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Phosphoryl Transfer Processes Promoted by a Trifunctional Calix[4]arene inspired by DNA Topoisomerase I

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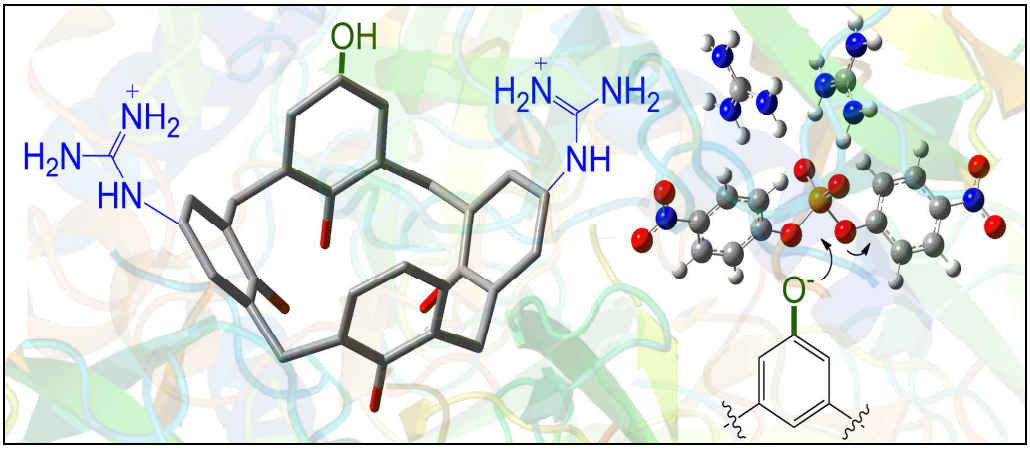


Keywords: reaction mechanisms • enzyme mimics • supramolecular catalysis • artificial nucleases • transphosphorylation

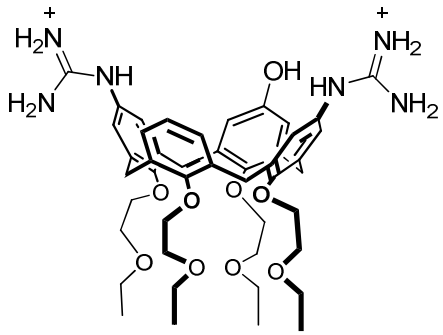
ABSTRACT

The *cone*-calix[4]arene derivative (**1**H_3)$^{2+}$, decorated at the upper rim with two guanidinium units and a phenolic hydroxyl in an ABAH functionalization pattern, effectively promotes the cleavage of the DNA model compound bis(*p*-nitrophenyl) phosphate (BNPP) in 80% DMSO solution, at pH values in the range 8.5 – 12.0. The pH-dependence of the kinetics was found to be fully consistent with the results of the potentiometric titration of the triprotic acid (**1**H_3)$^{2+}$. At pH 9.5 the rate enhancement of *p*-nitrophenol liberation from BNPP relative to background hydrolysis is 6.5×10^4-fold at 1 mM concentration of the calix[4]arene derivative. Experimental data clearly point to the effective cooperation of the three active units and to the involvement of the phenolate moiety as a nucleophile in the phosphoryl transfer step. Subsequent liberation of a second equivalent of *p*-nitrophenol from the phosphorylated calixarene intermediate is conceivably promoted by the "built-in" guanidine/guanidinium catalytic dyad.

INTRODUCTION

Design of effective catalysts of the cleavage of the highly inert phosphodiester function of nucleic acids and model compounds has been a challenging target since many decades.[1] The strategic presence of arginine residues in the active site of many nucleases has inspired the design of artificial phosphodiesterases endowed with one or more guanidinium/guanidine units that proved crucial in the catalytic mechanism.[2] Among systems based on the guanidinium motif, many examples of metal-free organocatalysts,[3,4] as well as of mimics of metalloenzymes such as staphylococcal nuclease, have been reported.[5] Still quite rare, on the other hand, are examples of mimics of the catalytic triad at the active site of *Human DNA topoisomerase I*[6] composed of a tyrosine and two arginine residues.[4] It was found that effective cleavage of DNA substrates proceeds *via* a transphosphorylation mechanism that involves the hydroxyl of the tyrosine unit, and activation by the guanidinium moiety of the two arginine residues.

The upper rim of *cone*-calix[4]arenes has been widely exploited as a suitable platform for the design of supramolecular catalysts.[7] As a convenient strategy for the development of trifunctional mimics of the active site of *Human DNA topoisomerase I*, here we focus on the preorganization of the target catalytic triad on a calix[4]arene scaffold. The development of synthetic strategies for the heterofunctionalization of the upper rim of calix[4]arenes is of crucial importance for the design of enzyme mimics featuring the cooperation of different active units. We report herein the synthesis of calix[4]arene **1**H, decorated at the upper rim with two guanidinium units and a phenolic hydroxyl group in a ABAH pattern of functionalization, and a kinetic investigation of the activity of this system in the cleavage of the DNA model compound bis(*p*-nitrophenyl) phosphate (BNPP).



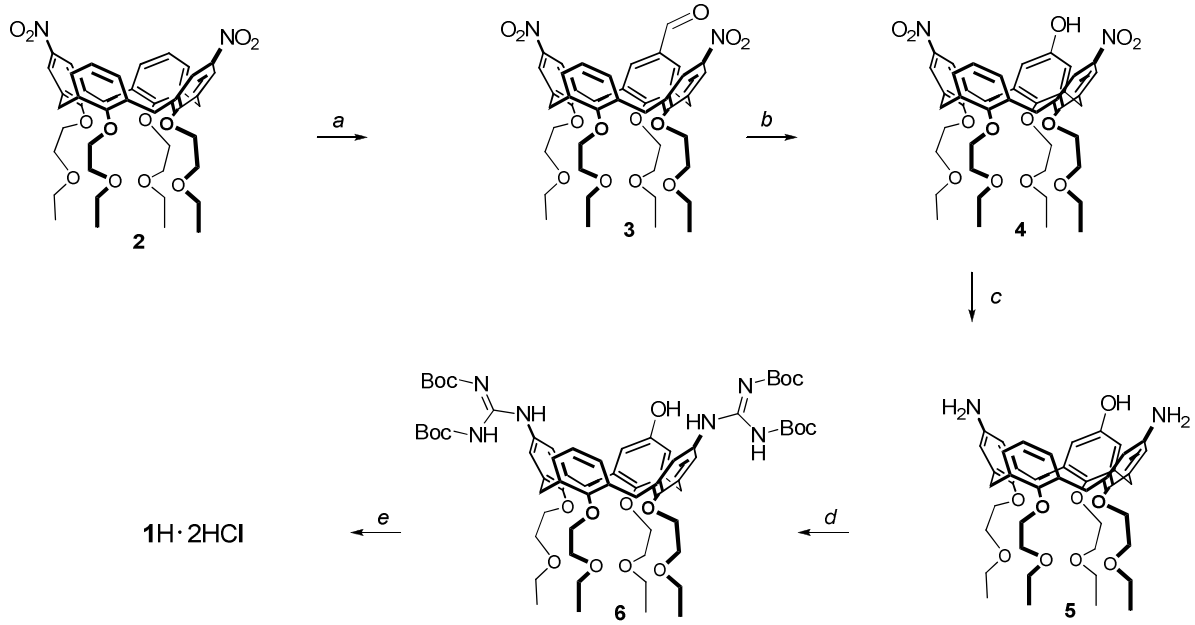
$(\mathbf{1}H_3)^{2+}$

RESULTS AND DISCUSSION

Calixarene Synthesis Compound **1**H was synthesized as the corresponding bis(hydrochloride) starting from 5,17-dinitro-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene **2**,[8] according to Scheme 1.

In the first step of the synthesis, the dinitroformyl derivative **3** was obtained from calixarene **2** by Gross formylation under controlled conditions (-10 °C, 2 h).[9] Interestingly, **3** was obtained as the main product (59% isolated yield), in an amount larger than the expected statistical distribution, yet lower than the 84% yield obtained in the monoformylation of the analogous dinitrotetrapropoxycalix[4]arene derivative under similar conditions.[10] This finding can be ascribed to the presence of the ethoxyethyl chains at the lower rim of **2** that can complex the Lewis acid and thus influence the electrophilic aromatic substitution at the upper rim. Subsequent Baeyer-Villiger oxidation,[11] followed by the hydrolysis of the resulting formate ester (not isolated), led to the dinitrohydroxy derivative **4** (81% yield). In the following step calixarene **4** was reduced[12] to **5** in quantitative yield. Compound **5** was converted into the Boc-protected diguanidino derivative **6** (42% yield) according to a published procedure[3a,3c,3d] by using [*N*,*N*'–bis(Boc)]thiourea and $HgCl_2$. The target compound **1**H was obtained as bis(hydrochloride) (93% yield) after removal of the Boc protecting groups. All these newly synthesized ABAH derivatives show HR-MS, ^{1}H and ^{13}C NMR in agreement with their structures. The ABAH substitution pattern is clearly supported by the symmetry of the NMR spectra in the aromatic and methylene bridge regions (see SI, pp. S2-S6). In particular, the $ArCH_2Ar$ ^{1}H NMR signals displayed the two expected AX systems (in a 1:1 ratio) around 4.5 and 3.2 ppm for the axial and equatorial protons, respectively.

Scheme 1. Synthesis of 1H.[a]



[a] *a*: $SnCl_4$, Cl_2CHOCH_3; dry $CHCl_3$. *b*: (1) *m*-CPBA; DCM. (2) NaOH 2M, MeOH-H_2O 4:1. *c*: $NaBH_4$, $NiCl_2{\cdot}6H_2O$; MeOH. *d*: [*N*,*N*'–bis(Boc)]thiourea, $HgCl_2$, NEt_3; dry DMF. *e*: (1) TFA, TES; DCM. (2) HCl 1M; EtOH.

Acid-base Titrations The determination of acidity constants of calixarene $(\mathbf{1}H_3)^{2+}$ is a prerequisite for a rational investigation of its activity in phosphoryl transfer reactions. The medium used in the titration experiments was a mixture of DMSO/H_2O 80:20 (v/v), hereafter referred to as 80% DMSO. This mixture was successfully used for pK_a determination,[13,14] for the investigation of the hydrolysis of phosphodiesters,[3a-3d,5a,5b,15,16] and for the dephosphorylation of the terminal phosphate of ATP.[17] The autoprotolysis constant of water (pK_w) in 80% DMSO is as high as 18.4.[18] This implies that the water dissociation is significantly inhibited and that the pH value of a neutral solution is 9.2 in this medium.

The dihydrochloride of **1**H (2.0 mM) was potentiometrically titrated with a standard solution of Me_4NOH in the same solvent mixture. Figure 1 shows the titration plot and the distribution diagram calculated on the basis of the given pK_a values.

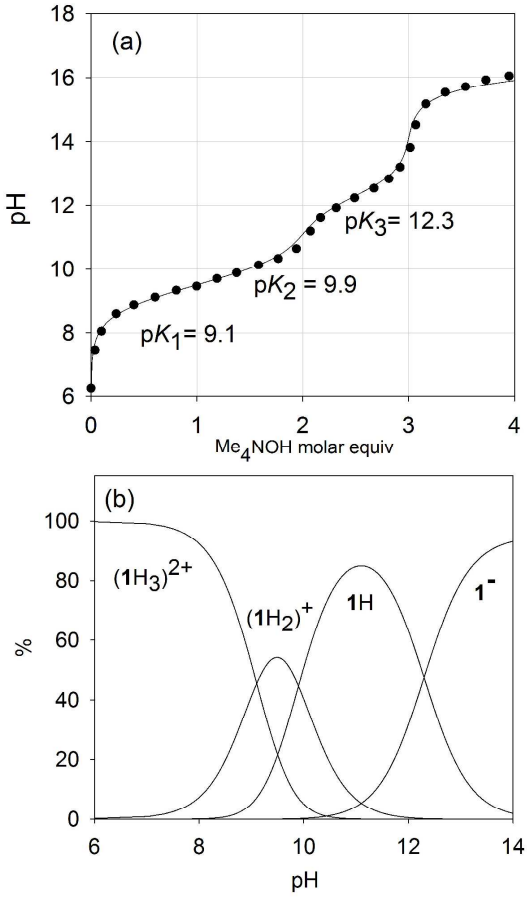
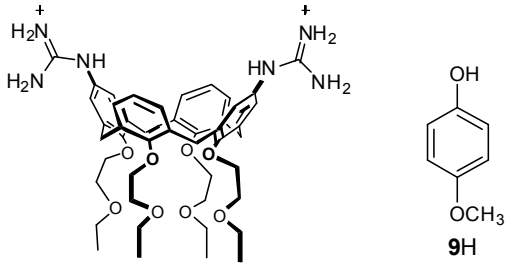


Figure 1. Titration of (**1**H$_3$)$^{2+}$ (2.0 mM) with Me$_4$NOH in 80% DMSO, 25 °C, in the presence of 10 mM Me$_4$NClO$_4$ (*a*), and distribution diagram of the species as a function of pH (*b*).

Titration of (**1**H$_3$)$^{2+}$ with Me$_4$NOH showed, as expected, the presence of three titratable protons. Comparison with the pK_a values of model compounds (Chart 1) would suggest that the most acidic proton (pK_{a1}=9.1) belongs to a guanidinium unit, whose acidity is strongly enhanced by electrostatic repulsion with the other guanidinium unit, and presumably unaffected by the presence of the neutral phenolic hydroxyl. Another possibility, however, is that the most acidic proton belongs to the phenolic hydroxyl, whose acidity is expected to be much higher than that of the model compound *p*-methoxyphenol (Chart 1) on account of the strong electrostatic stabilization of the phenolate ion provided by the neighboring guanidinium units. The above hypotheses do not represent mutually exclusive alternatives, as it might well be that (**1**H$_2$)$^+$ is actually a mixture of tautomeric forms *A* and *B* (Figure 2).[19]



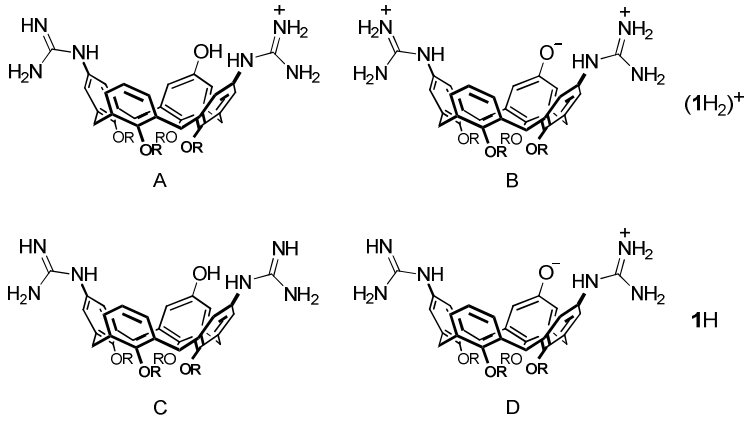
$(\mathbf{7}H_2)^{2+}$

pK_{a1} = 9.3
pK_{a2} = 11.5

9H

pK_a = 12.7

Chart 1. pK_a values of model compounds in 80% DMSO at 25 °C (data from ref. 3d for $(\mathbf{7}H_2)^{2+}$; for compound **9**H see SI, p. S9).



$(\mathbf{1}H_2)^+$

1H

Figure 2. Possible tautomeric structures of $(\mathbf{1}H_2)^+$, (top), and **1**H, (bottom).

As to the second deprotonation step leading to the neutral species **1**H, comparison of the pK_{a2} value of 9.9 with the corresponding value of the model compound lacking the phenolic hydroxyl, pK_a=11.5 (Chart 1), rules out the possibility that the phenolic hydroxyl acts as an innocent spectator also in this deprotonation step. It seems more likely that ionization of the phenolic hydroxyl is extensive and, consequently, that the equilibrium between tautomers *C* and *D* (Figure 2) is mostly shifted towards the zwitterionic form *D*.

The question of the involvement of the phenolic hydroxyl during the first and second titration step was decided by UV-Vis spectrophotometric titrations of $(\mathbf{1}H_3)^{2+}$ with Me_4NOH (Figure 3). Whereas addition of 1 mol equiv of base caused a complete conversion of *p*-methoxyphenol into *p*-methoxyphenolate ion, (Figure 3*a*), the same amount of base transformed only ca. 55% of the phenolic hydroxyl of $(\mathbf{1}H_3)^{2+}$ into the corresponding phenolate ion, (Figure 3*b*). Proton removal from the phenolic hydroxyl was complete, or very nearly so, on addition of a second equivalent of base. Thus, combination of potentiometric and spectrophotometric titration data indicates that

the equilibrium between the tautomers *A* and *B* is well balanced, with only a slight prevalence of *B*, while the equilibrium between *C* and *D* is strongly biased towards zwitterion *D*.

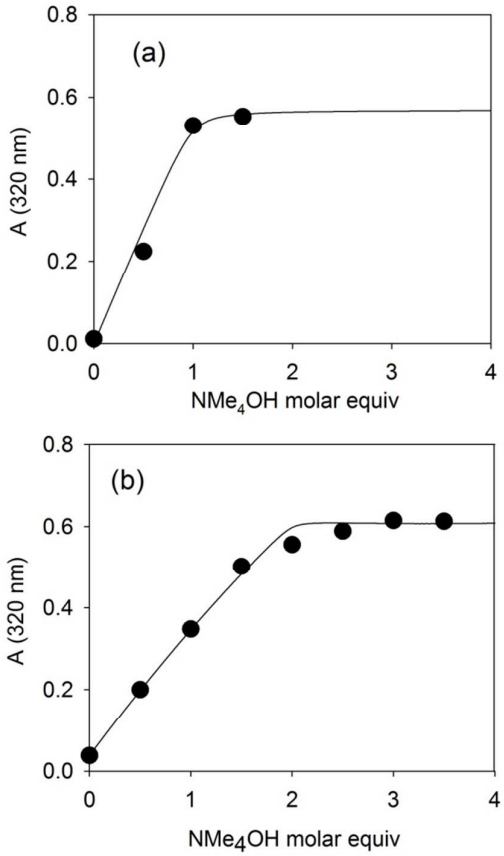
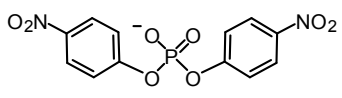


Figure 3. UV-Vis titration of 0.2 mM *p*-methoxyphenol (*a*) and 0.2 mM calixarene **1**H·2HCl (*b*) with Me_4NOH (25 °C, DMSO 80%). The full lines are calculated (see SI p. S7).

Cleavage of BNPP A set of kinetic experiments was carried out with the DNA model bis(*p*-nitrophenyl) phosphate (BNPP) substrate. Compound $(\mathbf{1}H_3)^{2+}$ was added to the reaction medium buffered in the pH range 8.5 – 12.0 with 0.10 M *N*,*N*-diisopropyl ethanolamine/perchlorate salt buffer.



The results of the kinetic experiments are listed in Table 1 (entries 1 – 7) as pseudo-first-order specific rates $k_{obs} = v_0/[BNPP]$, where v_0 is the spectrophotometrically determined initial rate of

p-nitrophenol (pNPOH) liberation. The pseudo-first- order rate constants for the cleavage of the same substrate in the presence of the diguanidinocalixarene $(\mathbf{7}H_2)^{2+}$, and phenol **9**H are also reported for comparison, (entries 8 and 9, respectively).

Table 1. Cleavage of BNPP in the Presence of Additives, (80% DMSO, 50 °C).

Entry	Additive	pH	$10^6 \times k_{obs}$ (s^{-1}) [a,b]	k_{obs}/k_{bg} [c]
1	**1**H·2HCl	8.5	8.8	1.3×10^5
2		9.0	23	1.1×10^5
3		9.5	44	6.5×10^4
4		10.0	42	1.9×10^4
5		10.5	35	5.1×10^3
6		11.1	26	9.6×10^2
7		12.0	19	8.8×10
8	**7**·2HCl	10.4	0.64	1.2×10^2
9	**9**H	12.7	0.50 [d]	

[a] From initial rate of PNPOH liberation measured in 0.20 mM BNPP, 1.0 mM additive, 0.10 M *N*,*N*'-diisopropyl ethanolamine buffer, 10 mM Me_4NClO_4 solutions. [b] Calculated as v_o/[BNPP]; maximum error = ±10%. [c] The background rate constant (k_{bg}, s^{-1}) for the hydroxide-catalyzed cleavage has been calculated by the expression $k_{bg} = 10^{(pH-18.67)}$, obtained from data reported in ref. 5a. [d] Corrected for the contribution of the background reaction OH^- + BNPP: $k_{bg} = 1.1 \times 10^{-6}$ s^{-1} at pH 12.7.

The trifunctional calixarene scaffold of $(\mathbf{1}H_3)^{2+}$ turned out to be very effective in the cleavage of BNPP in the investigated pH range (Table 1, entries 1 – 7), with accelerations over background (k_{obs}/k_{bg}) ranging from 10^5- to 10^2-fold. Comparison with the control experiments in entries 8 and 9 indicates that the phenolic hydroxyl of the trifunctional calix[4]arene is directly involved in the cleavage of BNPP, presumably via a transphosphorylation process, electrophilically/electrostatically assisted by the neighboring guanidinium units.[20]

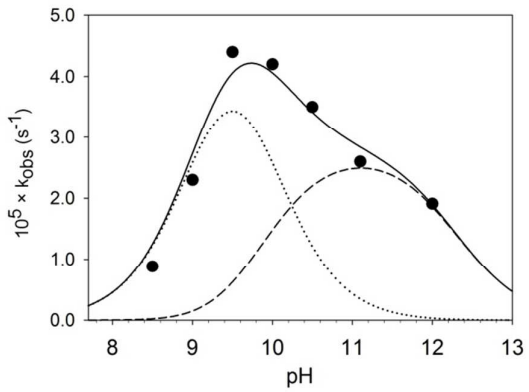


Figure 4. pH–rate profiles for the cleavage of 0.20 mM BNPP in the presence of 1.0 mM of $(\mathbf{1}H_3)^{2+}$, 0.10 M *N*,*N*-diisopropyl ethanolamine buffer, 10 mM Me_4NClO_4, (data points from Table 1). The solid line is the plot of eqn (1) with best fit values of k_1 and k_2. The dotted and dashed lines are the plot of the individual contribution of the species $(\mathbf{1}H_2)^+$ and **1**H, respectively, to the overall reactivity.

A plot of k_{obs} values in Table 1 (entries 1 – 7) *vs* pH (Figure 4) reveals a nonsymmetrical bell-shaped profile, which indicates that more than one species is kinetically active. Comparison with the distribution diagram in Figure 1 suggests that the active species are $(\mathbf{1}H_2)^+$ and **1**H , (eqn (1)),

$$k_{obs} = k_1[(\mathbf{1}H_2)^+] + k_2[\mathbf{1}H] \quad (1)$$

whose pH-dependent concentrations are given by standard equations for a triprotic acid, (eqns (2) and (3)), respectively, where C_T is the total concentration of **1**H·2HCl, and K_{a1}, K_{a2}, and K_{a3} are the acidity constants of the triprotic acid $(\mathbf{1}H_3)^{2+}$ (see SI, p. S8).

$$[(\mathbf{1}H_2)^+] = \frac{C_T[H^+]^2K_{a1}}{[H^+]^3+[H^+]^2K_{a1}+[H^+]K_{a1}K_{a2}+K_{a1}K_{a2}K_{a3}} \quad (2)$$

$$[\mathbf{1}H] = \frac{C_T[H^+]K_{a1}K_{a2}}{[H^+]^3+[H^+]^2K_{a1}+[H^+]K_{a1}K_{a2}+K_{a1}K_{a2}K_{a3}} \quad (3)$$

The potentiometrically determined K_a values (Figure 1) were used as known quantities in a nonlinear least-squares fit of kinetic data, in which k_1 and k_2 were treated as adjustable parameters. Best fit values of $k_1 = (6.2 \pm 0.4) \times 10^{-2}$ $M^{-1}s^{-1}$, and $k_2 = (2.8 \pm 0.2) \times 10^{-2}$ $M^{-1}s^{-1}$, were used to plot the overall pH-rate profile and the individual contributions of the species in Figure 4. Notably, the monocationic species $(\mathbf{1}H_2)^+$ is about twice as reactive as the neutral species **1**H. Remembering that about 1/2 of $(\mathbf{1}H_2)^+$ is in the tautomeric form *B* (Figure 2), whereas **1**H is almost

exclusively in form *D*, it turns out that tautomer *B*, featuring two guanidinium units, is about 4 times more reactive than tautomer *D*, where only one of the two guanidines is protonated. This observation clearly indicates that both guanidinium units in *B* cooperate in the stabilization of the dianionic transition state (Figure 5).

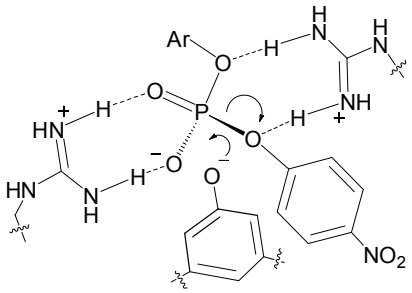


Figure 5. Suggested mechanism of BNPP cleavage promoted by $(\mathbf{1}H_2)^+$ involving two guanidinium units as electrophilic activators and a phenolate moiety acting as a nucleophile.

Time-course kinetics. To get further insights into the mechanism of action of calix[4]arene $(\mathbf{1}H_3)^{2+}$ as a phosphoryl transfer agent, pNPOH liberation was monitored by UV spectrophotometry in a time-course experiment (Figure 6) carried out under substrate-excess conditions on a 5.0 mM BNPP and 0.1 mM trifunctional calixarene **1**H·2HCl solution buffered at pH 9.5. An initial burst of pNPOH release is observed, followed by a much slower phase.

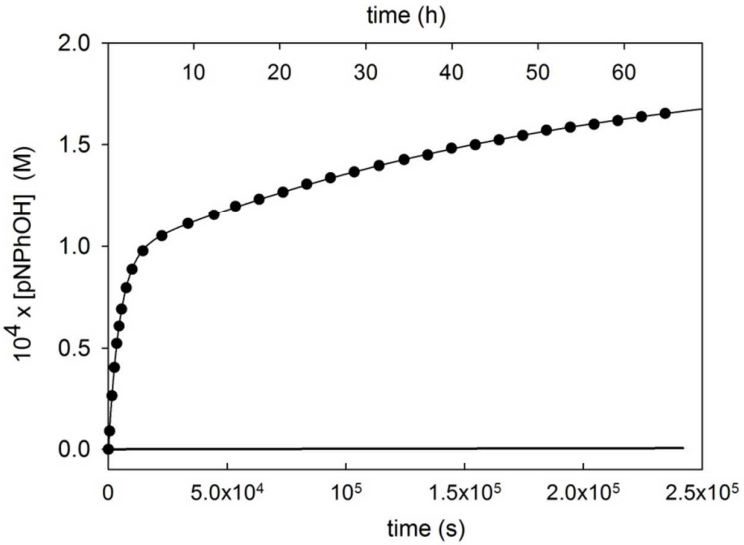
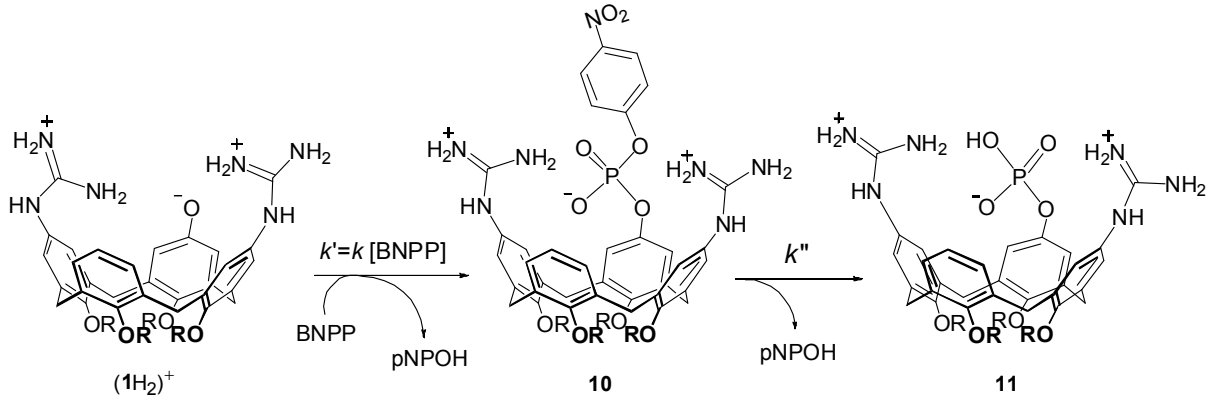


Figure 6. Liberation of pNPOH in a 5.0 mM solution of BNPP upon addition of 0.10 mM calixarene $(\mathbf{1}H_3)^{2+}$ (10 mM Me_4NClO_4, 0.10 M *N*,*N*-diisopropyl ethanolamine buffer, pH 9.5; 80% DMSO, 50 °C). Data points are experimental and the solid line is the plot of eqn (4) with best fit parameters $k' = 2.0 \times 10^{-4}$ s^{-1}, and $k'' = 5.4 \times 10^{-6}$ s^{-1}. The gray solid line corresponds to background pNPOH liberation calculated at pH 9.5.

This is consistent with the reaction sequence in Scheme 2, featuring a fast step of phosphorylation of the calixarene scaffold with the liberation of a first molar equivalent of pNPOH, followed by a much slower step with the liberation of a second equivalent of pNPOH from phosphodiester **10**. The latter step is presumably assisted by the neighboring guanidine/guanidinium catalytic dyad.[3c,16,21]



Scheme 2. BNPP cleavage promoted by $(\mathbf{1}H_2)^+$.

This kinetic scheme corresponds to the case of two consecutive irreversible first-order reactions.[22] From standard integrated equations, properly adapted to the investigated reaction system, eqn (4) follows for the time-dependent concentration of liberated pNPOH, where C_T is the total concentration of the calixarene derivative, k' = k[BNPP], and $\tau = (k'+k'')^{-1}$, (see Scheme 2).

$$[\text{pNPOH}]=C_T\,\tau\,k'\left[\tau\,k'\left(1-e^{-\frac{t}{\tau}}\right)+(1-e^{-k''t})\right] \quad (4)$$

When the first exponential term dies out, the second exponential decay becomes apparent and eqn (4) reduces to eqn (5).

$$[\text{pNPOH}]=C_T\,\tau^2\,k'^2+C_T\left(1-e^{-k''t}\right) \quad (5)$$

A nonlinear least-squares fit of experimental data to eqn (4) gave the following values: k' = (2.0 ± 0.2) × 10^{-4} s^{-1}, and k'' = (5.4 ± 0.2) × 10^{-6} s^{-1}.

It is interesting to compare the pseudo-first-order rate constants k' with the k_{obs} value of 4.4 × 10^{-5} s^{-1} (Table 1, entry 3) measured at the same pH, but with different reactant concentrations. Whereas the former corresponds to a second-order rate constant of 4.1 × 10^{-2} $M^{-1}s^{-1}$, the latter corresponds to a second-order rate constant of 4.4 × 10^{-2} $M^{-1}s^{-1}$. The good agreement between the two values shows that the rate constants are concentration-independent quantities and, consequently, that association between reactants is too weak to affect the kinetics, as previously found in related studies.[3c,3d,5b] In other words, there is no significant binding in the reactant state

(subsaturating conditions) and all of the available binding energy arising from the interaction of the guanidinium unit(s) with the phosphate group along the activation process is selectively utilized in transition state stabilization.

The operation of the mechanism presented in Scheme 2, with nucleophilic assistance in the cleavage of BNPP is also supported by ES-MS analysis of the reaction mixture of the experiment reported in Figure 6. The ES-MS spectrum of an aliquot of the reaction mixture withdrawn after 15 minutes confirmed the formation of a *p*-nitrophenylphosphoryl derivative, that we ascribe to the *O*-phosphorylated product **10** (Figure S2 (top), SI). A similar analysis carried out on a sample withdrawn after 3 days confirmed the presence of **10**, whose peak has in this sample an higher intensity than in the sample withdrawn after 15 minutes, and of the phosphoryl derivative **11** (Figure S2 (bottom), SI).

The whole set of experimental data points to the effective cooperation of the three functional groups of $(\mathbf{1}H_2)^+$ or **1**H in the promotion of BNPP cleavage, and to the crucial role of the nucleophilic phenolate unit (Figure 5), with the formation of a key *O*-phosphorylated intermediate.

CONCLUSIONS

To sum up, we have shown that the diguanidino derivative of a calix[4]arene featuring a phenolic hydroxyl at the upper rim effectively promotes the cleavage of the DNA model compound BNPP in 80% DMSO solution at pH values ranging from weakly acidic (8.5) to moderately basic (12.0) values. Comparison of the pH-dependent initial rates of pNPOH liberation with the results of potentiometric and spectrophotometric acid-base titrations, clearly indicates the involvement of two distinct kinetically active species, differing in composition for the presence of one acidic proton in one species, and lack in the other. The more reactive species was inferred to be the positively charged tautomer *B* (Figure 2), in which phosphorylation of the aryloxide oxygen is strongly assisted by the synergic action of the two neighboring guanidinium units, acting as electrophilic/electrostatic activators. Less active is the neutral species *D*, in which activation is provided by one guanidinium moiety only. Investigation of time-course kinetics, coupled with mass spectrometric analysis, confirmed that exhaustive cleavage of BNPP is paralleled by phosphorylation of the aryloxide oxygen of the calix[4]arene derivative. It was found that overall two equivalents of pNPOH were liberated in subsequent steps occurring on widely different time scales. We suggest that liberation of the second equivalent of pNPOH from the phosphorylated

intermediate **10** is assisted by the built-in guanidine/guanidinium catalytic dyad. On the other hand, the efficiency of the latter in the final liberation of the active form $(\mathbf{1}H_2)^+$ by dephosphorylation of **11** is still too low to assure turnover. This is of course at variance with the catalytic cycle of DNA Topoisomerase I,[6] and a key step to be improved in forthcoming mimics of this class of enzymes.

EXPERIMENTAL SECTION

Instruments. NMR spectra were recorded on either 400 or 300 MHz spectrometers. Partially deuterated solvents were used as internal standards to calculate the chemical shifts (δ values in ppm). High resolution mass spectra were obtained by an electrospray ionization (ES-MS) single-quadrupole spectrometer. Potentiometric titrations were performed by an automatic titrator equipped with a combined microglass pH electrode. The experimental details and the procedure for the electrode calibration were the same as previously reported.[23] Spectrophotometric measurements were carried out at 400 nm on a double beam spectrophotometer.

Materials and general procedures. Syntheses of compounds **3** and **6**, were carried out under a nitrogen atmosphere. Flash chromatography was carried out on 230-240 mesh silica gel. Anhydrous $CHCl_3$ was obtained by distillation over $CaCl_2$. DMSO, purged 30 min with argon, and mQ water were used in the preparation of 80% DMSO used in kinetic and acid-base titration experiments. HPNP,[24] 5,17-dinitro-25,26,27,28-tetrakis(2-ethoxyethoxy)-calix[4]arene **2**,[8] and the bis(hydrochloride) diguanidino derivative **7**·2HCl,[3d] were prepared according to literature procedures. All other solvents and reagents were commercial samples and used as such.
Warning! Care was taken when handling tetramethylammonium perchlorate because it is potentially explosive.[25] No accident occurred in the course of the present work.

5,17-Dinitro-11-formyl-25,26,27,28-tetrakis(2-ethoxyethoxy) calix[4]arene (3): To a solution of **2** (0.22 g, 0.27 mmol) in dry $CHCl_3$ (20 mL) cooled at -10°C, $SnCl_4$ (1.6 mL, 13.92 mmol) and Cl_2CHOCH_3 (1.26 mL, 13.81 mmol) were added. The reaction mixture was stirred for 2 hours at -10°C, then it was quenched with distilled water (30 mL) and vigorously stirred for additional 30 minutes. The organic layer was washed with a saturated solution of $NaHCO_3$ (2 x 20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude

material was purified by flash chromatography (hexane/AcOEt 5.5:4.5 – hexane/AcOEt 1:1) to give **3** as a white solid (0.13 g, 0.16 mmol; 59% yield) mp 65–67 °C.: ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.61 (s, 1H), 7.75 (s, 4H), 7.03 (s, 2H), 6.48 (m, 3H), 4.70 (d, 2H, *J* =12.6 Hz), 4.61 (d, 2H, *J* =13.8 Hz), 4.35-4.26 (m, 4H), 4.17 (t, 2H, *J*=4.8 Hz), 4.07 (t, 2H, *J*=4.8 Hz), 3.85-3.76 (m, 8H), 3.55-3.47 (m, 8H), 3.38 (d, 2H, *J*=12.6 Hz), 3.31 (d, 2H, *J*=13.8 Hz), 1.23-1.15 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 191.1, 162.7, 161.2, 155.6, 142.6, 137.1, 136.3, 134.8, 133.3, 131.6, 130.2, 128.5, 124.2, 123.6, 123.1, 74.1, 74.0, 73.7, 69.8, 69.6, 69.5, 66.6, 66.5, 66.4, 30.9, 15.3. . HR ES-MS: *m/z* Calcd for $C_{45}H_{55}O_{13}N_2$ [(**3**+H)$^+$] 831.36987, found 831.36950.

5,17-Dinitro-11-hydroxy-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (4): To a solution of **3** (0.13 g, 0.16 mmol) in DCM (15 mL), *m*-CPBA (0.19 g, 1.10 mmol) was added. The reaction mixture was stirred for 5 days at room temperature, then was quenched with a solution of $NaHSO_3$ 0.2 M and vigorously stirred for additional 30 minutes. The organic layer was washed with brine (2 x 20 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting material was taken with a solution of NaOH 2 M in MeOH:H_2O 4:1 (5 mL) and was stirred for 2 hours at room temperature, then the mixture was concentrated by evaporation of the MeOH under reduced pressure. The remaining aqueous layer was neutralized with a solution of 1M HCl and extracted with DCM (2 x 15 mL), then the combined organic layers were dried on anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was triturated in refluxing hexane overnight, for three times, to give **4** as a pale yellow oil (0.11 g, 0.13 mmol; 81% yield): ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.38 (d, 2H, *J*=2.7 Hz), 7.34 (d, 2H, *J*=2.7 Hz), 6.91 (dd, 2H, J_1=7.6 Hz, J_2=2.1 Hz), 6.86 (td, 1H, J_1=7.6 Hz, J_2=2.1 Hz), 6.36 (s, 2H), 4.67 (d, 2H, *J*=13.8 Hz), 4.60 (d, 2H, *J*=13.8 Hz), 4.20 (t, 6H, *J*=5.1 Hz), 4.14 (t, 2H, *J*=5.4 Hz), 3.90 (t, 8H, *J*=5.4 Hz), 3.60-3.55 (m, 8H), 3.33 (d, 2H, *J*=13.8 Hz), 3.22 (d, 2H, *J*=13.8 Hz), 1.24-1.17 (m, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 161.6, 156.7, 152.0, 149.7, 142.4, 136.5, 136.4, 135.3, 134.7, 128.7, 122.8, 122.7, 115.1, 74.1, 73.1, 73.0, 69.7, 69.6, 60.1, 66.0, 65.9, 30.6, 30.5, 14.3. HR ES-MS: *m/z* Calcd for $C_{44}H_{55}N_2O_{13}$ [(**4**+H)$^+$] 819.36987, found 819.36987; *m/z* Calcd for $C_{44}H_{54}N_2O_{13}Na$ [(**4**+Na)$^+$] 841.35181, found 841.35284.

5,17-Diamino-11-hydroxy-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (5): To a solution of **4** (0.053 g, 0.065 mmol) in MeOH (10 mL), $NiCl_2{\cdot}6H_2O$ (0.062 g, 0.072 mmol) and $NaBH_4$ (0.025 g, 0.66 mmol) were added. The reaction mixture was stirred for 3 hours at room temperature, then was quenched with a solution of 1 M HCl (15 mL) and the pH raised to 8-9 with a solution of 1 M NaOH. The resulting mixture was extracted with AcOEt (3 x 15 mL) and the combined organic

layers were washed with distilled water (2 x 20 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Compound **5** was obtained as an orange to brown oil (0.049 g, 0.065 mmol; quantitative yield), pure enough to avoid further purifications. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.10 (d, 2H, *J*=7.6 Hz), 6.92 (t, 1H, *J*=7.6 Hz), 6.56 (s, 2H), 5.99 (s, 2H), 5.89 (s, 2H), 4.58 (d, 2H, *J*=12.8 Hz), 4.52 (d, 2H, *J*=12.8 Hz), 4.26 (m, 2H), 4.17 (m, 2H), 3.94-3.83 (m, 12H), 3.63-3.55 (m, 8H), 3.17 (d, 2H, *J*=12.8 Hz), 3.06 (d, 2H, *J*=12.8 Hz), 1.26-1.19 (m, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 151.3, 140.7, 135.5, 135.4, 134.9, 127.9, 116.2, 114.3, 73.1, 69.6, 66.0, 30.6, 30.5, 14.3. HR ES-MS: *m/z* Calcd for $C_{44}H_{59}N_2O_9$ [(**5**+H)$^+$] 759.42151, found 759.42188.

5,17–Bis-[*N*,*N*'–bis(tert-butoxycarbonyl)guanidine]-11-hydroxy-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (6): To a solution of **5** (0.049 g, 0.065 mmol) and triethylamine (72 µL, 0.039 mmol) in dry DMF (5 mL), *N*,*N*'-bis(*tert*-butoxycarbonyl)thiourea (0.015 g, 0.039 mmol) and $HgCl_2$ (0.070 g, 0.26 mmol) were added. The reaction mixture was stirred for 2 days at room temperature, then was quenched by adding AcOEt (15 mL) and the precipitated HgS was filtered off. The filtrate was washed with brine (3 x 20 mL) and the organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude material was purified by flash chromatography (hexane/AcOEt 7:3) to give **6** as a colorless oil (0.034 g, 0.027 mmol; 42% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 11.62 (s, 2H), 10.18 (s, 2H), 7.25 (s, 4H), 6.38 (t, 1H, *J*=6.9 Hz), 6.28 (d, 2H, *J*=6.9 Hz), 5.68 (s, 2H), 4.49 (d, 2H, *J*=12.6 Hz), 4.42 (d, 2H, *J*=13.5 Hz), 4.24 (3 t, 4H, *J*=6.6 Hz), 4.22-4.04 (m, 8H), 3.90-3.84 (m, 8H), 3.78-3.72 (m, 4H), 3.60-3.48 (m, 8H), 3.15 (d, 2H, *J*=12.6 Hz), 3.09 (d, 2H, *J*=13.5 Hz), 1.60-1.49 (m, 36H), 1.25-1.15 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 163.6, 155.5, 155.0, 153.9, 153.4, 150.7, 137.1, 137.0, 133.9, 133.0, 127.8, 123.6, 123.3, 122.5, 114.7, 83.6, 79.5, 74.0, 72.5, 69.6, 66.5, 66.2, 30.9, 30.8, 29.7, 28.2, 28.1, 15.4, 15.3. HR ES-MS: *m/z* Calcd for $C_{66}H_{95}N_6O_{17}$ [(**6**+H)$^+$] 1243.67482, found 1243.67444; *m/z* Calcd for $C_{61}H_{87}N_6O_{15}$ [(**6**-Boc+H)$^+$] 1143.62239, found 1143.62122.

5,17–Diguanidine-11-hydroxy-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene bis(hydrochloride) (1·2HCl). In a mixture of DCM/TFA/TES 95:2.5:2.5 (10 mL), **6** (0.034 g, 0.027 mmol) was dissolved. The reaction mixture was stirred overnight at room temperature and quenched by removal of the solvent under reduced pressure. The residue was taken into a 1 M HCl EtOH solution (3 mL), vigorously stirred for 30 min and then the solvent removed under reduced pressure. This procedure was repeated three times to exchange the CF_3COO^- anion to chloride. Then **1**·2HCl was obtained as a colorless oil (0.021 g, 0.025 mmol; 93% yield), pure enough to avoid further purifications. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 6.89 (d, 2H, *J*=7.2 Hz), 6.77 (t, 1H, *J*=7.2

Hz), 6.55 (s, 2H), 6.51 (s, 2H), 6.32 (s, 2H), 4.63 (d, 2H, *J*=13.2 Hz), 4.57 (d, 2H, *J*=13.2 Hz), 4.60 (d, 2H, *J*=12.8 Hz), 4.26 (t, 2H, *J*=5.6 Hz), 4.18 (t, 2H, *J*=5.2 Hz), 4.12 (m, 4H), 3.94-3.91 (m, 8H), 3.67-3.56 (m, 8H), 3.26 (d, 2H, *J*=13.6 Hz), 3.15 (d, 2H, *J*=12.8 Hz), 1.27-1.18 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 156.6, 155.5, 151.8, 149.6, 136.6, 136.5, 135.5, 135.0, 128.4, 128.1, 124.9, 124.8, 122.5, 114.8, 73.9, 73.0, 72.9, 69.9, 69.8, 69.7, 66.2, 66.1, 66.0, 30.5, 30.4, 14.3 HR ES-MS: *m/z* Calcd for $C_{46}H_{63}N_6O_9$ [(**1**+H)$^+$] 843.46510, found 843.46593.

Acid-base titrations

Potentiometric acid-base titrations of 5mL solutions of 2.0 – 3.0 mM (**1**H_3)$^{2+}$ (or **9**H) and 10 mM Me_4NClO_4 was carried out according to a previously reported procedure,[3d] by addition in small increments under argon atmosphere of a freshly prepared solution of 50 – 100 mM Me_4NOH, (80% DMSO, 25 °C). Elaboration of the titration plot was carried out with the software HYPERQUAD 2000.[26] UV-Vis spectrophotometric titrations of a 0.2 mM *p*-methoxyphenol or of 0.2 mM calixarene (**1**H_3)$^{2+}$ solution with Me_4NOH was carried out analogously under argon atmosphere, (DMSO 80%, at 25 ^{o}C).

Kinetic measurements

Liberation of pNPOH was spectrophotometrically monitored at 400 nm. Initial-rate measurements (data in Table 1) were carried out on 0.20 mM BNPP, 1.0 mM additive ((**1**H_3)$^{2+}$, or (**7**H_2)$^{2+}$, or **9**H), 0.10 M *N*,*N*-diisopropyl ethanolamine buffer, 10 mM Me_4NClO_4 solutions, (80% DMSO, 50.0 °C). The pH of the solution was adjusted at the selected pH value with a 50−100 mM solution of $HClO_4$ in 80% DMSO. The time-course experiment was carried out on a 5.0 mM BNPP, 0.1 mM (**1**H_3)$^{2+}$, 10 mM Me_4NClO_4, 0.10 M *N*,*N*-diisopropyl ethanolamine buffer solution, (pH 9.5; 80% DMSO, 50.0 °C). The pH of the solution was adjusted at pH 9.5 by addition of the proper volume of the 50 – 100 mM solution of $HClO_4$ in 80% DMSO. Non-linear least-square fit of experimental data to eqn (4) was carried out with the software SigmaPlot 12.0 (Systat Software, Inc.).

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ASSOCIATED CONTENT

Supporting information

^{1}H and ^{13}C NMR spectra of compounds, potentiometric acid-base titration of compound **9**H, spectrophotometric acid-base titrations of $(\mathbf{1}H_3)^{2+}$ and **9**H (equations for the calculated lines in Figure 3 of main text), standard equations for the distribution diagram of the species of a triprotic acid, ESI-MS spectra of the reaction mixture in the time-course experiment in Figure 6 of main text (BNPP cleavage in the presence of $(\mathbf{1}H_3)^{2+}$).

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(19) It can be shown that $K_{a1} = (K_{a1})_A + (K_{a1})_B$, , where and $(K_{a1})_A$ and $(K_{a1})_B$ are the acidity constants for the deprotonation equilibrium producing tautomer *A* or tautomer *B*, respectively.

(20) The rates of cleavage of BNPP by the trifunctional calix[4]arene (entries 1 – 7) are from more than one to nearly two orders of magnitude higher than the rate of cleavage by *p*-methoxyphenoxide ion (entry 9). These rate enhancements strongly underestimate the effect of the neighboring guanidinium units because *p*-methoxyphenoxide ion is a much stronger base and, conceivably, a stronger nucleophile than the phenoxide unit in $(\mathbf{1}H_2)^+$ and **1**H.

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