JOC The Journal of Organic Chemistry

Article

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

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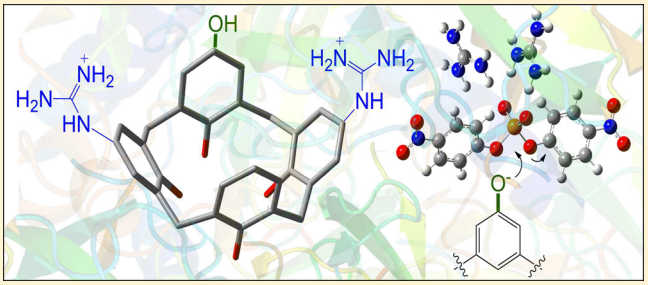
Supporting Information

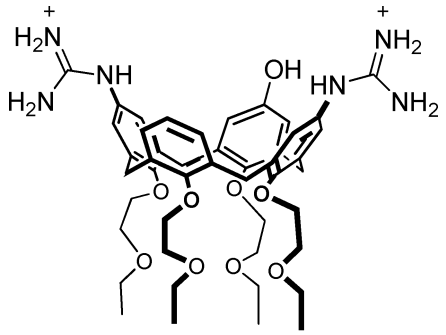
ABSTRACT: The *cone*-calix[4]arene derivative $(\mathbf{1}H_3)^{2+}$, decorated at the upper rim with two guanidinium units and a phenolic hydroxyl in an ABAH functionalization pattern, effectively promotes the cleavage of the DNA model compound bis(*p*-nitrophenyl) phosphate (BNPP) in 80% DMSO solution 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 $(\mathbf{1}H_3)^{2+}$. 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

The design of effective catalysts for cleavage of the highly inert phosphodiester function of nucleic acids and model compounds has been a challenging target for 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, we focus here 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 an 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).

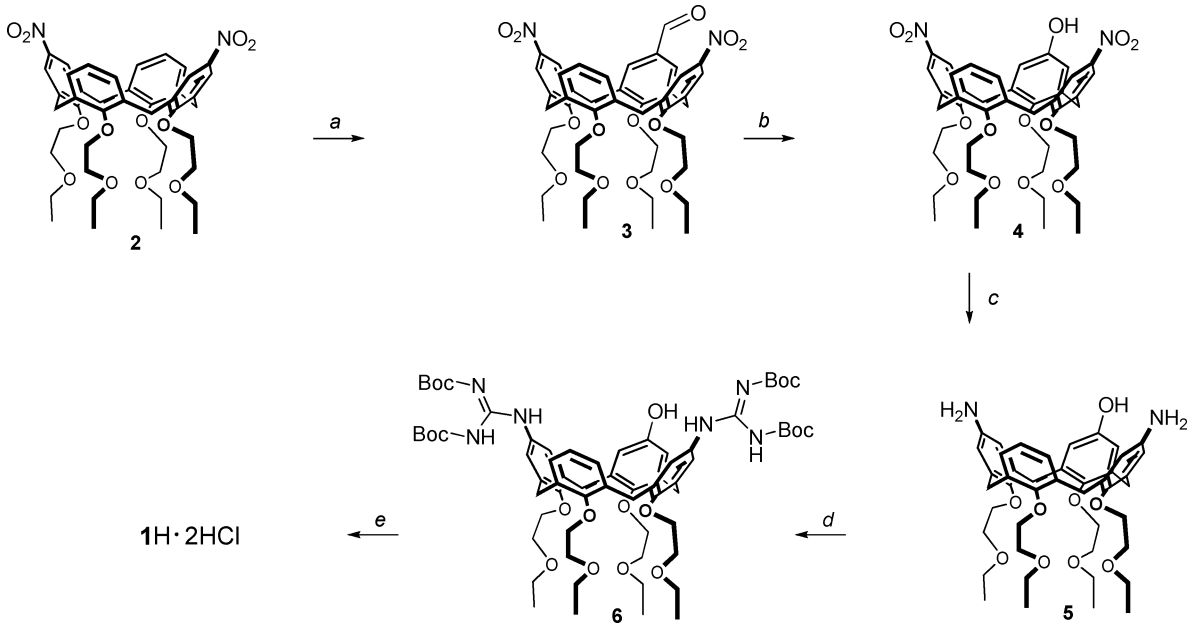


$(\mathbf{1}H_3)^{2+}$

Received: July 8, 2016
Published: August 31, 2016

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019

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.

RESULTS AND DISCUSSION

Calixarene Synthesis. Compound **1H** 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, 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 to the Boc-protected diguanidino derivative **6** (42% yield) according to a published procedure[3a,c,d] by using [*N*,*N*′-bis(Boc)]thiourea and $HgCl_2$. The target compound **1H** was obtained as bis(hydrochloride) (93% yield) after removal of the Boc protecting groups. All these newly synthesized ABAH derivatives show HR-MS and ^1H 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 Supporting Information (SI), pp S2−S6). In particular, the $ArCH_2Ar$ ^1H 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.

Acid−Base Titrations. The determination of acidity constants of calixarene $(\mathbf{1H_3})^{2+}$ 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 investigation of the hydrolysis of phosphodiesters,[3a−d,5a,b,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 **1H** (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.

Titration of $(\mathbf{1H_3})^{2+}$ with Me_4NOH 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 $(\mathbf{1H_2})^{+}$ is actually a mixture of tautomeric forms A and B (Figure 2).[19]

As for the second deprotonation step leading to the neutral species **1H**, a comparison of the pK_{a2} value of 9.9 of $(\mathbf{1H_3})^{2+}$ with the corresponding pK_{a2} value of 11.5 of the model compound $(\mathbf{7H_2})^{2+}$ lacking the phenolic hydroxyl (Chart 1) rules out the possibility that the phenolic hydroxyl also acts as an innocent spectator also in this deprotonation step. It seems

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019

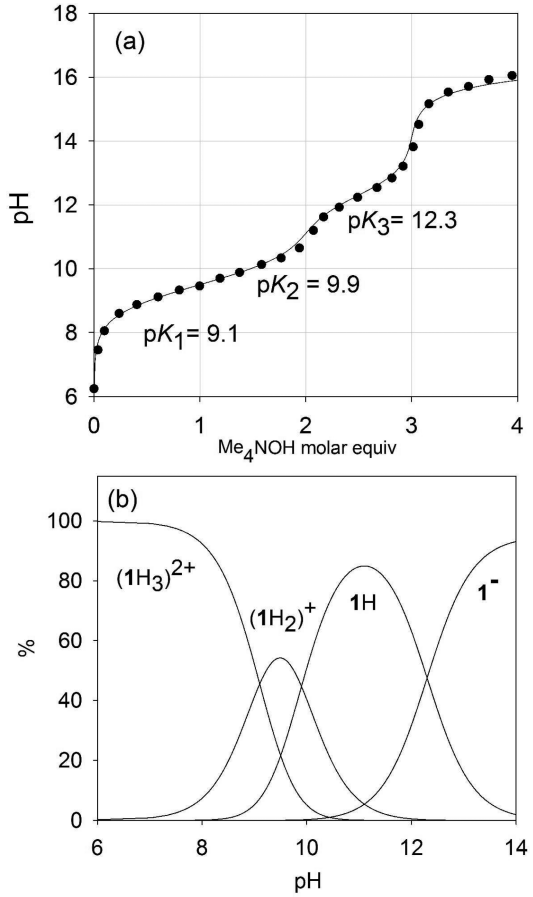
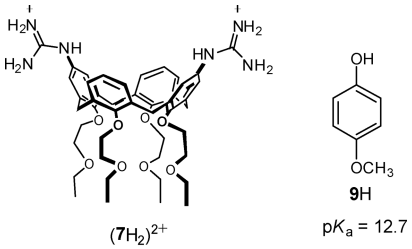


Figure 1. Titration of $(\mathbf{1}H_3)^{2+}$ (2.0 mM) with Me_4NOH in 80% DMSO, 25 °C, in the presence of 10 mM Me_4NClO_4 (a) and a distribution diagram of the species as a function of pH (b).

Chart 1. pK_a Values of Model Compounds in 80% DMSO at 25 °C[a]



[a]Data from ref 3d for $(7H_2)^{2+}$; for compound **9**H, see SI p S9).

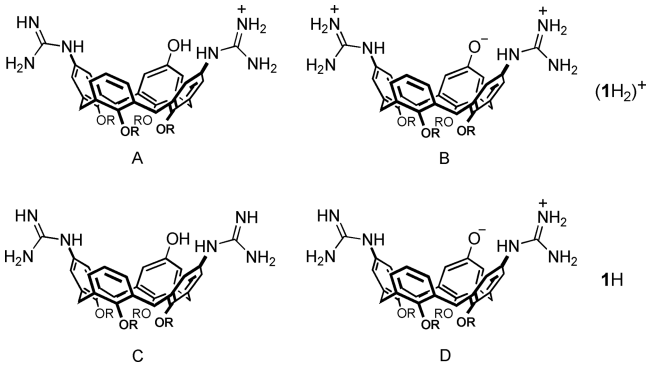


Figure 2. Possible tautomeric structures of $(\mathbf{1}H_2)^+$ (top) and **1**H (bottom).

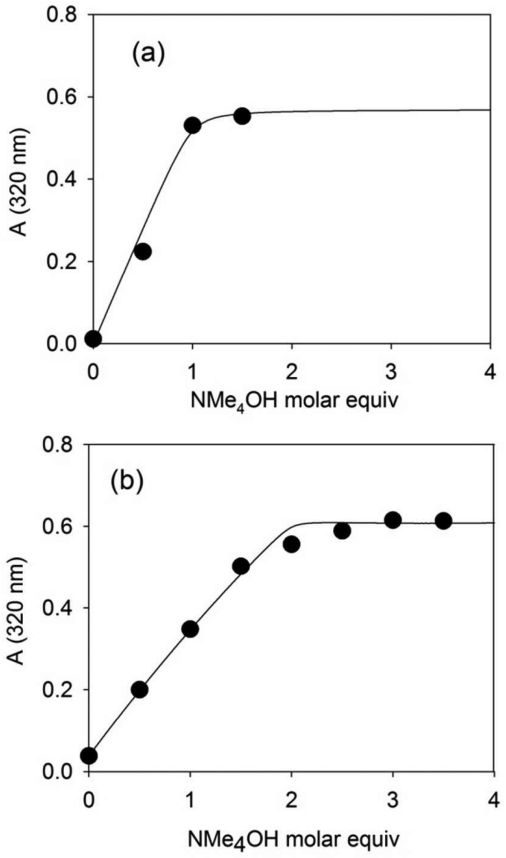


Figure 3. UV−vis titration of 0.2 mM *p*-methoxyphenol (a) and 0.2 mM calixarene **1**H·2HCl (b) with Me_4NOH (25 °C, DMSO 80%). The full lines are calculated (see SI, p S7).

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 toward the zwitterionic form D.

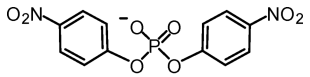
The question of the involvement of the phenolic hydroxyl during the first and second titration steps was decided by UV−vis spectrophotometric titrations of $(\mathbf{1}H_3)^{2+}$ with Me_4NOH (Figure 3).

Whereas addition of 1 mol equiv of base caused a complete conversion of *p*-methoxyphenol to *p*-methoxyphenolate ion (Figure 3a), the same amount of base transformed only ∼55% of the phenolic hydroxyl of $(\mathbf{1}H_3)^{2+}$ into the corresponding phenolate ion (Figure 3b). Proton removal from the phenolic hydroxyl was complete, or very nearly so, upon addition of a second equivalent of base. Thus, the combination of potentiometric and spectrophotometric titration data indicates that the equilibrium between tautomers A and B is well balanced, with only a slight prevalence of B, whereas the equilibrium between C and D is strongly biased toward zwitterion D.

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

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019

8.5−12.0 with 0.10 M *N*,*N*-diisopropyl ethanolamine/perchlorate salt buffer.



BNPP

The results of the kinetic experiments are listed in Table 1 (entries 1−7) as pseudo-first-order specific rates

Table 1. Cleavage of BNPP in the Presence of Additives in 80% DMSO at 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 the 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 $v_0/[BNPP]$; 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.

$k_{obs} = v_0/[BNPP]$, 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 $(7H_2)^{2+}$ and phenol **9**H are also reported for comparison (entries 8 and 9, respectively).

The trifunctional calixarene scaffold of $(1H_3)^{2+}$ 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]

A plot of k_{obs} values in Table 1 (entries 1−7) relative to 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 $(1H_2)^+$ and **1**H (eq 1)

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

whose pH-dependent concentrations are given by standard equations for a triprotic acid (eqs 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 $(1H_3)^{2+}$ (see SI, p S8).

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

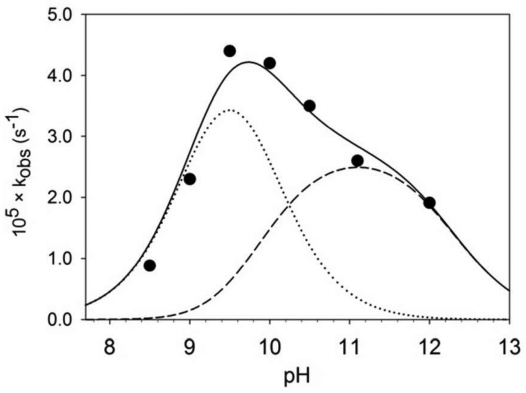


Figure 4. pH rate profiles for the cleavage of 0.20 mM BNPP in the presence of 1.0 mM $(1H_3)^{2+}$, 0.10 M *N*,*N*-diisopropyl ethanolamine buffer, and 10 mM Me_4NClO_4 (data points from Table 1). The solid line is the plot of eq 1 with best fit values of k_1 and k_2. The dotted and dashed lines are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

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

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 $(1H_2)^+$ is approximately twice as reactive as neutral species **1**H. Remembering that approximately 1/2 of $(1H_2)^+$ 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 approximately 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).

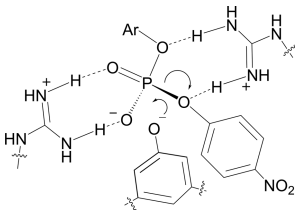


Figure 5. Suggested mechanism of BNPP cleavage promoted by $(1H_2)^+$ involving two guanidinium units as electrophilic activators and a phenolate moiety acting as a nucleophile.

Time-Course Kinetics. To obtain further insights into the mechanism of action of calix[4]arene $(1H_3)^{2+}$ 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 in 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.

This is consistent with the reaction sequence in Scheme 2, featuring a fast step of phosphorylation of the calixarene

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019

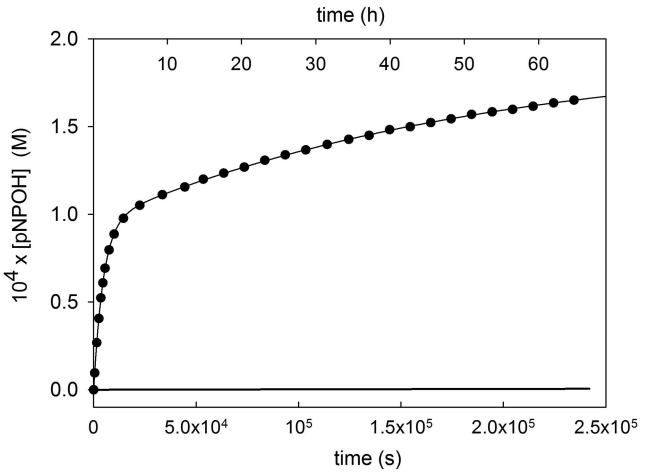


Figure 6. Liberation of pNPOH in a 5.0 mM solution of BNPP upon addition of 0.10 mM calixarene $(\mathbf{1}H_3)^{2+}$ (10 mM Me_4NClO_4, 0.10 M *N*,*N*-diisopropyl ethanolamine buffer, pH 9.5, 80% DMSO, 50 °C). Data points are experimental, and the solid line is the plot of eq 4 with best fit parameters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

scaffold with the liberation of the 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]

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, eq 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).

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

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

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

A nonlinear least-squares fit of experimental data to eq 4 gave the following values: $k' = (2.0 \pm 0.2) \times 10^{-4}$ s^{-1} and $k'' = (5.4 \pm 0.2) \times 10^{-6}$ 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 4.0×10^{-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,d,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.

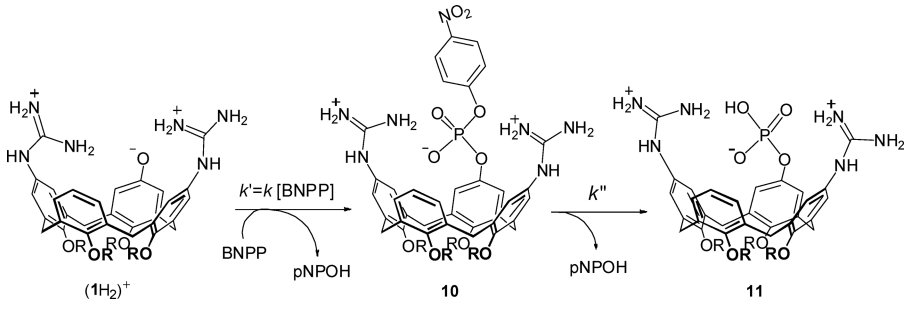
The operation of the mechanism presented in Scheme 2, with nucleophilic assistance in the cleavage of BNPP, is also supported by ES-MS analysis of the reaction mixture of the experiment reported in Figure 6. The ES-MS spectrum of an aliquot of the reaction mixture withdrawn after 15 min confirmed the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

The whole experimental data set 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

In summary, 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). 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 lacking 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 neutral species D in which activation is provided by one guanidinium moiety only. Investigation of

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



DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012–9019

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 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 ensure turnover. This is of course at variance with the catalytic cycle of DNA topoisomerase I[6] and is 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 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 were added $SnCl_4$ (1.6 mL, 13.9 mmol) and Cl_2CHOCH_3 (1.26 mL, 13.8 mmol). The reaction mixture was stirred for 2 h at −10 °C; then, it was quenched with distilled water (30 mL) and vigorously stirred for an additional 30 min. The organic layer was washed with a saturated solution of $NaHCO_3$ (2 × 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 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 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) was added *m*-CPBA (0.19 g, 1.10 mmol). The reaction mixture was stirred for 5 days at room temperature and was then quenched with a solution of 0.2 M $NaHSO_3$ and vigorously stirred for an additional 30 min. The organic layer was washed with brine (2 × 20 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The resulting material was taken with a solution of 2 M NaOH in 4:1 MeOH:H_2O (5 mL) and was stirred for 2 h 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 1 M HCl and extracted with DCM (2 × 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 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 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 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) were added $NiCl_2·6H_2O$ (0.062 g, 0.26 mmol) and $NaBH_4$ (0.025 g, 0.66 mmol). The reaction mixture was stirred for 3 h at room temperature and then quenched with a solution of 1 M HCl (15 mL), and the pH was raised to 8−9 with a solution of 1 M NaOH. The resulting mixture was extracted with AcOEt (3 × 15 mL), and the combined organic layers were washed with distilled water (2 × 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) that was pure enough to avoid further purification. ^{1}H NMR (400 MHz, CD_3OD): δ 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 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, 0.51 mmol) in dry DMF (5 mL) were added *N*,*N*′-bis(*tert*-butoxycarbonyl)thiourea (0.072 g, 0.26 mmol) and $HgCl_2$ (0.070 g, 0.26 mmol). The reaction mixture was stirred for 2 days at room temperature and then quenched by adding AcOEt (15 mL), and the precipitated HgS was filtered off. The filtrate was washed with brine (3 × 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 (7:3 hexane/AcOEt) to give **6** as a colorless oil (0.034 g, 0.027 mmol, 42% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 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 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-ethoxy-ethoxy)calix[4]arene Bis(hydrochloride) (**1**·2HCl).* In a mixture of 95:2.5:2.5 DCM/TFA/TES (10 mL) was dissolved **6** (0.034 g, 0.027 mmol). The reaction mixture was stirred overnight at room temperature and quenched by removal of the solvent under reduced

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019

pressure. The residue was taken into a 1 M HCl EtOH solution (3 mL) and vigorously stirred for 30 min, and then the solvent was 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 purification. ^{1}H NMR (400 MHz, CD_3OD): δ 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), 4.26 (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 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 5 mL solutions of 2.0−3.0 mM $(\mathbf{1}H_3)^{2+}$ (or **9**H) and 10 mM Me_4NClO_4 were 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 in 80% DMSO at 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 $(\mathbf{1}H_3)^{2+}$ solution with Me_4NOH was carried out analogously under an argon atmosphere (DMSO 80%, at 25 °C).

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

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01643.

^{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 (eqs for the calculated lines in Figure 3 of the 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 the main text (BNPP cleavage in the presence of $(\mathbf{1}H_3)^{2+}$) (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Thanks are due to the Ministero dell'Istruzione e dell'Università e della Ricerca (PRIN 2010JMAZML Multi-NanoIta), Ateneo 2015, and Consiglio Nazionale delle Ricerche (CNR) for financial support. Thanks are also due to CIM (Parma University) for the use of NMR and mass spectrometry facilities.

■ REFERENCES

(1) (a) Joshi, T.; Graham, B.; Spiccia, L. *Acc. Chem. Res.* **2015**, *48*, 2366−2379. (b) Raynal, M.; Ballester, P.; Vidal-Ferran, A.; van Leeuwen, P. W. *Chem. Soc. Rev.* **2014**, *43*, 1734−1787. (c) Mancin, F.; Scrimin, P.; Tecilla, P. *Chem. Commun.* **2012**, *48*, 5545−5559. (d) Lönnberg, H. *Org. Biomol. Chem.* **2011**, *9*, 1687−1703. (e) Aiba, Y.; Sumaoka, J.; Komiyama, M. *Chem. Soc. Rev.* **2011**, *40*, 5657−5668. (f) Morrow, J. R.; Amyes, T. L.; Richard, J. P. *Acc. Chem. Res.* **2008**, *41*, 539−548.

(2) (a) Salvio, R. *Chem. - Eur. J.* **2015**, *21*, 10960−10971. (b) Blondeau, P.; Segura, M.; Pérez-Fernández, R.; de Mendoza, J. *Chem. Soc. Rev.* **2007**, *36*, 198−210.

(3) (a) Salvio, R.; Moliterno, M.; Caramelli, D.; Pisciottani, L.; Antenucci, A.; D'Amico, M.; Bella, M. *Catal. Sci. Technol.* **2016**, *6*, 2280−2288. (b) Salvio, R.; Cacciapaglia, R.; Mandolini, L.; Sansone, F.; Casnati, A. *RSC Adv.* **2014**, *4*, 34412−34416. (c) Salvio, R.; Mandolini, L.; Savelli, C. *J. Org. Chem.* **2013**, *78*, 7259−7263. (d) Baldini, L.; Cacciapaglia, R.; Casnati, A.; Mandolini, L.; Salvio, R.; Sansone, F.; Ungaro, R. *J. Org. Chem.* **2012**, *77*, 3381−3389. (e) Gnaccarini, C.; Peter, S.; Scheffer, U.; Vonhoff, S.; Klussmann, S.; Göbel, M. W. *J. Am. Chem. Soc.* **2006**, *128*, 8063−8067. (f) Scheffer, U.; Strick, A.; Ludwig, V.; Peter, S.; Kalden, E.; Göbel, M. W. *J. Am. Chem. Soc.* **2005**, *127*, 2211−2217. (g) Kneeland, D. M.; Ariga, K.; Lynch, V. M.; Huang, C. Y.; Anslyn, E. V. *J. Am. Chem. Soc.* **1993**, *115*, 10042−10055. (h) Jubian, V.; Dixon, R. P.; Hamilton, A. D. *J. Am. Chem. Soc.* **1992**, *114*, 1120−1121.

(4) (a) Wirth-Hamdoune, D.; Ullrich, S.; Scheffer, U.; Radanovic, T.; Durner, G.; Gobel, M. W. *ChemBioChem* **2016**, *17*, 506−514. (b) Ullrich, S.; Nazir, Z.; Busing, A.; Scheffer, U.; Wirth, D.; Bats, J. W.; Durner, G.; Gobel, M. W. *ChemBioChem* **2011**, *12*, 1223−1229. (c) Muche, M. S.; Göbel, M. W. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2126−2129.

(5) (a) Salvio, R.; Volpi, S.; Cacciapaglia, R.; Sansone, F.; Mandolini, L.; Casnati, A. *J. Org. Chem.* **2016**, *81*, 4728−4735. (b) Salvio, R.; Volpi, S.; Cacciapaglia, R.; Casnati, A.; Mandolini, L.; Sansone, F. *J. Org. Chem.* **2015**, *80*, 5887−5893. (c) Tjioe, L.; Meininger, A.; Joshi, T.; Spiccia, L.; Graham, B. *Inorg. Chem.* **2011**, *50*, 4327−4339. (d) Tjioe, L.; Joshi, T.; Brugger, J. l.; Graham, B.; Spiccia, L. *Inorg. Chem.* **2011**, *50*, 621−635. (e) Sheng, X.; Lu, X.; Chen, Y.; Lu, G.; Zhang, J.; Shao, Y.; Liu, F.; Xu, Q. *Chem. - Eur. J.* **2007**, *13*, 9703−9712. (f) Zelder, F. H.; Salvio, R.; Rebek, J., Jr. *Chem. Commun.* **2006**, 1280−1282. (g) Ait-Haddou, H.; Sumaoka, J.; Wiskur, S. L.; Folmer-Andersen, J. F.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **2002**, *41*, 4014−4016.

(6) Redinbo, M. R.; Stewart, L.; Kuhn, P.; Champoux, J. J.; Hol, W. G. J. *Science* **1998**, *279*, 1504−1513.

(7) (a) Rebilly, J.-N.; Colasson, B.; Bistri, O.; Over, D.; Reinaud, O. *Chem. Soc. Rev.* **2015**, *44*, 467−489. (b) Cacciapaglia, R.; Di Stefano, S.; Mandolini, L.; Salvio, R. *Supramol. Chem.* **2013**, *25*, 537−554. (c) Homden, D. M.; Redshaw, C. *Chem. Rev.* **2008**, *108*, 5086−5130.

(8) van Loon, J. D.; Heida, J. F.; Verboom, W.; Reinhoudt, D. N. *Recl. Trav. Chim. Pays-Bas* **1992**, *111*, 353−359.

(9) Arduini, A.; Fanni, S.; Manfredi, G.; Pochini, A.; Ungaro, R.; Sicuri, A. R.; Ugozzoli, F. *J. Org. Chem.* **1995**, *60*, 1448−1453.

(10) Struck, O.; Verboom, W.; Smeets, W. J. J.; Spek, A. L.; Reinhoudt, D. N. *J. Chem. Soc., Perkin Trans. 2* **1997**, 223−227.

(11) Morita, Y.; Agawa, T.; Nomura, E.; Taniguchi, H. *J. Org. Chem.* **1992**, *60*, 3658−3672.

(12) Nose, A.; Kudo, T. *Chem. Pharm. Bull.* **1988**, *36*, 1529−1533.

(13) Georgieva, M. *Anal. Chim. Acta* **1977**, *90*, 83−89.

(14) Wróbel, R.; Chmurzyński, L. *Anal. Chim. Acta* **2000**, *405*, 303−308.

(15) Corona-Martinez, D. O.; Taran, O.; Yatsimirsky, A. K. *Org. Biomol. Chem.* **2010**, *8*, 873−880.

(16) Salvio, R.; Cincotti, A. *RSC Adv.* **2014**, *4*, 28678−28682.

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019

(17) Salvio, R.; Casnati, A.; Mandolini, L.; Sansone, F.; Ungaro, R. *Org. Biomol. Chem.* **2012**, *10*, 8941−8943.
(18) Kreevoy, M. M.; Baughman, E. H. *J. Phys. Chem.* **1974**, *78*, 421−423.
(19) It can be shown that $K_{a1} = (K_{a1})_A + (K_{a1})_B$, where $(K_{a1})_A$ and $(K_{a1})_B$ are the acidity constants for the deprotonation equilibrium producing tautomer A or 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 2 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.
(21) Savelli, C.; Salvio, R. *Chem. - Eur. J.* **2015**, *21*, 5856−5863.
(22) Frost, A. A; Pearson, R. G. In *Kinetics and Mechanism*, 2nd ed.; John Wiley & Sons: New York, 1961; Ch. 8.
(23) Salvio, R.; Cacciapaglia, R.; Mandolini, L. *J. Org. Chem.* **2011**, *76*, 5438−5443.
(24) Brown, D. M.; Usher, D. A. *J. Chem. Soc.* **1965**, 6558−6564.
(25) *Hazards in the Chemical Laboratory*, 5th ed.; Luxon, S. G., Ed.; The Royal Society of Chemistry: Cambridge, UK, 1992; p 524.
(26) (a) Gans, P.; Sabatini, A.; Vacca, A. *Talanta* **1996**, *43*, 1739−1753. (b) Alderighi, L.; Gans, P.; Ienco, A.; Peters, D.; Sabatini, A.; Vacca, A. *Coord. Chem. Rev.* **1999**, *184*, 311−318.

DOI: 10.1021/acs.joc.6b01643
J. Org. Chem. 2016, 81, 9012−9019