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Calix[4]arene methylenebisphosphonic acids as calf intestine alkaline phosphatase inhibitors

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Calix[4]arenes bearing one or two methylenebisphosphonic acid fragments were prepared *via* addition of diethylphosphite to the parent calix[4]arene aldehydes. The resulting compounds displayed stronger inhibition of calf intestine alkaline phosphatase than simple methylenebisphosphonic or 4-hydroxyphenyl methylenebisphosphonic acids. The action of these phosphorylated calix[4]arenes is concordant with partial mixed-type inhibition. The inhibition constants K_i and K_i' for the calix[4]arene bis(methylenebisphosphonic) acid in Tris-HCl buffer at pH 9 are 0.38 μM and 2.8 μM respectively. The replacement of the phosphoric acid moieties on the macrocycle with diethylphosphonates results in a sharp decrease of its inhibitory action. Preorganizing phosphonic acid fragments using a calixarene platform therefore provides a promising approach for the design of efficient alkaline phosphatase inhibitors.

Introduction

With our increasing understanding of the role of phosphorylation in regulating biochemical events, there is growing interest in the bioactivities associated with natural and synthetic phosphonic acids.[1] The mode of action of these compounds is generally associated with the metabolic pathways involving monoalkylphosphates.

Non-specific alkaline phosphatases catalyze phosphoryl transfer in the hydrolysis or the transphosphorylation of phosphate monoesters.[2,3] These are involved in a variety of biological phenomena, including recent studies demonstrating their heightened expression in osteoblasts,[4] with possible consequences for osteoporotic patients. Single crystal X-ray analyses have revealed the presence of a magnesium and two zinc ions in the active site of the alkaline phosphatase of *Escherichia coli*, which are able to bind inorganic phosphate,[5] vanadate,[6] phosphonoacetic or mercaptomethylphosphonic acids.[7] Mammalian alkaline phosphatases display greater variety in their structural features, including a diversity of loop regions located near the active site.[8] The hydrophobic isoforms of these enzymes bear lipophilic anchors of importance to their membrane-associated function.[9]

Despite their structural similarities with natural monoalkylphosphates, alkylphosphonic acids are only weak inhibitors of alkaline phosphatases. By taking advantage of the greater affinity of sulfur for the metal ions in the active site, the introduction of a thiol group into the structure of the phosphonic acid was found to considerably improve the binding of the inhibitor to bovine alkaline phosphatase.[10] Methylenebisphosphonic acid **1**, however, causes only insignificant inhibition of alkaline phosphatase.[10]

The preorganization of several high affinity groups on the surface of a molecular platform is often used in the design of highly efficient and selective ligands and receptors. Calixarenes represent a very commonly used class of molecular platforms readily available through the cyclocondensation of *p*-substituted phenols with formaldehyde.[11] They were recently employed for the preorganization of multiple biologically relevant groups such as amino acids and small peptides.[12] Hence peptidocalixarenes are able to complex cytochrome C and other proteins,[13] while calixarenes bearing several permethylammonium residues interact with nucleotides and nucleic acids.[14] Amphiphilic sulfo- and sulfonatocalix[*n*]arenes were demonstrated to bind to bovine serum albumin.[15]

We hypothesized that the preorganization of two fragments of methylenebisphosphonic acid at the wide rim of calix[4]arene (Fig. 1) would result in improved affinity, and hence inhibitory activity, through their simultaneous coordination with metal ions at the binding site of alkaline phosphatase. Herein we report on the synthesis and inhibitory activity of calix[4]arene bisphosphonic acids **2** and **3** (Fig. 1).

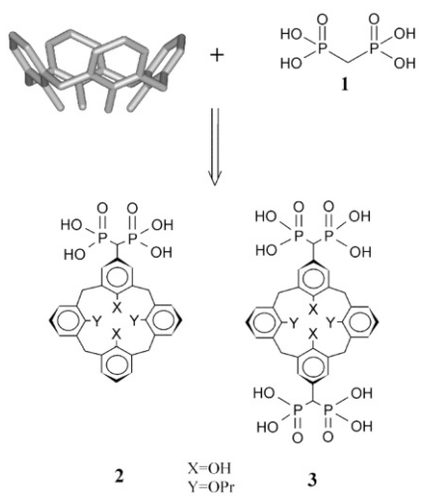

Fig. 1 Molecular design of novel alkaline phosphatase inhibitors.

Results and discussion

Synthesis

Methylenebisphosphonates are conventionally obtained by the Arbuzov reaction of geminal dihaloalkanes with alkylphosphites.[16] 4-Hydroxy-3,5-di-*tert*-butylbenzaldehyde has been reported to react with two equivalents of dialkylphosphite in the presence of diethylamine as a base to give 4-hydroxy-3,5-di-*tert*-butylphenyl methylenebisphosphonate in moderate yield.[17] We employed a slightly modified procedure for the synthesis of calix[4]arene bisphosphonic acids **2** and **3** as well as the model 4-hydroxyphenyl methylenebisphosphonic acid **4** from the readily available mono- and diformyl calixarenes **5** and **6**[18] and 4-hydroxybenzaldehyde **7**, respectively.

Scheme 1 highlights the sequence of events in the transformation. The reaction of 4-hydroxybenzaldehyde **7** with

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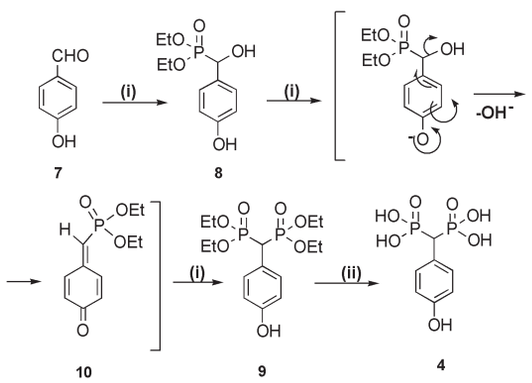
one equivalent of sodium diethylphosphite generated *in situ* in dioxane gives hydroxyphosphonate **8** in 80% yield. Subsequently **8** is converted quantitatively to bisphosphonate **9** by a large excess of $(EtO)_2P(O)Na$ in diethylphosphite/dioxane solution.[19] A possible mechanism for this reaction involves the base-mediated elimination of hydroxide ion from hydroxyphosphonate **8**, yielding the highly reactive quinonemethide **10**, to which the addition of diethylphosphite leads to bisphosphonate **9**. The treatment of either benzaldehyde or anisaldehyde under the same conditions fails to give the respective bisphosphonates, in agreement with the proposed intermediates.[19] The use of a large excess of sodium diethylphosphite in dioxane/diethylphosphite allows the conversion of aldehyde **7** to bisphosphonate **9** in a single step. The standard dealkylation of bisphosphonate **9** with Me_3SiBr and subsequent methanolysis of the silyl esters affords bisphosphonic acid **4**[16b] in almost quantitative yield.



Scheme 1 Synthesis of 4-hydroxyphenyl methylenebisphosphonic acid **4**. Reagents and conditions: (i) $(EtO)_2P(O)H$, sodium metal; (ii) 1) bromotrimethylsilane, room temperature, 30 h, 2) MeOH, 50 °C, 2 h.

Under identical conditions, calixarene aldehydes **5** and **6** provided bisphosphonate **11** and bis(bisphosphonate) **12** respectively (Scheme 2), with hydroxyphosphonates **13** and **14**[20] as intermediates.[21] Calixarene bisphosphonate **11** and bis(bisphosphonate) **12** were converted quantitatively to the corresponding acids **2** and **3** by subsequent treatment with Me_3SiBr and methanol (Scheme 2).

The ^{1}H NMR spectra of calix[4]arenes **2**, **3**, **11** and **12** contain pairs of AB doublets (J = 13 Hz) for the methylene protons of the bridges indicative of the *syn* orientation of all four aromatic rings. These doublets are separated by 0.75–0.85 ppm, indicating a C_{2v}-symmetrical pinched cone conformation.[22] Although two pinched cone conformations are theoretically possible for molecules **3** and **12** – one with quasi-parallel and the other with quasi-coplanar arylbisphosphonate fragments – the ^{1}H NMR spectrum does not allow us to distinguish these two structures.

Assay results

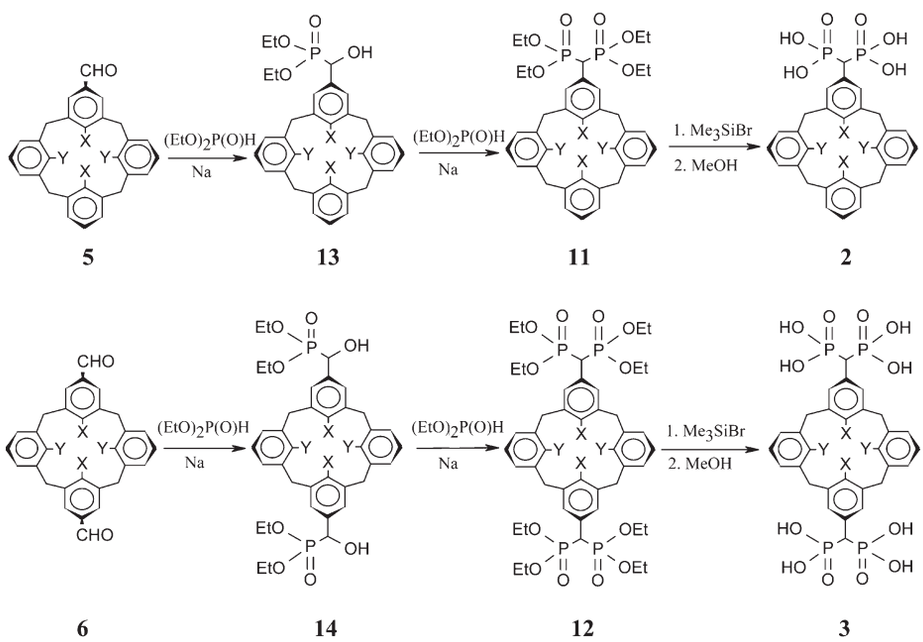
The inhibitory activities of compounds **1–4** and **12** were investigated in the calf intestine alkaline phosphatase catalyzed hydrolysis of *p*-nitrophenylphosphate. The K_m and V_{max} values for the control reaction, and apparent values K_m' and V_{max}' for the reactions at fixed inhibitor concentrations were determined from the Lineweaver–Burk plots (Fig. 2). The influence of compounds **1–4** and **12** on the enzyme activity is in agreement with mixed-type or partial mixed-type inhibition. The correlations between K_m' and V_{max}' and the concentration of compounds **1**, **4** and **12** are linear, whereas in the case of compounds **2** and **3**, there are negative deviations from linearity (Fig. 3). Thus compounds **1–4** and **12** not only result in a reduction of the apparent substrate–enzyme affinity, but also affect the ability of the enzyme–substrate complex to undergo its transformation into the reaction products *via* full or partial inhibition (Scheme 3).

The dissociation constants of the enzyme inhibitor (K_i) and the enzyme–substrate inhibitor (K_i') complexes with **1**, **4** and **12** were calculated using the kinetic model for mixed-type inhibition ($k_2' = 0$). In the case of compounds **2** and **3**, K_i and K_i' values were obtained according to the kinetics for partial mixed-type inhibition ($k_2'/k_2 < 1$). The rate of the formation of *p*-nitrophenol during hydrolysis of *p*-nitrophenylphosphate in the presence of an inhibitor (Scheme 3) is given by eqn. (1):[23]

$$V = \frac{(k_2 + k_2'[I]/K_i')[E][S]}{K_S(1+[I]/K_i) + [S](1+[I]/K_i')} \quad (1)$$

The ratios V_{max}'/V_{max} and K_m'/K_m can be expressed ($K_S \approx K_m$) by eqn. (2) and eqn. (3).[24]

$$1/(V_{max}'/V_{max} - 1) = \frac{K_i'}{(k_2'/k_2 - 1)[I]} + \frac{1}{k_2'/k_2 - 1} \quad (2)$$



Scheme 2 Synthesis of calix[4]arene methylenebisphosphonic acids **2** and **3**.

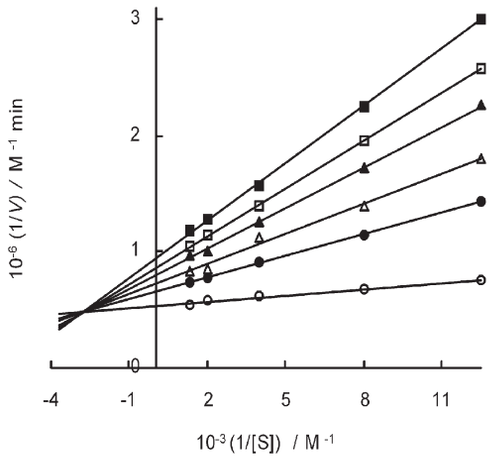


Fig. 2 Lineweaver–Burk graphical representation of calf intestine alkaline phosphatase inhibition by calix[4]arene **3**. The reaction mixtures contain 0.1 M Tris-HCl buffer (pH 9) and various substrate concentrations. The concentrations of the inhibitor were 0 (○), 1.1 μM (●), 2 μM (△), 3 μM (▲), 4 μM (□) and 5 μM (■).

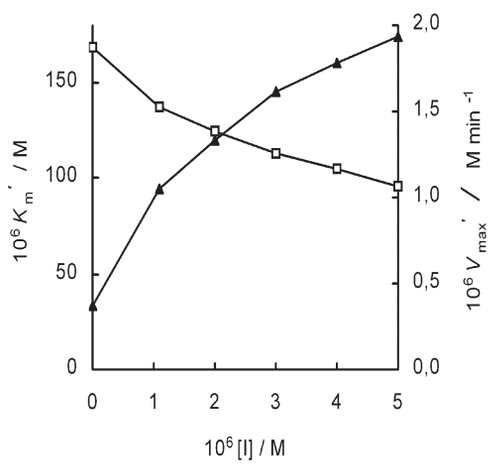
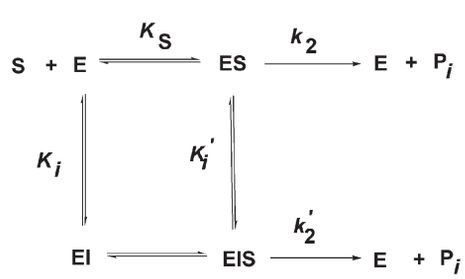


Fig. 3 Dependencies of K_m' (▲) and V_{max}' (□) values on the concentration of calix[4]arene **3**.



Scheme 3

$$1/(K_m'/K_m - 1) = \frac{1}{K_i'/K_i - 1} + \frac{K_i'}{[I](K_i'/K_i - 1)} \qquad (3)$$

The inhibition constants K_i and K_i' are obtained from the ordinate intercept and the slope of the plot of $\{(K_m'/K_m) - 1\}^{-1}$ *versus* 1/[I]. The ordinate intercept of plot of $\{(V_{max}'/V_{max}) - 1\}^{-1}$ *versus* 1/[I] gives the relative value k_2'/k_2.

The inhibition constants for calix[4]arene **2** were found to be considerably lower than those of methylenebisphosphonic acid **1**. Remarkably, the K_i value for calix[4]arene bisphosphonic acid **2** is about 10 times smaller than that of model compound **4** (Table 1). As expected, though, it is calixarene **3**, bearing two methylenebisphosphonic acid motifs, which displays the strongest inhibition with K_i = 0.38 μM, K_i' = 2.8 μM and the relative value k_2'/k_2 = 0.3. The affinity of alkaline phosphatase for calix[4]arene **3** is approximately two orders of magnitude higher than for *p*-nitrophenylphosphate, and three orders of magnitude higher than for the protected calixarene **12** (Table 1).

Table 1 Inhibition constants for the inhibitors **1–4** and **12** of calf intestine alkaline phosphatase[a]

Inhibitor	K_i/μM	K_i'/μM
1	67 ± 5	750 ± 100
2	2.5	46
3	0.38	2.8
4	22 ± 4	290 ± 110
12	820 ± 180	8500 ± 2100

[a] 0.1 M Tris-HCl buffer, pH 9; K_m value 36 ± 9 μM.

In the active site of the bacterial alkaline phosphatase, the distance separating the two zinc cations is known to be 3.9 Å, whereas the magnesium ion is positioned at a distance of 5 Å and 7 Å respectively from each of these atoms.[5] The active site of calf intestine alkaline phosphatase is believed to share these structural features.[8] The mechanism of catalysis by alkaline phosphatase includes the formation of the enzyme–substrate complex, phosphoryl transfer from this complex to a nucleophilic serine group, the hydrolysis of the covalent phosphorylated enzyme intermediate, the formation of a non-covalent enzyme–phosphate complex and the release of inorganic phosphate.[3,5,8] Presumably, the activity of compounds **1–4** is a consequence of their coordination to the cations in the binding site. The lipophilicity of the calix[4]arene scaffold may tighten the binding of **2** and **3** through interactions with non-polar residues in the vicinity of the catalytic domain. The higher inhibitory activity of bis(bisphosphonic) acid **3** compared to bisphosphonic acid **2** can be attributed to better metal chelation or to synergistic interactions with two of the metal ions. A plausible alternative synergy may be the result of additional electrostatic interactions between the anions of **3** and a positively charged arginine[3,5] at the enzyme's active site. The main contribution to the stability of the enzyme–inhibitor complex remains that of the phosphoryl groups of calix[4]arenes **2** and **3**, as the protected analogous phosphonate **12** displays no inhibitory activity.

In conclusion, calix[4]arenes prove to be versatile molecular platforms for the construction of efficient alkaline phosphatase inhibitors. The efficient inhibitory activity of calixarene **3** is the consequence of the preorganization of two methylenebisphosphonic acid substructures. The efficient coordination of **3** with the metal cations in the enzyme's active site and the additional van der Waals interactions brought about by the scaffold itself are probably responsible for its high activity.

Experimental

Materials

^{1}H and ^{31}P NMR spectra were recorded on a VXP 300 spectrometer operating at 300 MHz and 121.5 MHz respectively. The chemical shifts are reported using internal tetramethylsilane and external 85% H_3PO_4 as references. The melting points were determined on a Boetius apparatus and are uncorrected. Bromotrimethylsilane was freshly distilled. All reactions were carried out under dry argon. Formylcalix[4]arenes **5** and **6** were synthesized according to known procedures.[25] Calf intestine alkaline phosphatase was purchased from Fluka and used without additional purification. The activity of enzyme was 1.1 U mg^{-1}. *p*-Nitrophenylphosphate was obtained from Sigma.

General procedure for synthesis of bisphosphonates 9, 11 and 12

To diethyl phosphite (5 ml) was cautiously added sodium metal (0.3 g, 13 mmol) in small portions at room temperature. Compound **5** (1 mmol), **7** (1 mmol) or **6** (0.5 mmol) was added to the resulting solution, as appropriate. The reaction mixture was stirred at room temperature for 48 h and then quenched with water (100 ml) and extracted with chloroform (3 × 50 ml). The chloroform layer was concentrated under vacuum and the residue was washed with hexane and dried *in vacuo.* Yields were almost quantitative.

Tetraethyl 4-hydroxyphenylmethylene-1,1-bisphosphonate 9

White powder. Yield 99%, mp 88–89 °C (from hexane) (mp 89 °C[16a]). Found: C, 47.45; H, 6.75; P, 16.41. $C_{15}H_{26}O_7P_2$ requires C, 47.37; H, 6.89; P, 16.29%; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.05, 1.28 (two t, 6 H + 6 H, *J* 6.9 Hz, diastereotopic $POCH_2CH_3$), 3.61 (t, 1 H, *J* 25 Hz, P_2CH), 3.89, 4.04, 4.14 (three m, 2 H + 2 H + 4 H, diastereotopic $POCH_2CH_3$); 6.80 (d, 2 H, *J* 7.5 Hz, Ar*H*), 7.21 (d, 2 H, *J* 7.5 Hz, Ar*H*), 8.42 (s, 1 H, ArO*H*); δ_P (121.421 MHz; $CDCl_3$; H_3PO_4) 20.4.

5-Bis(diethoxyphosphoryl)methyl-25,27-dipropoxycalix[4]-arene 11

Light oil. Yield 98%. Found: C, 61.42; H, 5.80; P, 9.15. $C_{43}H_{56}O_{10}P_2$ requires C1, 61.58; H, 5.91; P, 9.07%. δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.86, 1.22 (two t, 6 H + 6 H, *J* 6.9 Hz, diastereotopic $POCH_2CH_3$), 1.24 (t, 6 H, *J* 7.5 Hz, $OCH_2CH_2CH_3$), 2.04 (m, 4 H, $OCH_2CH_2CH_3$), 3.36, 3.38 (two d, 2 H + 2 H, *J* 13.2 Hz, $ArCH_{2eq}$), 3.58 (t, 1 H, *J* 25 Hz, P_2CH), 3.70, 3.91 (two m, 2 H + 2 H, diastereotopic $POCH_2CH_3$), 3.98 (t, 4 H, J_{HH} 6.9 Hz, $OCH_2CH_2CH_3$), 4.07 (m, 4H, diastereotopic $POCH_2CH_3$), 4.30 (d, 4 H, *J* 3.2 Hz, $ArCH_{2ax}$); 6.88, 6.91, 7.06 (three d, 2 H + 2 H + 2 H, *J* 7.5 Hz, *m*-Ar*H*), 6.67 (m, 3H, *p*-Ar*H*), 7.20 (s, 2H, *m*-Ar*H*), 8.15, 8.21 (two s, 1 H + 1 H, ArO*H*); δ_P (121.421 MHz; $CDCl_3$; H_3PO_4) 19.7; *m*/*z* (FAB MS) 795.5 ($[M + H]^+$, 100%).

5,17-Bis[bis(diethoxyphosphoryl)methyl]-25,27-dipropoxycalix[4]arene 12

Light oil. Yield 99%. Found: C, 57.69; H, 7.15; P, 11.53. $C_{52}H_{76}O_{16}P_4$ requires C, 57.77; H, 7.09; P, 11.46%. δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.84, 1.24 (two t, 12 H + 12 H, *J* 6.9 Hz, diastereotopic $POCH_2CH_3$), 1.31 (t, 6 H, *J* 7.5 Hz, $OCH_2CH_2CH_3$), 3.60 (t, 2 H, *J* 25 Hz, P_2CH), 2.05 (m, 4H, $OCH_2CH_2CH_3$), 3.38 (d, 4 H, *J* 13.2 Hz, $ArCH_{2eq}$), 3.71, 3.89 (two m, 4 H + 4 H, diastereotopic $POCH_2CH_3$), 3.98 (t, 4 H, *J* 6.9 Hz, $OCH_2CH_2CH_3$), 4.08 (m, 8 H, diastereotopic $POCH_2CH_3$), 4.28 (d, 4 H, *J* 13.2 Hz, $ArCH_{2ax}$), 6.61 (t, 2 H, *J* 7.5 Hz, *p*-Ar*H*), 6.87 (d, 4 H, *J* 7.5 Hz, *m*-Ar*H*); 7.22 (s, 4 H, *m*-Ar*H*), 8.13 (s, 2 H, ArO*H*); δ_P (121.421 MHz; $CDCl_3$; H_3PO_4) 19.36; *m*/*z* (FAB MS) 1081.4 ($[M + H]^+$, 100%).

General procedure for synthesis of bisphosphonic acids 2, 3 and 4

An eight-fold molar excess of bromotrimethylsilane per phosphonate group was added to a solution of bisphosphonate **9**, **11** or **12** (0.1 mmol) in dry chloroform (5 ml). The reaction mixture was stirred at room temperature for 30 h and then was concentrated under reduced pressure. The residue was dissolved in absolute methanol (15 ml), the resulting mixture stirred at 50 °C for 2 h, and then concentrated and dried *in vacuo* (0.05 mmHg) for 10 h.

5-Bis(dihydroxyphosphoryl)methyl-25,27-dipropoxycalix[4]-arene 2

Light powder. Yield 99%, mp 132–134 °C (from methanol). Found: C, 64.85; H, 7.15; P, 7.82. $C_{35}H_{40}O_{10}P_2$ requires C, 64.98; H, 7.10; P, 7.79%. δ_H (300 MHz; DMSO-d_6; Me_4Si) 1.31 (t, 6 H, *J* 7.5 Hz, $OCH_2CH_2CH_3$), 2.04 (m, 4 H, $OCH_2CH_2CH_3$), 3.37, 3.44 (two d, 4H, *J* 13.2 Hz, $ArCH_{2eq}$), 3.25 (t, 1 H, *J* 25 Hz, P_2CH), 3.98 (wide s, 4H, $OCH_2CH_2CH_3$), 4.19 (d, 4 H, *J* 13.2 Hz, $ArCH_{2ax}$), 6.57, 6.78 (two t, 2 H + 1 H, *J* 7.5 Hz, *p*-Ar*H*), 7.1 (m, 2 H + 2 H + 2 H, *m*-ArH); 7.23 (s, 2 H, *m*-Ar*H*), 8.46, 8.52 (two s, 1 H + 1 H, ArO*H*); δ_P (121.421 MHz; DMSO-d_6; H_3PO_4)18.2; *m*/*z* (FAB MS) 683.4 ($[M + H]^+$, 75%).

5,17-Bis[bis(dihydroxyphosphoryl)methyl]-25,27-dipropoxycalix[4]arene 3

Light powder. Yield 99%, mp 145–147 °C (from methanol). Found: C, 50.61; H, 5.12; P, 14.52. $C_{36}H_{44}O_{16}P_2$ requires C, 50.48; H, 5.18; P, 14.46%. δ_H (300 MHz; DMSO-d_6; Me_4Si) 1.31 (t, 6 H, *J* 7.5 Hz, $OCH_2CH_2CH_3$), 2.05 (m, 4 H, $OCH_2CH_2CH_3$), 3.25 (t, 2 H, *J* 25 Hz, P_2CH), 3.43 (d, 4 H, *J* 13.2 Hz, $ArCH_{2eq}$), 3.93 (t, 4 H, *J* 6.9 Hz, $OCH_2CH_2CH_3$), 4.21 (d, 4 H, $ArCH_{2ax}$, *J* 13.2 Hz), 6.58 (t, 2 H, *J* 7.5 Hz, *p*-Ar*H*), 6.92 (d, 4 H, *J* 7.5 Hz, *m*-Ar*H*); 7.26 (s, 4 H, *m*-Ar*H*), 8.46 (s, 2 H, ArO*H*); δ_P (121.421 MHz; DMSO-d_6; H_3PO_4) 18.61.

4-Hydroxyphenylmethylene-1,1-bisphosphonic acid 4

Light powder. Yield 98%, mp 148–150 °C (from methanol). Found: C, 31.45; H, 3.64; P, 23.22. $C_7H_{10}O_7P_2$ requires C, 31.36; H, 3.76; P, 23.11%. δ_H (300 MHz; DMSO-d_6; Me_4Si) 3.46 (t, 1 H, *J* 25 Hz, P_2CH), 6.67 (d, 2 H, *J* 7.5 Hz, Ar*H*), 7.26 (d, 2 H, *J* 7.5 Hz, Ar*H*); δ_P (121.421 MHz; DMSO-d_6; H_3PO_4) 18.5; *m*/*z* (FAB MS) 537.9 ($[2M + H]^+$, 10%), 269.0 ($[M + H]^+$, 30).

General procedure for the enzymatic assays of alkaline phosphatase activity

The influence of compounds **1–4** and **12** on the rate of *p*-nitrophenylphosphate hydrolysis catalyzed by alkaline phosphatase was determined in 0.1 M Tris-HCl buffer at pH 9. The kinetics of inhibition were studied at various concentrations for each inhibitor: **1**: 50 μM, 100 μM, 150 μM, 200 μM; **2** and **4**: 10 μM, 20 μM, 30 μM, 40 μM; **3**: 1.1 μM, 2 μM, 3 μM, 4 μM; 5 μM; **5**: 250 μM, 500 μM, 750 μM, 1000 μM. The mixtures containing the buffer, the substrate (0.08–1.0 mM) and inhibitor were incubated for 5 min at 23 °C, and reactions were initiated by the addition of enzyme (3 μg ml^{-1}). 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 18300 M^{-1} cm^{-1}.

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