J Incl Phenom Macrocycl Chem (2010) 66:271–277
DOI 10.1007/s10847-009-9607-9

ORIGINAL ARTICLE

Thiacalix[4]arene as molecular platform for design of alkaline phosphatase inhibitors

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Received: 9 April 2009 / Accepted: 10 June 2009 / Published online: 24 June 2009
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Abstract Effect of thiacalix[4]arene platform on inhibition of alkaline phosphatase by macrocyclic phosphonate is presented in this article. Using tetrakis(dihydroxyphosphorylmethyl) derivatives we have found that phosphonate inhibitor on thiacalix[4]arene platform has displayed stronger inhibition properties towards alkaline phosphatases from bovine intestine mucosa, shrimp and human placenta than its structural calix[4]arene analogue. For elucidation of the molecular mechanism of the inhibition the tested macrocyclic compounds were docked computationally to the active site of alkaline phosphatase from shrimp. The role of thiacalix[4]arene platform in formation of the enzyme-inhibitor complex is discussed.

Keywords Calix[4]arene · Thiacalix[4]arene · Phosphonic acid · Alkaline phosphatase · Inhibition · Molecular docking

Introduction

Phosphonic acids are known to function as bioisosteric analogues of numerous natural products including monoalkylphosphates [1–3]. The biological properties of synthetic phosphonic acids as well as the therapeutic potential of bisphosphonates [4–6] generate interest in designing and studying these compounds. We have already shown that preorganizing the phosphonic acid fragments on calix[4]-arene platform provides a promising approach for the design of efficient alkaline phosphatase inhibitors [7]. Calix[4]arenes bearing one or two methylenebisphosphonic acid residues exhibited in vitro stronger inhibition of calf intestine alkaline phosphatase than simple methylenebisphosphonic acid. Chiral calix[4]arene α-aminophosphonic acids were found to have inhibitory activity toward porcine kidney alkaline phosphatase which depends strongly on the absolute configuration of the α-carbon atoms [8].

Alkaline phosphatase is a known metal-dependent enzyme which catalyzes the hydrolysis and transphosphorylation of monoalkylphosphates and regulates the functions of many biological systems [9–11]. Enhanced activity of the enzyme leads to the calcification of soft tissues [12], development of inflammatory [13] and other diseases [14]. Single crystal X-ray analyses of the alkaline phosphatases from *Escherichia coli* [15, 16] and human placenta [17] have revealed the presence of a magnesium and two zinc ions in the enzyme active site. The zinc ions and positively charged amino acids residues are involved in mechanisms of substrate or inhibitor binding. The efficient coordination of calix[4]arene methylenebisphosphonic and α-aminophosphonic acids with the metal cations and amino acids residues in the enzyme's active site and the additional interactions brought about by the scaffold itself are probably responsible for high activity of macrocyclic inhibitors [7, 8]. Obviously, improved affinity to the binding site of the enzyme can be achieved as a result of the modification of phosphonate part as well as the change in framework of macrocyclic scaffold of the inhibitor.

In context of our investigation of bioactive macrocyclic compounds we used the thiacalix[4]arene as molecular scaffold in the design of new effective inhibitors of alkaline

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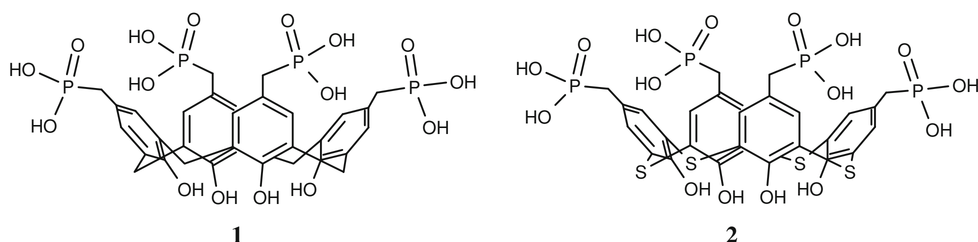


Fig. 1 Chemical structures of compounds **1** and **2**

phosphatase. We assumed that the presence of four bridging sulfur atoms in the macrocyclic skeleton of the thiacalixarene, instead of four methylene groups in the classical calix[4]arene, can result in enhanced inhibitory properties of the functionalized macrocycle. Herein we report on the activity of thiacalix[4]arene and calix[4]arene tetrakismethylphosphonic acids **1** and **2** as inhibitors of alkaline phosphatases from bovine intestine mucosa, shrimp and human placenta (Fig. 1).

Experimental

Materials

The alkaline phosphatase from bovine intestinal mucosa (type VII-L, with an activity of 3,800 units/mg protein), alkaline phosphatase from human placenta (type XXIV, with an activity of 14 units/mg solid), alkaline phosphatase from shrimp (buffered aqueous glycerol solution with an activity 1,000 DEA units/ml), 4-nitrophenylphosphate (disodium salt, hexahydrate) and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Sigma Chemical Co. (USA).

Methods

Synthesis of calix[4]arene and thiacalix[4]arene derivatives

Compounds **1**, **3** [18] and **4**, **6** [19] were synthesized according to known procedures. ^{1}H and ^{31}P NMR spectra were recorded on a Varian VXR 300 and Gemini-200 spectrometers operating at 300 MHz and 80.95 MHz, respectively. The chemical shifts are reported using internal tetramethylsilane (^{1}H NMR) and external 85% H_3PO_4 (^{31}P NMR) as references.

Tetrakis(dihydroxyphosphorylmethyl) thiacalix[4]arene ***2*** Tetrakis(diethoxyphosphorylmethyl) thiacalix[4]arene **6** (0.186 g, 0.17 mmol) was boiled for 26 h with 20 mL of 6 N aqueous hydrochloric acid. The precipitate formed was filtered off and washed with water (2 × 5 mL). The product was dried in vacuo (0.01 mm Hg) at 100 °C for 1 h. Compound **2** was obtained as light powder. Yield 0.15 g (98%). Being heated above 400 °C, the compound decomposes without melting. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 2.90 (d, 8H, $J = 21.7$ Hz, CH_2–P), 7.54 (s, 8H, H-arom.). ^{31}P NMR (121 MHz, DMSO-d$_6$): δ (ppm) 22.6. Anal. Calcd. for $C_{28}H_{28}O_{16}P_4S_4$: C, 38.54; H, 3.23; P, 14.20; S, 14.70. Found: C, 38.71; H, 3.32; P, 13.96; S, 14.62.

Tetrakis(diethoxyphosphorylmethyl) calix[4]arene ***5*** Triethyl phosphite (0.7 g, 4.2 mmol) was added dropwise to solution of compound **3** (0.62 g, 1 mmol) in chloroform (10 mL). The mixture was stirred for 22 h. The solvent was removed under vacuum and the residue was washed with hexane. Yield 1.02 g (100%). Satisfactory elemental analysis and spectroscopic data were obtained for compound **5** [18].

Effect of inhibitors on the alkaline phosphatase activity

A mixture containing 0.1 M Tris–HCl buffer (pH 9), *p*-nitrophenylphosphate as a substrate, and an inhibitor had been preliminarily thermostated for 5 min at 25 °C. The inhibitors **1** and **2** as tetrasodium salts were preliminary dissolved in dimethyl sulfoxide (the concentration of DMSO in reaction mixture was 2.5 vol.%). In experiments with the inhibition of alkaline phosphatase from bovine intestine mucosa, the concentration of *p*-nitrophenylphosphate was 0.016–0.25 mM. The concentration of inhibitors was varied in the range of 50–200 nM in the case of compound **1** and 10–40 nM in the case of compounds **2**. In experiments with the inhibition of alkaline phosphatase from human placenta, the concentration of substrate was 0.02–0.25 mM; the concentration of inhibitors was varied in the range of 0.15–0.60 mM in the case of compound **1** and 20–80 μM in the case of compounds **2**. In experiments with the inhibition of alkaline phosphatase from shrimp, concentration of substrate was 0.007–0.033 mM; the concentration of inhibitors was varied in the range of 50–200 μM in the case of compound **1** and 1.5–6 μM in the case of compounds **2**. The reaction was started by adding the enzyme. The rate of formation of *p*-nitrophenol during

the hydrolysis of *p*-nitrophenylphosphate was determined from an increase in the absorbance of the reaction mixture at 400 nm using the molar absorption coefficient of 18,300 M^{-1} cm^{-1}.

Kinetic determinations

The type of inhibition was determined using Lineweaver–Burk double reciprocal plots of initial rates of substrate hydrolysis V_o (calculated from the linear part of time-curve) versus *p*-nitrophenylphosphate concentration. The values of Michaelis constant (K_m) and maximum velocity (V_{max}) for the control reaction, and apparent values $K_m{}'$ and $V_{max}{}'$ for the reactions at fixed inhibitor concentrations were determined from these plots. In agreement with a mixed-type inhibition, the ratios $V_{max}{}'/V_{max}$ and $K_m{}'/K_m$ can be expressed by equations:

$$V_{max}{}' = V_{max}/(1 + [I]/K_i{}')$$
$$V_{max}{}'/V_{max} = K_m{}'/K_m(1 + [I]/K_i),$$

where [I] is the inhibitor concentration; K_i and $K_i{}'$ are dissociation constants of enzyme–inhibitor and enzyme–substrate–inhibitor complexes, respectively [20].

For competitive inhibition K_i values were estimated according to equation [20]:

$$K_m{}' = K_m(1 + [I]/K_i)$$

The inhibition constants are the means of four values obtained from the kinetic data of the experiments performed at different inhibitor concentrations. The results given in Table 1 are presented as mean ± S.D.

Docking studies

Calculations of the docking of inhibitors were carried out using the program AutoDock (version 3.05) [21, 22]. For the preliminary preparation of molecules, the program AutoDockTools was used. The spatial structure of alkaline phosphatase from shrimp was used according to the data reported in [23] (the identifier 1SHN in RSCB Protein Data Bank). Hundred runs of the genetic algorithm [22] with a generation size of 200 and the maximum number of energy estimates of 50 millions were performed. The following parameters of van der Waals interactions were used: for zinc ions: r 1.1 Å, ε 0.25 kcal/mol [24] formal charge +2e [25]. The analysis of the docking results and preparation of figures were performed using the Swiss Pdb Viewer program [26].

Results and discussion

Synthesis

Tetrakis(dihydroxyphosphorylmethyl) derivatives of calix[4]arene (**1**) and thiacalix[4]arene (**2**) were obtained by the Ungaro method [18] from the synthetic accessible chloromethyl derivatives **3** and **4**, respectively (Scheme 1). The Arbuzov reaction of compounds **3** and **4** with triethyl phosphite afforded tetrakis(diethoxyphosphorylmethyl) calix[4]arene (**5**) [18] and thiacalix[4]arene (**6**) [19] in high yield. The esters **5**, **6** were converted to the corresponding tetrakismethylphosphonic acids **1**, **2** by subsequent treatment with 20% HCl solution. Compounds **1**, **2** were obtained quantitatively as colorless crystalline substances. As well as the parent esters **5**, **6**, compounds **1** and **2** exist in the *cone* conformations, where the benzene rings are oriented to one side with respect to the main plane of the macrocycle, formed by the four methylene groups or sulfur atoms, respectively. The stabilization of phosphonic acids **1** and **2** in the *cone* conformations may be caused by the formation of a system of intramolecular hydrogen bonds of phenolic OH group at the lower rim of the macrocycles [19, 27–29].

Table 1 Inhibition constants of calix[4]arene and thiacalix[4]arene tetrakismethylphosphonic acids **1** and **2** against the alkaline phosphatases and free energies for the formation of enzyme-inhibitor complexes

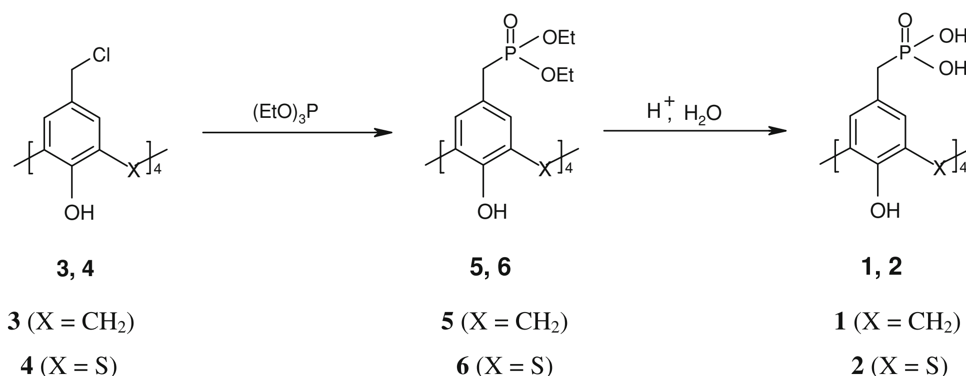
Inhibitor	Alkaline phosphatase[a]	K_i (μM)	$K_i{}'$ (μM)	ΔG[b] (kcal/mol)	ΔG^c_{doc} (kcal/mol)
1	From human placenta	940 ± 130	3600 ± 370	−4.13	–
2	From human placenta	59 ± 10	270 ± 21	−5.77	–
1	From bovine intestine	0.070 ± 0.003	0.92 ± 0.22	−9.76	–
2	From bovine intestine	0.021 ± 0.001	0.075 ± 0.007	−10.47	–
1	From shrimp	33 ± 3	–	−6.11	−9.66
2	From shrimp	3.0 ± 0.2	–	−7.53	−10.20

[a] 0.1 M Tris–HCl buffer (pH 9), 298.15 K

[b] Calculated from the equation: $\Delta G = -RT \ln(K_i^{-1})$

[c] Calculations were carried out using AutoDock 3.05

Scheme 1 Synthesis of calix[4]arene and thiacalix[4]arene tetrakismethylphosphonic acids **1** and **2**



Inhibition kinetics study

For the evaluation of calix[4]arene and thiacalix[4]arene tetrakismethylphosphonates **1**, **2** as inhibitors of alkaline phosphatases, the spectrophotometric assays of enzyme activities were used. Enzymatic activities of alkaline phosphatases were determined by following the change in absorbance that accompanied the hydrolysis of *p*-nitrophenylphosphate. In the reaction medium at pH 9 the phosphate part of substrate and phosphoryl residue of inhibitor were mainly in forms of dianions. The influence of inhibitors **1** and **2** on the activity of alkaline phosphatase from bovine intestine mucosa was in agreement with a mixed-type inhibition. Results of the experiments in Lineweaver–Burk coordinates are presented in Figs. 2 and 3. The ordinate intercept and the slope of the straight lines from these figures linearly depend on the inhibitor concentration. These results correspond to the mechanism of inhibition by **1** and **2** that involves the binding of inhibitor to the enzyme with formation of enzyme–inhibitor or enzyme–substrate–inhibitor complexes. It is to note that in the case of calix[4]arene **1** the value of inhibition constant K_i is considerably smaller than value of K_i'. The similar mechanism of inhibition by calix[4]arene **1** and thiacalix[4]arene **2** was observed for the alkaline phosphatase from human placenta. On the contrary, the inhibition of alkaline phosphatase from shrimp was found to be of competitive type (Figs. 4 and 5). This type of inhibition supposes that macrocyclic phosphonate inhibitors **1** and **2** bind to enzyme only in the substrate binding site.

The obtained inhibition constants characterize differences in affinity of macrocyclic inhibitors to alkaline phosphatases from different sources. For example, the inhibitory activity of compound **2** toward alkaline phosphatase from bovine intestine mucosa is by three orders of

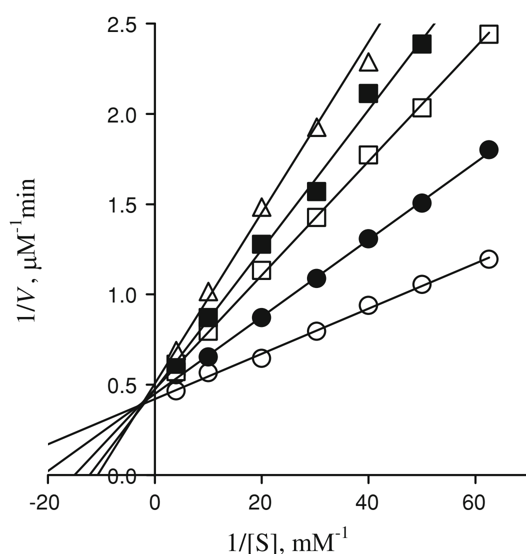


Fig. 2 Lineweaver–Burk graphical representation of inhibition of bovine intestine alkaline phosphatase by calix[4]arene **1**. The concentrations of the inhibitor were 0 (*open circle*), 0.05 μM (*filled circle*), 0.1 μM (*open square*), 0.15 μM (*filled square*) and 0.2 μM (*open triangle*)

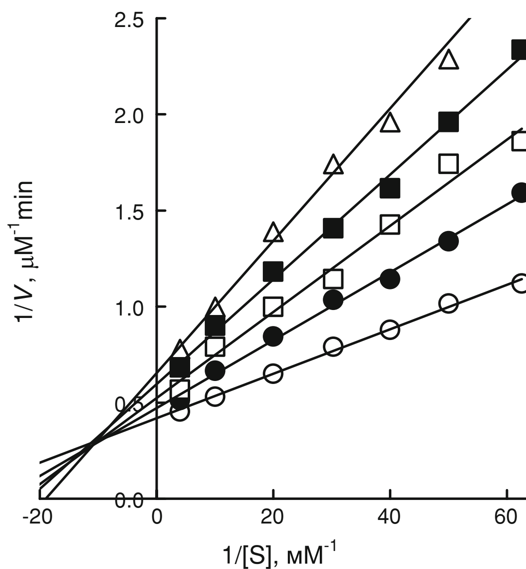


Fig. 3 Lineweaver–Burk graphical representation of inhibition of bovine intestine alkaline phosphatase by thiacalix[4]arene **2**. The concentrations of the inhibitor were 0 (*open circle*), 10 nM (*filled circle*), 20 nM (*open square*), 30 nM (*filled square*) and 40 nM (*open triangle*)

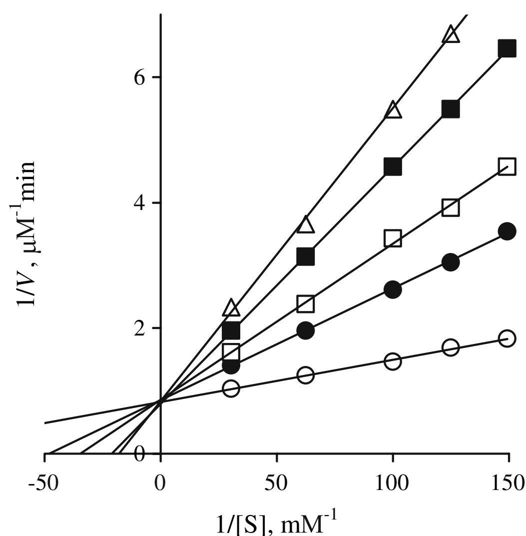


Fig. 4 Lineweaver–Burk plot of inhibition of shrimp alkaline phosphatase by calix[4]arene **1**. The concentrations of the inhibitor were 0 (*open circle*), 50 μM (*filled circle*), 100 μM (*open square*), 150 μM (*filled square*) and 200 μM (*open triangle*)

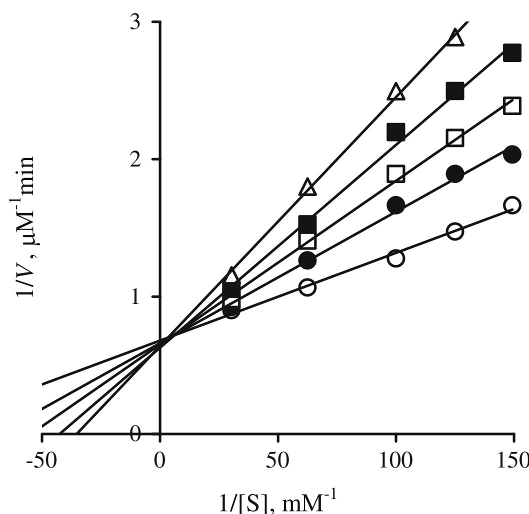


Fig. 5 Lineweaver–Burk plot of inhibition of shrimp alkaline phosphatase by thiacalix[4]arene **2**. The concentrations of the inhibitor were 0 (*open circle*), 1.5 μM (*filled circle*), 3.0 μM (*open square*), 4.5 μM (*filled square*) and 6.0 μM (*open triangle*)

magnitude higher than that in case of human placenta alkaline phosphatase (Table 1). This may be related to the macrocycle interaction with different amino acid environment in the active site of these enzymes. In the case of the enzyme from human placenta the active site is large in size, so that it enables the entry of the phosphorylated protein as a substrate. However, the residue Glu429 located near the active center of the enzyme [17] may be unfavorable for the binding of the negatively charged phosphonate fragments of a bulky macrocyclic ligand. The data on the structure of bovine intestinal isozyme indicate that the amino acid residues in the proximity of the active centre [30] can promote the binding of the hydrophobic macrocyclic compound. The sequences of alkaline phosphatases from human placenta and shrimp have 24% identical amino acids with conserved active-site residues, but the shrimp enzyme has a zinc triad in the active site, whereas alkaline phosphatases usually contain two zinc and one magnesium ion per monomer [23].

As is seen from Table 1, the thiacalix[4]arene tetrakismethylphosphonic acid **2** shows stronger inhibition effect than the compound **1** for all tested enzymes. The K_i value for inhibitor **2** in the case of alkaline phosphatase from bovine intestine mucosa is about three times smaller than for its analogue **1**. The free energy for the formation of the enzyme–inhibitor complex with compound **2** is lower approximately by 0.7 kcal/mol than the value of the free energy for the binding of the compound **1**. In the cases of alkaline phosphatases from human placenta and shrimp the thiacalix[4]arene derivative **2** binds to enzyme about 10 times stronger than the inhibitor **1**, which corresponds to differences in free energy of 1.6 and 1.4 kcal/mol, respectively. It should be noted that thiacalix[4]arene derivative **2** showed low-micromolar affinity for alkaline phosphatase from bovine intestine mucosa. The inhibition constant value for this inhibitor (21 nM) is approximately 10 times lower than that for calix[4]arene bis(methylene-bisphosphonic) acid which have been reported earlier [7].

Molecular docking study

For elucidation of the molecular mechanism of inhibition the compounds **1** and **2** were docked computationally to the active site of alkaline phosphatase from shrimp. The standard parameters of the AMBER force field for Zn ion were used in computer modeling of this metalloenzyme containing three zinc ions. The study was carried out on the A subunit of the enzyme. The ligand was removed from the active site of alkaline phosphatase and then the active site was examined with inhibitors **1** and **2**. The phosphoryl residues of inhibitors were in the form of dianions. From the results of calculations, we chose variants with the minimal energy of the enzyme–inhibitor complex. The binding free energies derived from docking (ΔG_{doc}) indicate that thiacalix[4]arene derivative **2** exhibits stronger binding affinity for alkaline phosphatase than inhibitor **1** (Table 1). According to the computations performed, the thiacalix[4]arene and calix[4]arene tetrakismethylphosphonates **1**, **2** are located in the active site of shrimp alkaline phosphatase, with phosphonate residue being oriented toward two zinc ions and with the macrocyclic scaffold being arranged nearer to the surface of the enzyme. The phosphorylated thiacalix[4]arene and calix[4]arene adopt conformations which can be described as *distorted cones* where

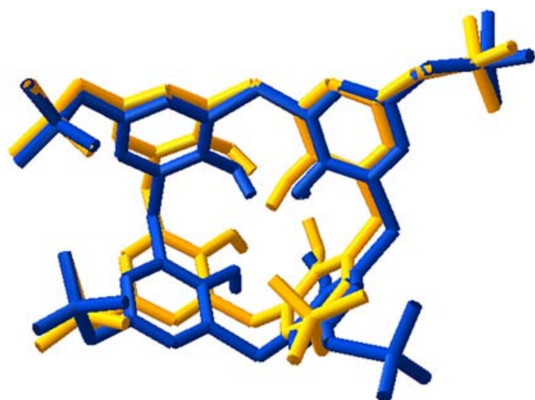
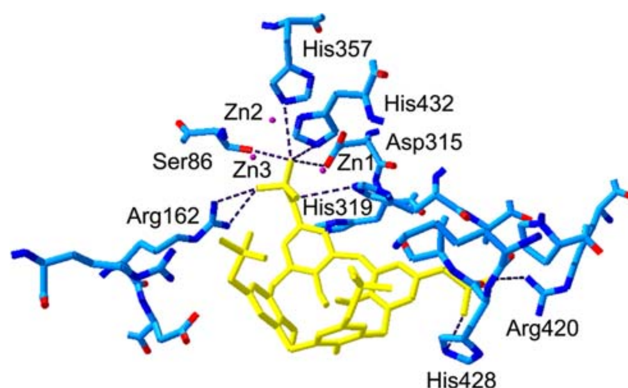


Fig. 6 Superposition of the docked conformations of inhibitor **1** (*in yellow*) and inhibitor **2** (*in blue*)



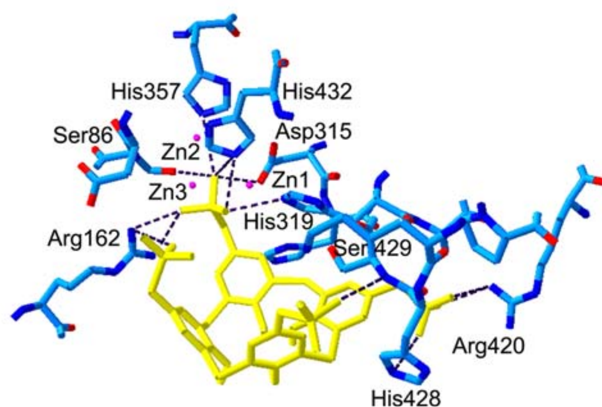


Fig. 7 The interacting modes of calix[4]arene **1** (*top*) and thiacalix[4]arene **2** (*bottom*) with the adjacent amino acid residues in the active site of shrimp alkaline phosphatase obtained after AutoDock 3.05 calculations. *Dotted lines* show the hydrogen bonds in which the inhibitor is involved

one of benzene rings is oriented almost in parallel to the main plane. The orientation of this benzene ring may be explained by the binding of its phosphonate substituent to the amino acid residues in active site of alkaline phosphatase. The calculated geometry of thiacalix[4]arene **2** was compared with the geometry of the calix[4]arene inhibitor **1** in the enzyme active site (Fig. 6). As a result, the positions of oxygen and phosphorus atoms of four phosphonate residues are characterized by average root mean square deviations of 0.45, 0.33, 1.18, and 3.06 Å, respectively. Accordingly, the deviations of 0.46, 0.57, 1.41 and 1.30 Å were obtained for corresponding carbon (and sulfur) atoms of macrocyclic cavity formed by the benzol rings and methylene or sulfide bridges. These data may reflect additional fixation of inhibitor **2** by means of two phosphonate groups which occupy the enzyme active site with an altered orientation in comparison to inhibitor **1**.

Examination of the data obtained shows that the two oxygens of phosphonate fragment of inhibitor **1** were positioned in close contact with Zn1 and one phosphonate oxygen was anchored to Zn2. These oxygens are also involved in the hydrogen bonds to Arg162, Ser86, His357, His432, Asp315 and His319. One more phosphonate fragment of inhibitor **1** provides additional fixation of inhibitor by means of hydrogen bonds with His428 and Arg420 (Fig. 7). The hydrogen bonds formed by two phosphonate fragments of inhibitor **2** with amino acid residues inside the enzyme active site are almost the same. Two oxygens of these phosphonate fragments were anchored to Zn1 and Zn2 in the similar way. However, other two phosphonate residues of more active inhibitor **2** occupy the enzyme active site to make the hydrogen bonds to Arg162 and NH-Ser429, respectively (Fig. 7). Thus, thiacalix[4]arene inhibitor **2** forms more hydrogen bonds than calix[4]arene inhibitor **1**. This is in accordance with findings that introducing sulfur atoms into structure of macrocycle leads to larger cavity size, accordingly longer hydrogen bonding between phenolic groups and greater flexibility of thiacalix[4]arene compared to calix[4]arene [31]. These properties of thiacalix[4]arene derivative **2** can provide the more effective zinc ion chelation in the enzyme active site and the more extensive network of hydrogen bonds between the oxygens of phosphonate groups and the amino acid residues of alkaline phosphatase.

Conclusions

In conclusion, we revealed the stronger inhibition activity of thiacalix[4]arene tetrakismethylphosphonate than its structural calix[4]arene analogue towards alkaline phosphatases from bovine intestine mucosa, shrimp and human placenta. Thus, the thiacalix[4]arenes can be exploited as molecular scaffold for the construction of potent phosphonate inhibitors of the enzymes.

Acknowledgments The authors greatly appreciate the possibility of using the calculation cluster of the Kyiv National Taras Shevchenko University. This research was supported by the National Academy of Sciences of Ukraine (A.I.V., L.A.K., V.P.K., grant 01/03-08). A.B.D.

and V·I.K. thank the NASU-RFBR grant for partial support of the work.

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