Chem. Biol. Lett. 2015, 2(1), 1-5 Article

Chemical Biology
LETTERS

Evaluation of inhibition of protein tyrosine phosphatase 1B by calixarene-based α-ketophosphonic acids

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Received on: 01-Dec-2014 Published on: 5-Feb-2015

ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) is known to be implicated in insulin resistance, and inhibitors of PTP1B were supposed to be useful to regulate insulin and leptin signalling pathways. In this report, calix[4]arene mono- and bis-α-ketophosphonic acids were tested *in vitro* as potential inhibitors of PTP1B. New calix[4]arene-based inhibitors were synthesized through the reaction of corresponding acyl chlorides with triisopropylphosphite followed by dealkylation of α-ketophosphonate diisopropyl ester groups. Inhibiting capacity of the synthesized compounds toward PTP1B was higher than that for other protein tyrosine phosphatases such as TC–PTP, LAR, MEG1, MEG2, and SHP2. Kinetic studies showed that the inhibitor can bind to PTP1B in competition with substrate. According to molecular docking results, phosphonate groups of the inhibitors form hydrogen bonds with amino acid residues in the active site. In addition, macrocyclic platform provides hydrophobic and van der Waals contacts with the enzyme.

Keywords: calix[4]arene; α-ketophosphonic acid; protein tyrosine phosphatase; inhibition; molecular docking

Enzymatic phosphorylation and dephosphorylation of proteins are known to be one of the main regulatory mechanisms of signal transduction. The stages of biochemical phosphoryl transfer reactions are governed by protein kinases and protein phosphatases.[1] Protein tyrosine phosphatases (PTPs) form a family of enzymes, which catalyze the hydrolysis of phosphotyrosine residues in a number of proteins and are implicated in regulation of different cell activities including proliferation, differentiation, and metabolism.[2] Abnormal activities of PTPs have been associated with several diseases such as cancer, leukaemia, Noonan syndrome, and immune disorders.[3-5] PTP1B, the first protein tyrosine phosphatase which was purified and characterized,[6] plays a crucial role in type 2 diabetes and obesity.[7,8] More then 300 million people worldwide are known to have diabetes and this number is growing rapidly. Type 2 diabetes covers more than 90 % of all cases of diabetes mellitus.[9] In addition, several studies have confirmed that PTP1B expression contributes to breast[10] and gastric[11] cancers. Thus, PTP1B can be important medicinal target,[12] and there is a great need for developing the inhibitors which could serve as drugs for treatment of type 2 diabetes or cancer.

Over the past years, a lot of compounds have been designed as inhibitors of PTP1B and other protein tyrosine phosphatases. Among different phosphotyrosine mimics,[13] phosphonic acid derivatives including difluoromethylene phosphonates and α,α-difluoro-β-ketophosphonates displayed potent inhibition.[14-16] We have previously reported that covalent attachment of the phosphonic acid fragments to calix[4]arene scaffold provides an efficient approach to create inhibitors of alkaline phosphatase[17] and *Yersinia* protein tyrosine phosphatase.[18] Calix[4]arenes are considered to be promising scaffolds for designing biologically active compounds,[19-22] and calix[4]arene-based phosphonates were also found to inhibit human protein tyrosine phosphatases.[23] The macrocycles with methylenebisphosphonate or α-hydroxymethylenebisphosphonate groups at the wide rim exhibited micromolar inhibitory activity against PTP1B showing some selectivity over TC-PTP and only modest selectivity over SHP2.[23] In case of α-hydroxymethylphosphonic acid derivatives of calix[4]arene,[24] good inhibition was

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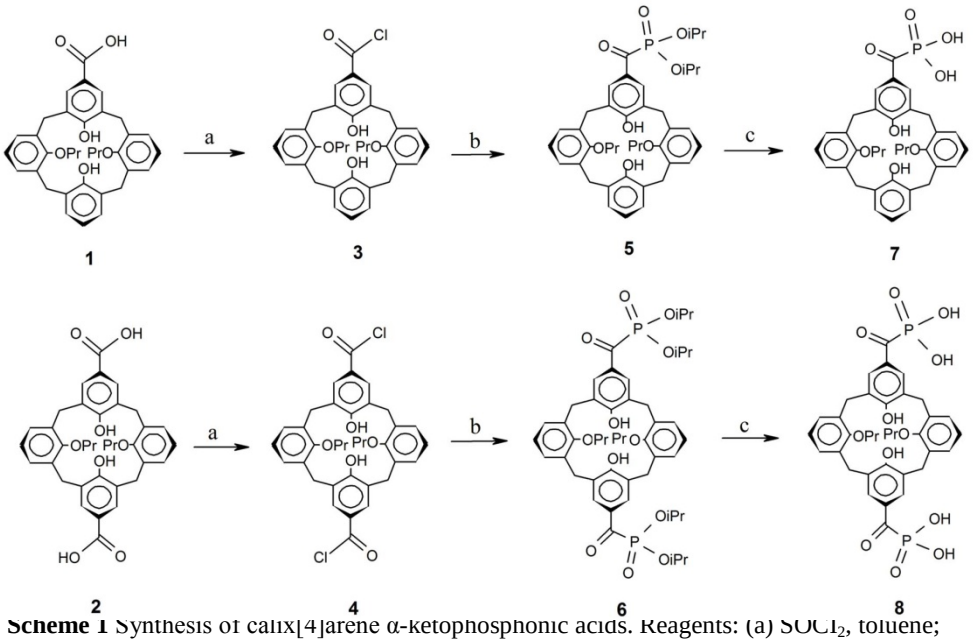
Cite as: *Chem. Biol. Lett., 2015, 2(1), 1-5.*



Scheme 1 Synthesis of calix[4]arene α-ketophosphonic acids. Reagents: (a) $SOCl_2$, toluene; (b) $(iPrO)_3P$, CH_2Cl_2; (c) $(Me_3)_3SiBr$, MeOH.

observed against CD45 with low selectivity over PTP1B.

Extending these approaches we present here new calix[4]arene derivatives bearing one or two α-ketophosphonic acid fragments. It should be noted that the C(O)–P bonds of α-ketophosphonic acids can be stable in aqueous solution, in contrast to their dialkyl esters.[25] In this report, we examined *in vitro* the effects of the calix[4]arene-based α-ketophosphonates on PTP1B and the selectivity of their action in comparison with some other protein tyrosine phosphatases.

The Arbuzov reaction is a general method for the preparation of α-ketophosphonates from acyl chlorides.[26] Calix[4]arene α-ketophopshonic acids **7** and **8** were synthesized in several stages (Scheme 1). The treatment of calix[4]arene carboxylic acids **1** and **2** with thionyl chloride gave corresponding acyl chlorides **3** and **4**[23] which were converted to calix[4]arene α-ketophosphonic acid esters **5** and **6** using reaction with triisopropylphosphite. The standard dealkylation of calix[4]arene derivatives **5** and **6** with Me_3SiBr and subsequent methanolysis of the silyl esters led to isolation of α-ketophosphonic acids **7** and **8** in almost quantitative yields. The ^1H NMR spectra of calix[4]arene acids **7** and **8** display AB spin system doublets of axial and equatorial protons for the methylene bridges ($\Delta\delta$ = 0.75 – 0.85 ppm, J_{HH} = 13 Hz), indicating C2v-symmetrical *pinched cone* conformation of the macrocyclic skeleton.[27]

Inhibitory activities of the new compounds were evaluated *in vitro* against PTPs with *p*-nitrophenyl phosphate as a substrate. At physiological pH, calix[4]arene α-ketophopshonic acids **7** and **8** exist in $P(O)(OH)O^-$ and partially in $P(O)(O^-)_2$ anionic forms, named 'phosphonates'. Under assay conditions, both inhibitors significantly suppressed PTP1B activity in a dose–dependent manner. Kinetic curves of PTP1B inhibition by calix[4]arene α-ketophosphonates **7** and **8** showed a time-dependent decrease in the reaction rate (Fig. 1), which can be explained by reversible slow–binding inhibition mechanism.[28] The activity of the enzyme was measured using linear kinetics of steady-state phase of the substrate hydrolysis. Values of IC_{50} were calculated from dose-dependent curves as concentrations of the tested compound which decreased the enzyme activity to 50 %. The values of IC_{50} obtained for all PTPs are summarized in Table 1.

The inhibiting effects of compounds **7** and **8** on PTP1B activity were observed at micromolar concentrations of the inhibitors. At the same time, diethyl ester of the calix[4]arene α-ketophosphonic acid (compound **7**) did not change the activity of PTP1B at the concentrations up to 50 μM. This indicates that α-ketophosphonate monoanion as non-hydrolysable mimic of the phosphate group is essential for the inhibition.

The mono-substituted macrocycle was found to be slightly more effective as compared to the bis-substituted one. The IC_{50} values were 3.2 and 6.7 μM for compounds **7** and **8**, respectively. The data obtained show only small differences in PTP1B inhibiting activities of methylenebisphosphonate,[24] α-hydroxymethylphosphonate,[23] and α-ketophosphonate bearing derivatives of calix[4]arenes. However, the α-ketophosphonate bearing inhibitors of PTP1B may contribute to their selectivity

Table 1 Inhibitory potency (IC_{50}, μM) of compounds **7** and **8** against PTP1B and other protein tyrosine phosphatases.

PTPs	Compound **7**	Compound **8**
PTP1B	3.2 ± 0.4	6.7 ± 0.2
TC-PTP	27 ± 3	51 ± 10
PTP-MEG2	26 ± 1	46 ± 6
PTP-MEG1	60 ± 10	>100
SHP2	40 ± 6	53 ± 5
CD45	11 ± 3	16 ± 3
PTPβ	32 ± 4	30 ± 10
PTP-LAR	72 ± 5	56 ± 3

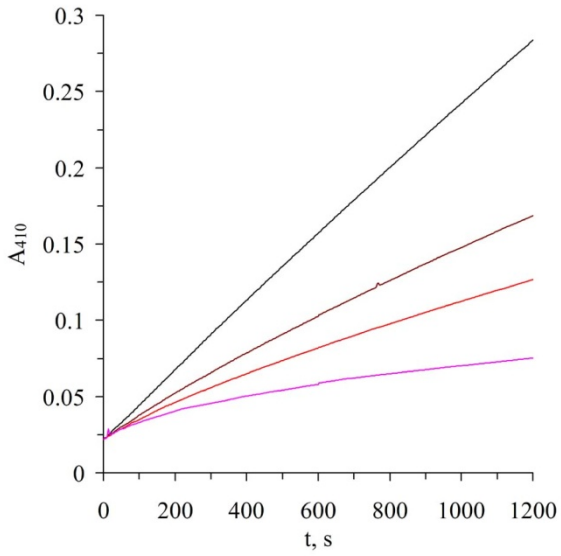


Figure 1 Progress curves for the hydrolysis of *p*-nitrophenyl phosphate (2mM) catalyzed by PTP1B in absence of the inhibitor (upper curve) and in presence of 6, 12 and 24 μM bis-substituted derivative **8**.

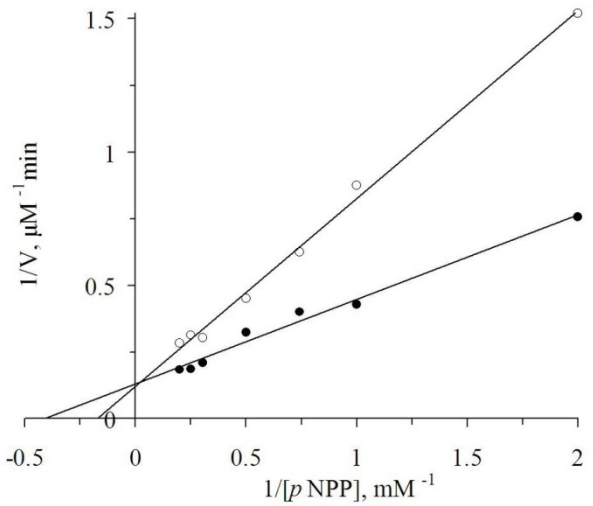
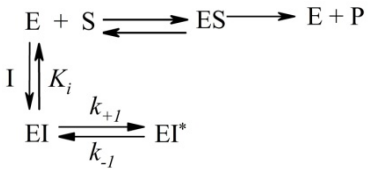


Figure 2 The double-reciprocal plot of inhibition of PTP1B by bis-substituted calix[4]arene-α-ketophosphonic acid **8**. The concentration of the inhibitor was 0 μM (●) and 6 μM (○).

as compared to other protein tyrosine phosphatases which share the same signature motif Cys(XXXXX)Arg in the catalytic domains.[29] The introduction of α-ketophosphonate group instead of α-hydroxymethylphosphonate resulted in increased selectivity towards PTP1B over SHP2 and CD45. Calix[4]arene derivatives **7** and **8** are about 8-fold more effective inhibitors of PTP1B in comparison with MEG2 and TC-PTP which is essential for T-cell functions.[22] Compound **7** showed approximately 10-fold selectivity over SHP2, LAR, PTP-β, MEG1, and 3-fold selectivity over CD45 (Table 1).

The double-reciprocal plot of steady-state velocity versus substrate concentration was applied to characterize the mechanism of PTP1B inhibition on example of bis-substituted macrocycle **8** (Fig. 2). The kinetic data revealed that the calix[4]arene derivative acts as competitive inhibitor with apparent inhibition constant of 4.4 ± 0.4 μM. Thus, substrate and inhibitor may compete for the enzyme active site. As it is seen from Fig. 1, the initial velocity of the enzymatic reaction depends on inhibitor concentration. This indicates a possible two-step inhibition mechanism[28] with rapid formation of the first enzyme-inhibitor complex and its slow isomerization to the more stable complex EI^* (Scheme 2). The apparent inhibition constant can be represented by the equation $K_i^* = K_i\, k_{-1} / (k_{-1} + k_{+1})$. The rate constant k_{-1} was calculated using relationship $k_{-1} = (V_s/V_o)\, k_a$ where V_o and V_s are initial and steady-state velocities, respectively, and k_a is the apparent first-order rate constant. The values of k_{+1} and K_i were determined from dependence of $1 / (k_a - k_{-1})$ on $1 /$ [I].[28] The calculated k_{-1} and k_{+1} values were 0.0017 and 0.0042 c^{-1}; the inhibition constants K_i and K_i^* were 12.3 and 3.6 μM, respectively.



Scheme 2 Kinetic scheme for possible two-step mechanism of enzyme-inhibitor complex formation.

Molecular docking of the inhibitors into the active site of PTP1B showed that the synthesized compounds may interact with the enzyme more tightly when its WPD-loop (Thr177-Pro185) is closed. In case of inhibitors **7** and **8,** the calculated free energies ΔG_{doc} were -9.76 and -9.32 kcal/mol for the closed WPD-loop conformation (PDB code 2CNF) and -7.18 and -8.09 kcal/mol for open WPD-loop (PDB code 1NL9), respectively. Both inhibitors were found to interact with P-loop (His214-Arg221) and WPD-loop in closed or open forms. In a more probable complex, when WPD-loop is closed, the phosphonate group of mono-substituted inhibitor **7** makes hydrogen bonds with Ala217, Gly220, and Arg221. The propoxy groups on the lower rim of the macrocycle provide hydrophobic interactions with Arg47, which is known to be important for substrate specificity,[30] Tyr46 and Asp48. The calix[4]arene skeleton is involved in hydrophobic interaction with Phe182 and may provide van der Waals contacts with Asp181 and Tyr46 (Fig. 3, *a*). According to ΔG_{doc} values, the binding mode of bis-substituted compound **8** in the active site of the closed conformation is almost the same as that of mono-substituted inhibitor **7**, taking into account that the second phosphonate group of inhibitor **8** does not interact with PTP1B active surface. However, when WPD-loop is open, one phosphonate group of compound **8** makes hydrogen bonds with residues in the P-loop while another group interacts with Ala264. The calix[4]arene platform of inhibitor **8** is also involved in van der Waals contacts with amino acid residues at the active site (Fig. 3, *b*).

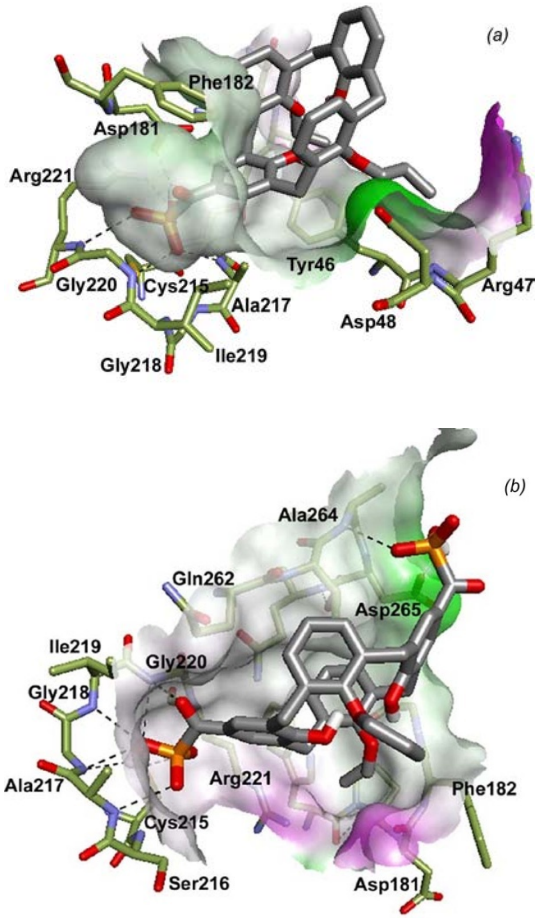


Figure 3 (*a*) Interaction of calix[4]arene-α-ketophosphonate **7** with adjacent amino acid residues in the active site of PTP1B when WPD-loop is closed. (*b*) Possible binding mode of bis-α-ketophosphonate **8** to PTP1B with open WPD-loop.

In conclusion, we have synthesized and evaluated the properties of mono- and bis-substituted α-ketophosphonic acid derivatives of calix[4]arene as possible inhibitors of PTP1B. The macrocyclic α-ketophosphonates were found to be able to inhibit PTP1B with IC_{50} values in the micromolar range displaying selectivity over TC-PTP, MEG1, MEG2, SHP2, LAR and PTP-β. The α-ketophosphonate moiety together with macrocyclic platform was supposed to bind to the active site of PTP1B by reversible slow-binding inhibition mechanism. Thus, α-ketophosphonates as mimics of phosphate biomolecules may be exploited for designing new inhibitors of protein tyrosine phosphatases.

SYNTHETIC AND EXPERIMENTAL MATERIALS

The synthesis and experimental details are available as supplementary data and can be downloaded from journal site.

ACKNOWLEDGMENTS

This research was supported by the State Program "Nanotechnology and nanomaterials" (grants of National Academy of Sciences of Ukraine 5.18.2.8 and 5.16.1.2).

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