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Protein tyrosine phosphatase 1B inhibitors isolated from *Morus bombycis*

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ABSTRACT

Bioassay-guided fractionation of the chloroform-soluble fraction of *Morus bombycis*, using an in vitro PTP1B inhibitory assay led to the identification of three 2-arylbenzofurans, albafuran A (**1**), mulberrofuran W (**2**) and mulberrofuran D (**6**), along with three chalcone-derived Diels–Alder products, kuwanon J (**3**), kuwanon R (**4**), and kuwanon V (**5**). Compounds **1**–**6** showed remarkable inhibitory activity against PTP1B with IC_{50} values ranging from 2.7 to 13.8 μM. Inhibition kinetics were analyzed by Lineweaver–Burk plots, which suggested that compounds **1**–**6** inhibited PTP1B in a mixed-type manner. The present results indicate that the respective lipophilic and hydroxyl groups of 2-arylbenzofurans and chalcone-derived Diels–Alder products play an important role in inhibition of PTP1B.

The world-wide incidence of type 2 diabetes mellitus (T2DM) and obesity in the population is increasing at an alarming rate. What was previously observed primarily in adult populations and associated with a sedentary lifestyle has now also become a medical problem in children.[1,2] The relationship between obesity and T2DM has a polygenetic component and is associated with insulin resistance.[3] Insulin resistance is evident in many tissues that are important for glucose homeostasis, including muscle, liver and, more recently, in fat and at the level of the central nervous system. Metabolic insulin signal transduction occurs through activation of the insulin receptor (IR), including autophosphorylation of tyrosine (Tyr) residues in the insulin receptor activation loop.[4] Several protein tyrosine phosphatases (PTPs), such as receptor protein tyrosine phosphatase (rPTP-α), leukocyte antigen-related tyrosine phosphatase (LAR), SH2-domain-containing phosphotyrosine phosphatase (SHP2), and protein tyrosine phosphatase 1B (PTP1B) have been implicated in the dephosphorylation of the IR.[5] There is substantial evidence supporting PTP1B as the critical PTP controlling insulin signaling pathway. PTP1B seems to be a key regulator of insulin receptor activity, acting at the insulin receptor and at downstream signaling insulin receptor substrate proteins.[6] Therefore, it has been suggested that compounds that reduce PTP1B activity or expression levels could not only be used for the treatment of type 2 diabetes, but also obesity.

Mori Cortex Radicis, the root cortex of some *Morus* species, has been used in traditional medicine for treating diabetes, as well as in diuretic, expectorant, and laxative agents.[7] Chemical and pharmacological investigations of *Morus* sp. have resulted in the isolation of a series of prenylated flavonoids, benzofurans, and other phenolic compounds,[8–10] which are cytotoxic agents that inhibit COX-1, COX-2, NO production, and HIF1.[11–13] During the course of our program to screen for PTP1B inhibitors from plants, it was found that the $CHCl_3$-soluble fraction of the root cortexes of *Morus bombycis* exhibited strong inhibitory activity (>80% inhibition at 30 μg/mL). Since it has been reported that PTP1B inhibitors may offer a potential treatment for obesity, diabetes, and related diseases, a phytochemical investigation was carried out. This Letter describes the isolation and characterization of six compounds from a $CHCl_3$ extract of *M. bombycis*, as well as the evaluation of the inhibitory activity of isolated compounds **1**–**6** against PTP1B.

The dried root cortex of *M. bombycis* (2.5 kg) was collected in August 2007, from Vinh Phuc province, Vietnam, and authenticated by Prof. Pham Thanh Ky, Department of Pharmacognosy, Hanoi College of Pharmacy, Vietnam where the voucher specimens were deposited.

The dried root bark of *M. bombycis* (2.5 kg) was powdered and extracted with MeOH (3 × 5 L) and then the MeOH extract was concentrated. The residue (72 g) was suspended in H_2O (1 L) and partitioned with *n*-hexane (3 × 0.5 L) and $CHCl_3$ (3 × 0.5 L), successively. The $CHCl_3$-soluble extract was concentrated in vacuo to give

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a brown solid (15.5 g), which was then chromatographed on a silica gel column using a $CHCl_3$–MeOH gradient as the mobile phase to afford two fractions, C1 and C2. Fraction C2 was subjected to preparative HPLC (YMC Pack Pro C18, 250 × 10 mm, 60% ACN in H_2O, flow rate 10 mL/min) to afford **1** (7.5 mg), **2** (7.2 mg), **3** (80 mg), and **4** (8.7 mg). Fraction C1 was chromatographed on a silica gel column eluted with an *n*-hexane–EtOAc gradient to give five subfractions, C1.1–C1.5. Purification of fractions C1.4 and C1.5 by preparative HPLC (YMC Pack Pro C18, 250 × 10 mm, 60% CH_3CN in H_2O, flow rate 10 mL/min) gave **5** (41 mg) and **6** (160 mg), respectively.

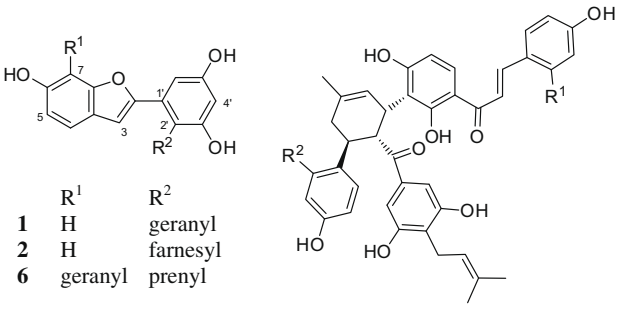
Compound **1** was obtained as yellow powder. The UV spectrum exhibited maximal absorptions at λ_{max} 219, 312, and 335 nm, indicating a 2-arylbenzofuran-type skeleton.[14] The ^1H and ^{13}C NMR spectra showed characteristic signals of the 3′,5′,6-trihydroxy-2-arylbenzofuran substructure along with the addition of a geranyl moiety at C-7. The ESI-MS data displayed molecular ion peaks at *m*/*z* 377.2 $[M-H]^-$, 3′,5′,6-trihydroxy-2-arylbenzofuran fragment at *m*/*z* 241.3, and *m*/*z* 136.3 of the geranyl fragment. The structure of compound **1** was identified as albafuran A by comparison with previous reported spectral data.[15]

Compound **2** was obtained as yellow powder, and its UV spectrum also displayed a maximal absorption pattern characteristic of a 2-arylbenzofuran skeleton. The ^1H and ^{13}C NMR data of **2** were very similar to those of **1**, with the exception of signals representative of a farnesyl group. ESI-MS data from **2** showed ion peaks at *m*/*z* 445.4, 241.3, and 204.3 corresponding to the parent ion, the 2-arylbenzofuran moiety, and the farnesyl fragment, respectively. Compound **2** was identified as mulberrofuran W by comparison with previously published data.[16]

Compounds **3**–**5** were isolated as yellow powders, with $[\alpha]_D^{25}$ +125.0, +65.8, and +110.0, respectively. Its UV spectrum showed maximal absorption at λ_{max} 225, 295, and 380 nm suggesting a chalcone skeleton. The ^1H NMR spectra of **3** revealed the chalcone skeleton along with a methylcyclohexenyl moiety. The conformation of the methylcyclohexene ring was determined to be of the *cis*–*trans* type based on the coupling constant around 5–6 Hz between H-3″/H-4″ and H-4″/H-5″. The ^1H NMR spectra indicated the location of the OH groups at C-2 and C-16″ for **3**, and C-16″ for **4**, were different from **5**. The ESI-MS molecular ion peaks at *m*/*z* 677.4, 661.5, and 645.5 $[M-H]^-$ of **3**–**5**, respectively, confirmed the existence of different hydroxylated units. Structures of **3**–**5** were identified as chalcone-derived Diels–Alder-type compounds as kuwanon J, R, and V, respectively, by comparison with previously reported data for the *Morus* genus.[17]

Compound **6** was obtained as a yellow powder, and its UV spectrum showed maximal absorptions at λ_{max} 245, 269, and 315 nm of 2-arylbenzofuran derivative. The ^1H and ^{13}C NMR data of **6** were closely comparable with those of **1**, except for the signals indicating the location of a prenyl group at C-2′. This was supported by the long-range correlations observed in the HMBC spectrum. The structure of **6** was identified as mulberrofuran D by comparison with previous literature data.[16]

All isolated compounds **1**–**6** (Fig. 1), together with RK-682 (3-hexadecanoyl-5-hydroxymethyl tetronic acid) and ursolic acid as positive controls,[18] were examined for their inhibitory effects on PTP1B activity in an in vitro assay. 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. To each well of a 96-well plate (final volume: 200 μL), 2 mM *p*-NPP and PTP1B (0.1 μg) were added 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 by the addition of 10 M NaOH. The amount of produced *p*-nitro phenol was estimated by measuring the absorbance at 405 nm.



	R^1	R^2
1	H	geranyl
2	H	farnesyl
6	geranyl	prenyl

	R^1	R^2
3	OH	OH
4	H	OH
5	H	H

Figure 1. Structures of isolated compounds **1**–**6**.

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.[19] The results are presented in Table 1. It appears that isolated compounds **1**–**6** exhibited strong inhibition of PTP1B activity with IC_{50} values ranging from 2.7 to 13.8 μM. The positive controls, RK-682 and ursolic acid (IC_{50} values of 4.5 and 3.9 μM, respectively) are used as known phosphatase inhibitors.

Inhibition kinetics studies were carried out in the absence and presence of active compounds with various concentrations of *p*-NNP (0.5, 1.0, 2.0, 5.0, and 10.0 mM) as substrate. The initial rate was determined on the basis of the rate of increase in absorbance at 405 nm. The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) of PTP1B were determined by Lineweaver–Burk Plot analysis for competitive inhibition and the intercept on vertical axis for noncompetitive inhibition. Under the conditions employed in this present investigation, the hydrolysis of *p*-NPP catalyzed by PTP1B follows Michaelis–Menten kinetics. The kinetic parameters K_m and V_{max} for hydrolysis obtained from an analysis of Lineweaver–Burk plots are listed in Table 1. As illustrated in Figure 2, the reciprocal plots of the isolated compounds are intersected to the left of the $1/V$ axis, suggesting compounds **1**–**6** inhibited PTP1B in a mixed-type manner. This indicates that they may both displace at the active site and an additional binding site of the PTP1B enzyme.

The active site of PTP1B contains a structural motif common to many PTPs. The base of the catalytic site is defined by the 214–221 PTP signature motif, a loop of eight amino-acid residues that forms a rigid, cradle-like structure that coordinates to the aryl-phosphate moiety of the substrate. The substrate-binding and catalytic mechanism of PTP1B has been explored using hydrophobic, salt-bridge, hydrogen-bonding, and electrostatic interactions, as well as by

Table 1
Inhibition effects of isolated compounds **1**–**6** against PTP1B

Compounds	Protein tyrosine phosphatase[a]			
	IC_{50} (μM)	K_m (mM)	V_{max} (μM/min)	Type of inhibition
1	9.2 ± 0.7	1.25 ± 0.05	6.58 ± 0.18	Mixture
2	2.7 ± 0.3	3.17 ± 0.03	5.49 ± 0.13	Mixture
3	2.7 ± 0.6	2.81 ± 0.08	5.41 ± 0.15	Mixture
4	8.2 ± 0.9	1.64 ± 0.03	5.95 ± 0.14	Mixture
5	13.8 ± 1.1	1.19 ± 0.04	6.71 ± 0.20	Mixture
6	4.3 ± 0.5	2.08 ± 0.02	5.78 ± 0.17	Mixture
RK-682[b]	4.7 ± 0.4	N.D.	N.D.	N.D.
Ursolic acid[b]	3.8 ± 0.3	N.D.	N.D.	N.D.

N.D.: Not determined.
[a] Values present mean ± SD of triplicate experiments.
[b] Positive control.

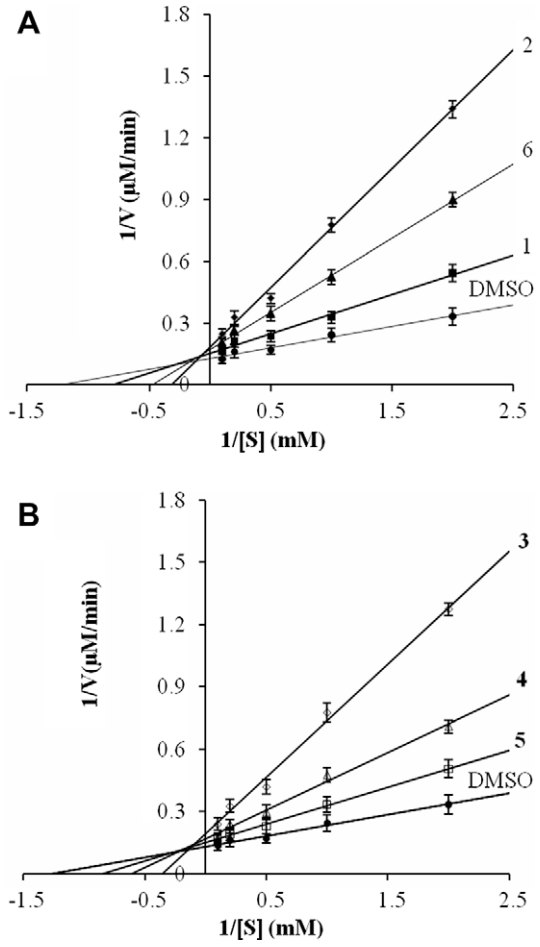


Figure 2. The Lineweaver–Burk plots of PTP1B inhibitory activity with *p*-NPP as substrate, in the presence of **1–6**. Symbols: (A)—**1**, (■) 5 μM; **2**, (♦) 5 μM; **6,** (▲) 5 μM and (B)—**3**, (◇) 5 μM, **4** (Δ) 5 μM, **5** (□) **5** μM, and DMSO (●) as control. The data represent the mean ± SD of triplicate difference experiment.

several N-termini that are capable of binding to an acidic site.[1] The need to have compounds with improved physico-chemical properties that are closely related to PTP1B has become a central issue that needs to be addressed in the development of PTP inhibitors. The isolated compounds **1–6** showed significantly differences in with respect to their biological activities and chemical structures. The 3′,5′,6-trihydroxy-2-arylbenzofuran derivatives, albafuran A (**1**), which possesses a geranyl group at C-2′, showed a strong inhibitory effect against PTP1B. Mulberrofuran W (**6**), which contains an additional prenyl unit at C-2′, was more inhibitory against PTP1B activity than **1**. The former had an IC_{50} value of 4.3 μM, while the latter had an IC_{50} value of 9.2 μM. On the other hand, the farnesyl unit at C-2′ in mulberrofuran W (**2**) seemed to impart the most inhibitory activity against PTP1B, with a low IC_{50} value of 2.7 μM. These results suggest that increasing the lipophilicity and decreasing the charge of aliphatic side chain leads to stronger binding. The prenyl, geranyl, and/or farnesyl moieties found in **1**, **2** and **6** increase cellular permeability, and thus make **1–6** potential treatments for PTP1B and obesity. It appears that the lipophilic group plays a role in direct modulation consisted of Meth258 and Phe52,[20] but the precise reason for this effect remains unclear. The methyl cyclohexene substituted chalcone-derived Diels–Alder-type compounds **3–5** displayed strong inhibition effects against PTP1B with IC_{50} values of 2.7, 9.2, and 13.8 μM, respectively. Interestingly, compounds **3–5** contain the same basic skeleton, only differing in the number of OH function groups. Compound **5**, which possesses OH groups at C-4, C-2′, C-4′, C-11″, C13″, and C-18″, exhibited strong dose-independent inhibition with an IC_{50} value of 13.8 μM, but was less potent than **4**, which contained one more OH group at C-16″, with the IC_{50} value of 9.2 μM. Compound **3** contained a very similar structure **4**, except with an additional OH group at C-2. This additional functionality increased the potency of **3** 3.4-fold with respect to **4**. Hence, the order of inhibitory activity of tested chalcone-derived Diels–Alder-type compounds was **3** > **4** > **5**. The hydroxyl groups not only provided the needed penetration into the active site and likely produced a hydrogen-bonding interaction with the amide backbone of the active-site loop, but also could have protonated or donated a hydrogen bond to the more acidic of inhibitors that extend into the adjacent aryl-phosphate binding site.[1,20] The above results suggest that increasing the number of OH groups in chalcone-derived Diels–Alder-type compound improves potential inhibitory effects against PTP1B.

Previous investigations have shown that a variety of biological activities such as antioxidant, hepatoprotective, anticancer, and as well as inhibitory effects against on tyrosinase, glucosidase, and PTP1B have been reported for the constituents of *Morus* species. Bioassay-guide fractionation of a MeOH extract of *Morus* sp. revealed a class of flavonoids that act as PTP1B inhibitors.[14] This report describes the 2-arylbenzofuran- and chalcone-derived Diels–Alder-type compounds isolated from a $CHCl_3$ fraction of the root bark of *M. bombycis* as new mixed-type inhibitors of PTP1B and thus offers an additional natural PTP1B inhibitor resource for the treatment of diabetes and obesity.

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