Bioorganic & Medicinal Chemistry Letters 20 (2010) 483–487

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Inhibition of *Yersinia* protein tyrosine phosphatase by phosphonate derivatives of calixarenes

Andriy I. Vovk [a,*], Lyudmyla A. Kononets [a], Vsevolod Yu. Tanchuk [a], Sergiy O. Cherenok [b], Andriy B. Drapailo [b], Vitaly I. Kalchenko [b], Valery P. Kukhar [a]

[a] *Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Sciences of Ukraine, Murmanska, 1, 02660 Kyiv-94, Ukraine*
[b] *Institute of Organic Chemistry, National Academy of Sciences of Ukraine, Murmanska, 5, 02660 Kyiv-94, Ukraine*

ARTICLE INFO

Article history:
Received 1 October 2009
Revised 20 November 2009
Accepted 21 November 2009
Available online 3 December 2009

Keywords:
Protein tyrosine phosphatase
Phosphonic acid
Calixarene
Inhibition
Molecular docking
Binding mode

ABSTRACT

Inhibition of *Yersinia* protein tyrosine phosphatase by calix[4]arene mono-, bis-, and tetrakis(methylenebisphosphonic) acids as well as calix[4]arene and thiacalix[4]arene tetrakis(methylphosphonic) acids have been investigated. The kinetic studies revealed that some compounds in this class are potent competitive inhibitors of *Yersinia* PTP with inhibition constants in the low micromolar range. The binding modes of macrocyclic phosphonate derivatives in the enzyme active center have been explained using computational docking approach. The results obtained indicate that calix[4]arenes are promising scaffolds for the development of inhibitors of *Yersinia* PTP.

Protein tyrosine phosphatases (PTPs) are known to be signal transduction enzymes that catalyze dephosphorylation of phosphotyrosine residues in proteins. The PTP termed YopH is a key outer membrane protein H secreted by pathogenic bacteria *Yersinia*. The species of these bacteria cause several diseases ranging from gastrointestinal syndromes to bubonic plague.[1] In infected cells, YopH can dephosphorylate multiple focal adhesion proteins such as focal adhesion kinase and focal adhesion protein p130Cas to disrupt the signaling pathways and to escape the immune responses.[2] It was established that YopH can also be responsible for dephosphorylation of the phosphotyrosine residues of paxillin, Fyn-binding protein, and other substrates.[3] Natural reservoirs of *Yersinia pestis* still exist[4] and there is a risk of outbreak of diseases or the threat of misuse of the patogenic bacteria for bioterroristic attacks.[5] Because the YopH, one of the most active PTPs known, is an essential virulence factor of the *Yersinia pestis*, there is growing interest in developing inhibitors of this enzyme.

Derivatives of furanyl salicylate compounds,[6] α-ketocarboxylic acid,[7] squaric acid,[7a] hexapeptide,[8] and phosphotyrosyl mimetic-containing tripeptides[9] have been identified as effective inhibitors of the *Yersinia* PTP. Some of vinyl sulfonates and sulfones may serve as mechanism-based enzyme inactivators.[10] Crystal structures were determined for complexes of the YopH with specific small molecule inhibitor, *p*-nitrocatechol sulfate,[11] and with hexapeptide mimetic as enzyme substrate analogue.[8] Many inhibitors of several PTPs contain non-hydrolysable phosphonate fragments, which are known as phosphotyrosine bioisosteres.[9a,12] However, benzylphosphonate,[11] aryloxymethano-, and aryloxyethanophosphonates[13] were found to be weak inhibitors of the *Yersinia* PTP.

Data in the literature indicate that inhibitors with two or more anionic groups show enhanced binding to PTPs when compared to the related monodentate compounds.[7c] We have reported[14] that preorganization of some phosphonate groups on the platform of calix[4]arene, macrocycle with unique structure and properties,[15] may be an efficient strategy for the design of powerful enzyme inhibitors. For example, calix[4]arene bearing two methylenebisphosphonic acid fragments at the wide rim exhibits high affinity for alkaline phosphatase.[14a] Present research was undertaken to evaluate the activity of phosphonate derivatives clustered by a macrocyclic calix[4]arene platform toward *Yersinia* PTP. To elucidate possible enzyme–inhibitor interactions, the functionalized macrocycles have been docked computationally to the active site of this phosphatase.

Calix[4]arene methylenebisphosphonic acids **1**–**3**, calix[4]arene and thiacalix[4]arene methylphosphonic acids **5**, **6**, and model 4-hydroxyphenyl methylenebisphosphonic acid **4** (Fig. 1) were synthesized according to previously developed synthetic procedures.[14,16,17] The compounds obtained were evaluated in vitro as inhibitors of recombinant phosphatase Yop51*.[18] The activities of

* Corresponding author. Fax: +38 044 573 2552.
E-mail address: vovk@bpci.kiev.ua (A.I. Vovk).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.11.126

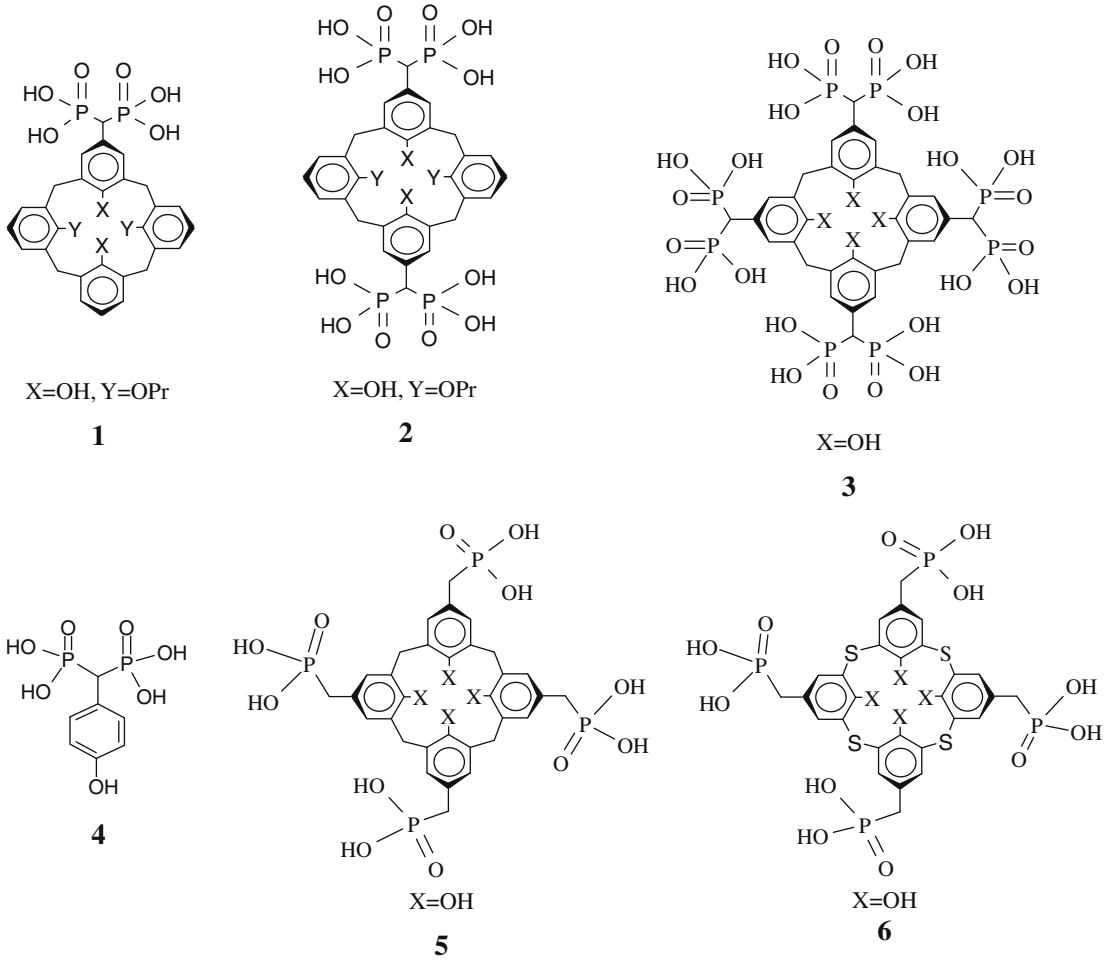

Figure 1. Chemical structures of inhibitors **1–6**. At physiological pH, the inhibitors called as phosphonates exist in $P(O)(OH)O^-$ and partially in $P(O)(O^-)_2$ anionic forms.

the enzyme were assayed by monitoring the rate of hydrolysis of *p*-nitrophenylphosphate used as substrate.[19]

The kinetic analysis revealed that the influence of compounds **1–6** on the activity of *Yersinia* PTP was in agreement with competitive type inhibition. The results correspond to the mechanism that involves the competitive binding of the inhibitor in the active site with formation of enzyme–inhibitor complex. Under assay conditions in the presence of EDTA and dithiothreitol, the Lineweaver–Burk double reciprocal plots were used to determine the values of inhibition constants for phosphorylated calix[4]arenes **2**, **5**, and thiacalix[4]arene **6** (Fig. 2) as well as compounds **1**, **3**, and **4**. The data indicates that the macrocyclic bisphosphonate **1** with K_i = 7.1 μM was approximately 80-fold more potent than the model compound **4** (Table 1). Introduction of two methylenebisphosphonic acid groups into the structure of calix[4]arene leads to the analog **2** being 400 times more effective inhibitor (K_i = 1.4 μM) as compared to the compound **4.** Corresponding tetrakis(methylenebisphosphonate) **3** showed a lower effect on the enzyme activity in comparison with mono- and bis(methylenebisphosphonate) **1** and **2**. Compounds **5** and **6** bearing four methylenephosphonate fragments at the wide rim of the macrocycle showed the highest affinities for the enzyme with inhibition constants in the low micromolar range (0.92 and 0.22 μM, respectively), whereas benzylphosphonate[11] exhibited no significant effect. Calixarene backbones, such as 25,26,27,28-tetrahydroxycalix[4]arene and 25,27-dipropoxy-26,28-dihydroxycalix[4]arene, did not change the enzyme activity at concentrations up to 20 μM. Thus, the higher inhibitory activities of phosphorylated derivatives of calix[4]arenes compared to model compounds can be attributed to synergistic effects of the phosphonate fragments and macrocyclic scaffold involved in the interaction with *Yersinia* PTP.

Although *Yersinia* PTP and mammalian PTPs have very similar catalytic cores, they differ in topology, charge distribution and lipophilicity profile of active surface.[6a] Based on such considerations, bulky macrocyclic phosphonate inhibitors appear to be capable of selective binding to the active center of a particular enzyme. The experiments, which have been carried out with *Yersinia* PTP and human PTPβ[20] show that the inhibitory activity of compound **2** toward *Yersinia* PTP is one order of magnitude higher than toward PTPβ (IC_{50} = 32 ± 7 μM). Noteworthy are more significant variations in IC_{50} values observed among macrocyclic inhibitors of PTPβ. As a result, compound **5** exhibited only modest selectivity for *Yersinia* PTP over PTPβ (IC_{50} = 3.8 ± 0.6 μM). Compound **6** was found to be more potent inhibitor of PTPβ (IC_{50} = 0.13 ± 0.02 μM) and showed an approximately threefold selectivity over *Yersinia* PTP. At the same time, compounds **2**, **5**, and **6** at concentration of 50 μM did not exhibit inhibitory activity against Ser/Thr protein phosphatase, such as human PP1.

To analyze the binding mode of macrocyclic phosphonate inhibitors in the active site of *Yersinia* PTP, computer-simulated docking studies were performed using QXP/FLO+ program.[21] A binding model was constructed automatically on the basis of known X-ray crystal structure of the enzyme in complex with *p*-nitrocatechol sulfate[11] (PDB code: 1PA9). Fulldock+ being the most exact in QXP/FLO+ was used as the method of the further optimization. The ligand was preliminary removed from the binding site of the enzyme, and

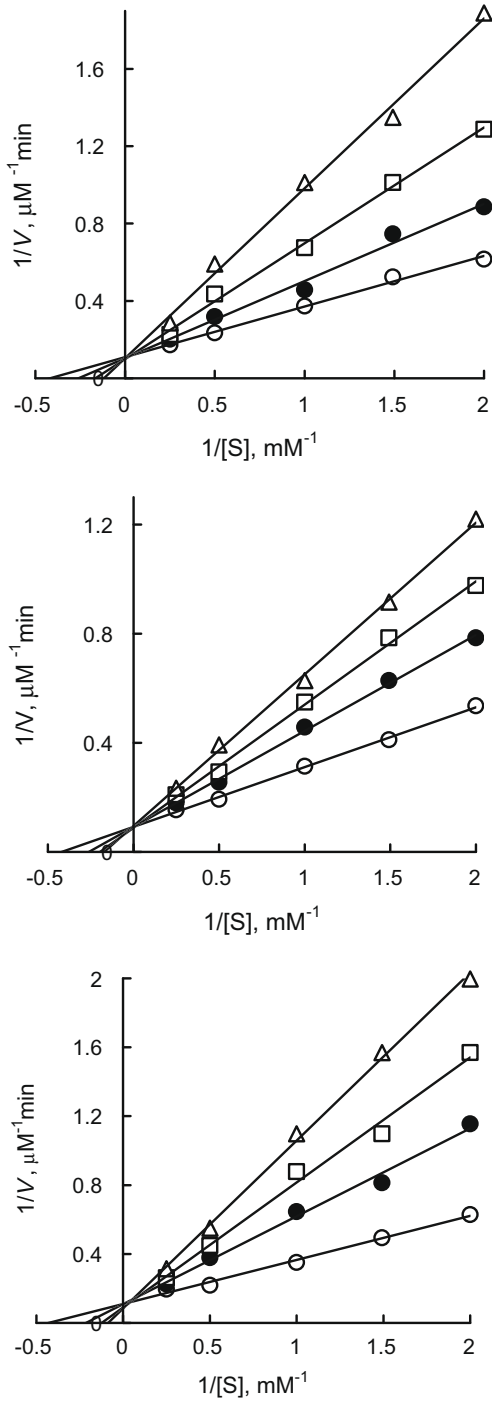

Figure 2. Lineweaver–Burk graphical representations of recombinant *Yersinia* PTP inhibition by calix[4]arene bis(methylenebisphosphonic) acid **2** (top panel), calix[4]arene tetrakis(methylphosphonic) acid **5** (middle panel) and thiacalix[4]arene tetrakis(methylphosphonic) acid **6** (bottom panel). The concentrations of the inhibitors were: top panel, no inhibitor (○),1 μM (●), 2 μM (□),3 μM (△); middle panel, no inhibitor (○), 0.5 μM (●), 1.0 μM (□),1.5 μM (△); bottom panel, no inhibitor (○), 0.25 μM (●), 0.50 μM (□), 0.75 μM (△).

then inhibitors were examined to explore their binding modes. The more favorable binding of the inhibitors was observed when their phosphoryl residues were in monoanionic form.

The docking calculations (Table 1) reveal that bis(methylenebisphosphonate) **2** exhibits stronger binding affinity for *Yersinia* PTP than methylenebisphosphonate **1** or tetrakis(methylene bisphosphonate) **3**. The predicted free energies ΔG_{doc} also indicate

Table 1
Experimental inhibition constants[a] and docking results (QXP/FLO+) for the inhibitors of *Yersinia* PTP

Inhibitor	K_i (μM)	ΔG_{doc}[b] (kJ/mol)	Cntc[c] (kJ/mol)	Hbnd[d] (kJ/mol)
1	7.1 ± 1.7	−26.0	−54.2	−4.3
2	1.4 ± 0.23	−30.5	−70.0	−17.5
3	40 ± 3	−25.3	−68.2	−12.4
4	570 ± 50	−12.2	−35.6	−4.2
5	0.92 ± 0.11	−34.2	−75.0	−8.2
6	0.22 ± 0.03	−36.8	−78.1	−10.0

[a] Mean values of K_i and standard deviations are given.
[b] Calculated binding free energy.
[c] Calculated ligand-site contact energy.
[d] Calculated hydrogen bond energy.

that the thiacalix[4]arene tetrakis(methylphosphonate) **6** binds more tightly to the enzyme than the calix[4]arene derivative **5**. The K_i values obtained from ΔG_{doc} for compounds **1**–**3** and **5**, **6** are in good agreement with the experimentally determined activity data. The correlation coefficient (r^2) between the free energies of binding and the corresponding experimental activities, expressed as pK_i, was 0.95 (Fig. 3). The experimental activities of the macrocyclic phosphonates correlate with contact energy contribution (r^2 = 0.76), but only weak correlation was observed between pK_i and hydrogen bond energy (r^2 = 0.17). This is in accordance with other studies showing that hydrophobic and electrostatic interactions dominate the binding of α-ketocarboxylate derivatives to the *Yersinia* PTP.[7b]

The calix[4]arene and thiacalix[4]arene molecules are characterized by flexibility at the level of aromatic cores,[15] and therefore different conformations of macrocyclic inhibitors have been achieved by their interactions with amino acid environment of the enzyme active site. Examination of the models shows that calix[4]arene methylenebisphosphonate **1** and calix[4]arene tetrakis(methylenebisphosphonate) **3** adopt *cone* and *partial cone* conformations, respectively. Calix[4]arene **2**, bearing two methylenebisphosphonate fragments at the upper rim, displays the structure which is described as *distorted cone* with one of benzene rings being oriented almost parallel to the main plane. Calix[4]arene and thiacalix[4]arene tetrakis(methylphosphonates) **5** and **6** are fixed in *flattened cone* conformation with angles between two opposite rings of approximately 160°, while other two aromatic rings are almost parallel to each other.

Molecular modelling results showed that all macrocyclic inhibitors occupy the active region, interfering with binding of enzyme substrate. The phosphonate residues of the macrocycles were

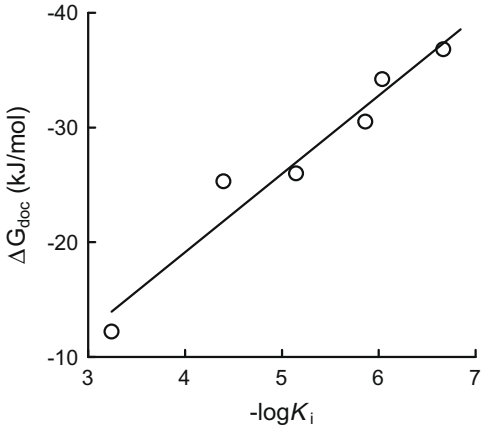

Figure 3. Correlation between the experimental activity (−log K_i) and binding free energy predicted by QXP/FLO+ for inhibitors **1**–**6**.

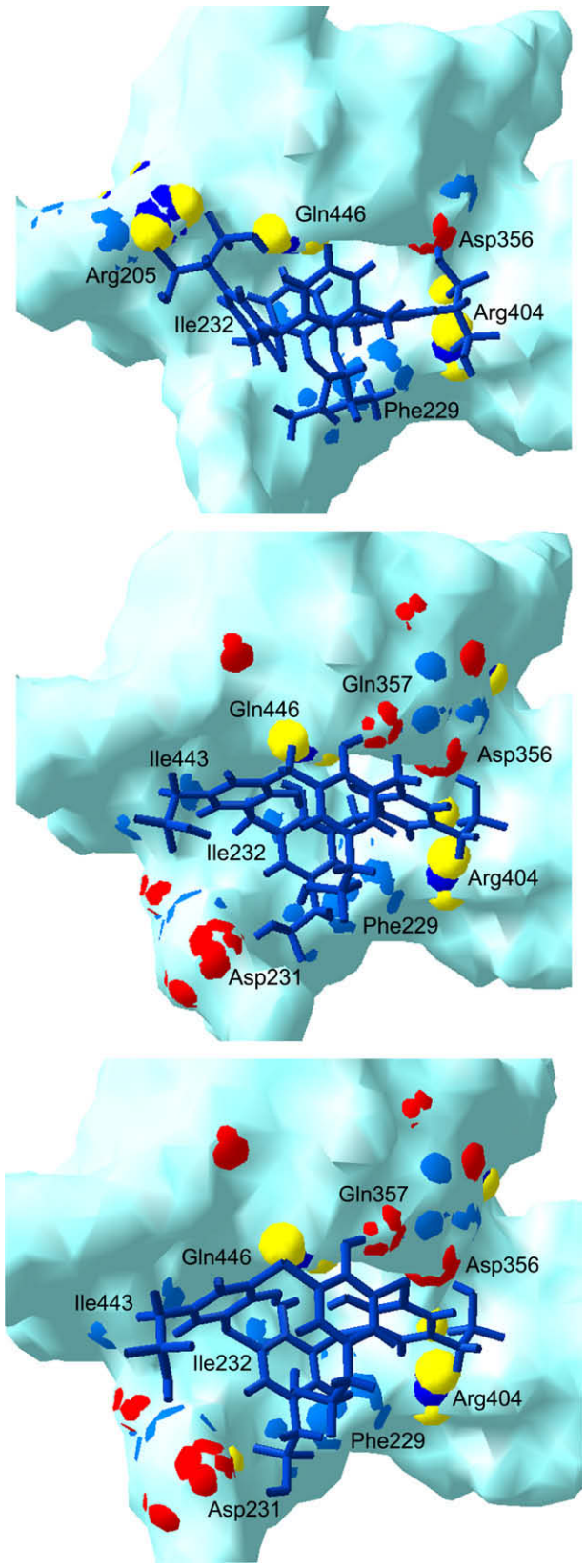

Figure 4. The possible binding modes of calix[4]arene bis(methylenebisphosphonate) **2** (top), calix[4]arene tetrakis(methylphosphonate) **5** (middle) and thiacalix[4]arene tetrakis(methylphosphonate) **6** (bottom) in the active site of *Yersinia* PTP.

remote from catalytic Cys403 located in the deep phosphotyrosine binding cavity. Figure 4 shows the computational models of the most effective inhibitors **2**, **5**, and **6** bound with the *Yersinia* PTP. As observed in case of compound **2**, the phosphonate oxygens of one of methylenebisphosphonate groups were positioned to make hydrogen bonds to Arg404 of P-loop (residues 403–411[22]). Closed conformation of WPD loop (residues 351–359)[23] provides the interaction of this methylenebisphosphonate group with Asp356. The second methylenebisphosphonate moiety of inhibitor **2** was found to be involved in interactions with Arg205 and Gln446. In addition, hydrophobic interactions of macrocyclic platform were observed with Phe229 and Ile232.

In contrast to compound **2**, the narrow rim of macrocycle **5** was oriented toward the enzyme surface. One of the phosphonate groups of the calix[4]arene tetrakis(methylphosphonate) **5** was bound to Asp356 of WPD loop and Arg404 of P-loop at the entry of the catalytic pocket. The favorable interactions were also found between phenol oxygens of this macrocycle and residues of Gln357 and Gln446. The macrocyclic platform of compound **5** is involved in hydrophobic interaction with Ile443, Phe229 and Ile232. The binding mode of compound **6** is similar to that observed for compound **5**. However, the calculated docking energies for these inhibitors (Table 1) indicate that the presence of sulfur atoms in the structure of the compound **6** provides more favorable contacts with the active surface. This can be explained by the larger cavity size of thiacalix[4]arene compared to calix[4]arene.[24]

In summary, we have found that calix[4]arenes are promising scaffolds for the design of inhibitors of the PTPs. The phosphorylated calix[4]arenes investigated in this paper exhibited strong competitive inhibition of *Yersinia* PTP. The phosphonate groups as well as macrocyclic structure are essential for the activities of these compounds. Model docking studies revealed similar binding mode for calixarene inhibitors covering the active site, with location of the phosphonate residues around the entry of the phosphotyrosine binding cavity. This provides molecular basis for understanding of mechanism of enzyme–inhibitor interaction and can be useful for the development of calixarene inhibitors of YopH.

Acknowledgement

This research was supported by the Science and Technology Center in Ukraine (Project No. 3558).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.126.

References and notes

1. (a) Guan, K. L.; Dixon, J. E. *Science* **1990**, *249*, 553; (b) Bliska, J. B.; Guan, K.; Dixon, J. E.; Falkow, S. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1187; (c) Cornelis, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8778.
2. (a) Persson, C.; Carballeira, N.; Wolf-Watz, H.; Fallman, M. *EMBO J.* **1997**, *16*, 2307; (b) Black, D. S.; Montagna, L. G.; Zitsmann, S.; Bliska, J. B. *Mol. Microbiol.* **1998**, *29*, 1263.
3. (a) Hamid, N.; Gustavsson, A.; Andersson, K.; McGee, K.; Persson, C.; Rudd, C. E.; Fallman, M. *Microb. Pathog.* **1999**, *26*, 231; (b) Black, D. S.; Marie-Cardine, A.; Schraven, B.; Bliska, J. B. *Cell. Microbiol.* **2000**, *2*, 401; (c) de la Puerta, M. L.; Trinidad, A. G.; del Carmen Rodríguez, M.; Bogetz, J.; Sánchez Crespo, M.; Mustelin, T.; Alonso, A.; Bayón, Y. *PLoS One* **2009**, *4*, e4431.
4. (a) Christie, A. B. *Ecol. Dis.* **1982**, *1*, 111; (b) Jayaraman, K. S. *Nature* **1994**, *371*, 467.
5. (a) McGovern, T. W.; Christopher, G. W.; Eitzen, E. M. *Arch. Dermatol.* **1999**, *135*, 311; (b) Inglesby, T. V.; Dennis, D. T.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Fine, A. D.; Friedlander, A. M.; Hauer, J.; Koerner, J. F.; Layton, M.; McDade, J.; Osterholm, M. T.; O'Toole, T.; Parker, G.; Perl, T. M.; Russell, P. K.; Schoch-Spana, M.; Tonat, K. *J. Am. Med. Assoc.* **2000**, *283*, 2281; (c) Hawley, R. J.; Eitzen, E. M., Jr. *Annu. Rev. Microbiol.* **2001**, *55*, 235.
6. (a) Tautz, L.; Bruckner, S.; Sareth, S.; Alonso, A.; Bogetz, J.; Bottini, N.; Pellecchia, M.; Mustelin, T. *J. Biol. Chem.* **2005**, *280*, 9400; (b) Vazquez, J.; Tautz, L.; Ryan, J. J.; Vuori, K.; Mustelin, T.; Pellecchia, M. *J. Med. Chem.* **2007**, *50*, 2137.

7. (a) Chen, Y. T.; Seto, C. T. *J. Med. Chem.* **2002**, *45*, 3946; (b) Hu, X.; Stebbins, C. E. *Bioorg. Med. Chem.* **2005**, *13*, 1101; (c) Xie, J.; Seto, C. T. *Bioorg. Med. Chem.* **2005**, *13*, 2981.
8. Phan, J.; Lee, K.; Cherry, S.; Tropea, J. E.; Burke, T. R., Jr.; Waugh, D. S. *Biochemistry* **2003**, *42*, 13113.
9. (a) Lee, K.; Gao, Y.; Yao, Z.-J.; Phan, J.; Wu, L.; Liang, J.; Waugh, D. S.; Zhang, Z.-Y.; Burke, T. R., Jr. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2577; (b) Lee, K.; Boovanahalli, S. K.; Nam, K.-Y.; Kang, S.-U.; Lee, M.; Phan, J.; Wu, L.; Waugh, D. S.; Zhang, Z.-Y.; No, K. T.; Leed, J. J.; Burke, T. R., Jr. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4037.
10. Liu, S.; Zhou, B.; Yang, H.; He, Y.; Jiang, Z.-X.; Kumar, S.; Wu, L.; Zhang, Z.-Y. *J. Am. Chem. Soc.* **2008**, *130*, 8251.
11. Sun, J.-P.; Wu, L.; Fedorov, A. A.; Almo, S. C.; Zhang, Z.-Y. *J. Biol. Chem.* **2003**, *278*, 33392.
12. (a) Frechette, R. F.; Ackerman, C.; Beers, R.; Moore, J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2169; (b) Li, X.; Bhandari, A.; Holmes, C. P.; Szardenings, A. K. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4301; (c) Holmes, C. P.; Li, X.; Pan, Y.; Xu, C.; Bhandari, A.; Moody, C. M.; Miguel, J. A.; Ferla, S. W.; De Francisco, M. N.; Frederick, B. T.; Zhou, S.; Macher, N.; Jang, L.; Irvine, J. D.; Grove, J. R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2719.
13. Iyer, S.; Younker, J. M.; Czyryca, P. G.; Hengge, A. C. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5931.
14. (a) Vovk, A.; Kalchenko, V.; Cherenok, S.; Kukhar, V.; Muzychka, O.; Lozynsky, M. *Org. Biomol. Chem.* **2004**, *2*, 3162; (b) Cherenok, S.; Vovk, A.; Muravyova, I.; Shivanyuk, A.; Kukhar, V.; Lipkowski, J.; Kalchenko, V. *Org. Lett.* **2006**, *8*, 549.
15. (a) Böhmer, V. *Angew. Chem., Int. Ed.* **1995**, *34*, 713–745; (b) Gutsche, C. D. Calixarenes Revisited. In *Monographs in Supramolecular Chemistry*; Stoddart, J. F., Ed.; Royal Society of Chemistry: Cambridge, 1998; (c) *Calixarenes 2001*; Asfari, Z., Böhmer, V., Harrowfield, J., Vicens, J., Eds.; Kluwer Academic: Dordrecht, 2001; (d) *Calixarenes in the Nanoworld*; Vicens, J., Harrowfield, J., Baklouti, L., Eds.; Springer: Dordrecht, 2007; (e) Rodik, R. V.; Boyko, V. I.; Kalchenko, V. I. *Curr. Med. Chem.* **2009**, *16*, 1630.
16. (a) Almi, M.; Arduini, A.; Casnati, A.; Pochini, A.; Ungaro, R. *Tetrahedron* **1989**, *45*, 2177; (b) Kasyan, O.; Swierczynski, D.; Drapailo, A.; Suwinska, K.; Lipkowski, J.; Kalchenko, V. *Tetrahedron Lett.* **2003**, *44*, 7167; (c) Vovk, A. I.; Kononets, L. A.; Tanchuk, V. Y.; Drapailo, A. B.; Kalchenko, V. I.; Kukhar, V. P. *J. Inclusion Phenom. Macrocycl. Chem.* **2009**. doi:10.1007/S10847-009-9607-9.
17. Gritsenko, P. G.; Lugovsky, E. V.; Kalchenko, V. I.; Komisarenko, S. V.; Koshel, T. A.; Cherenok, S. O. *Rep. Nat. Acad. Sci. Ukraine*, in press.
18. Zhang, Z.-Y.; Clemens, J. C.; Schubert, H. L.; Stuckey, J. A.; Fischer, M. W. F.; Hume, D. M.; Saper, M. A.; Dixon, J. E. *Biol. Chem.* **1992**, *267*, 23759.
19. The assay solutions contained 0.05 M BISTRIS buffer (pH 6.5), 100 mM NaCl, 0.05% Brij-35, 0.6 vol % of dimethyl sulfoxide, 0.4 mM EDTA, 1 mM DTT, *p*-nitrophenyl phosphate (0.5–4.0 mM) and inhibitor. The mixtures were preincubated for 5 min at 30 °C, and reactions were initiated by the addition of enzyme solution. The generation of *p*-nitrophenol during the hydrolysis of *p*-nitrophenylphosphate was measured by the increase of absorbance at 410 nm, using a molar absorption coefficient of 18,300 M^{-1} cm^{-1}. The K_m value was found to be 2.5 ± 0.3 mM. The kinetics of inhibition were studied at various concentrations for each inhibitor: **1**: 3 μM, 6 μM; **2**: 1 μM, 2 μM, 3 μM; **3**: 40 μM, 80 μM; **4**: 0.2 mM, 0.4 mM, 0.6 mM; **5**: 0.5 μM, 1.0 μM, 1.5 μM; **6**: 0.25 μM, 0.50 μM, 0.75 μM. The inhibition constants were determined measuring initial hydrolysis rates of enzyme substrate.
20. Compounds **2**, **5**, and **6** were evaluated as inhibitors of human recombinant PTPβ. In the assay, 1 mM *p*-nitrophenyl phosphate as enzyme substrate was preincubated for 5 min at 30 °C with varying concentrations of an inhibitor in 0.05 M BISTRIS buffer (pH 7.0) containing 100 mM NaCl, 0.05% Brij-35, 0.8 vol % of dimethyl sulfoxide and 1 mM diethylene triamine pentaacetic acid. The reaction was started by the addition of PTPβ solution and the change in absorbance was monitored over time at 410 nm. The value of IC_{50} was the concentration of the tested compound which decreased the enzyme activity to 50% after 5 min of reaction. For competitive inhibition, $K_i = 1/2\ IC_{50}$ at substrate concentration equivalent to the K_m value. Under assay conditions, K_m value of the PTPβ was estimated to be 1.0 ± 0.1 mM.
21. McMartin, C.; Bohacek, R. S. *J. Comput. Aided Mol. Des.* **1997**, *11*, 333.
22. Stuckey, J. A.; Schubert, H. L.; Fauman, E. B.; Zhang, Z.-Y.; Dixon, J. E.; Saper, M. A. *Nature* **1994**, *370*, 571.
23. Hu, X.; Stebbins, C. E. *Biophys. J.* **2006**, *91*, 948.
24. Hong, J.; Ham, S. *Tetrahedron Lett.* **2008**, *49*, 2393.