on ice and washed with ice-cold PBS. RIPA lysis buffer (Tris/HCl 50 mM, pH 7.4; NaCl 500 mM, NP40 5.044 mM, Na-deoxycholate 12.06 mM, SDS 3.47 mM, NaN_3 7.7 mM, Complete 4%, PMSF 1 mM, NaF 1 mM, $NaVO_3$ 1 mM) was added to each well, and the cells were scraped into small reaction tubes. Afterward, samples were sonicated with 10 pulses of 0.5 s and centrifuged at 4 °C and 13 000 rpm for 15 min. Protein determination, SDS-PAGE, and western blotting were performed as described previously.[27] The membranes obtained were blocked with 5% BSA in TBS-T and then incubated with the respective antibody overnight. As detection system, enhanced chemiluminescence was used.

Statistical Analysis. At least three independent experiments were performed. Data are presented as means ± standard error of the mean. If not stated otherwise, statistical differences were analyzed with GraphPad Prism using ANOVA and Dunnett's multiple comparison post-test. $p < 0.05$ was considered significant (*), $p < 0.01$ as highly significant (**).

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Supporting Information Available: PTP1B enzyme assay results from subfractions of the methanol extract of *P. lactiflora* roots as well as a HPLC trace of the crude methanol extract of *P. lactiflora* are available free of charge via the Internet at http://pubs.acs.org.

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