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Ribonuclease Activity of an Artificial Catalyst That Combines a Ligated Cu^{II} Ion and a Guanidinium Group at the Upper Rim of a *cone*-Calix[4]arene Platform

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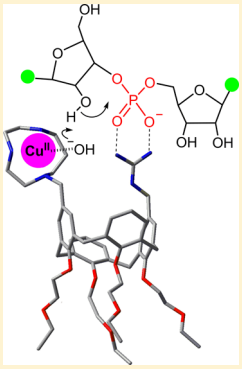
Supporting Information

ABSTRACT: A *cone*-calix[4]arene derivative, featuring a guanidinium group and a Cu^{II} ion ligated to a 1,4,7-triazacyclononane (TACN) ligand at the 1,3-distal positions of the upper rim, effectively catalyzes the cleavage of 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP) and a number of diribonucleoside 3′,5′-monophosphates (*NpN*′). Kinetic and potentiometric measurements support the operation of a general-base/general-acid mechanism and demonstrate that the hydroxo form of the ligated Cu^{II} ion is the sole catalytically active species. Rate enhancements relative to the background hydrolysis reaction at 1 mM catalyst concentration are 6×10^5-fold for HPNP and cluster around 10^7-fold with the most favorable catalyst−*NpN*′ combinations.

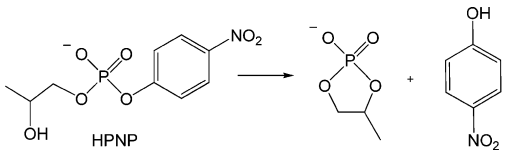


INTRODUCTION

In view of the biological importance of the phosphodiester bond, notably in DNA and RNA, a great effort has been devoted by many workers to the design and synthesis of supramolecular catalysts endowed with phosphodiesterase activity.[1−3] In its simplest expression, a supramolecular catalyst consists of a recognition unit and a catalytic unit connected by a suitable linker. In a large number of phosphodiesterases reported so far, the recognition unit consists of one or two guanidinium units,[3] whereas the catalytic unit is either a general base or a nucleophile, depending on whether the catalyst is expected to display RNAase or DNAase activity, respectively.

In recent works, we[4] and others[5] have used a *m*-xylylene spacer for connecting a guanidinium group to a 1,4,7-triazacyclononane (TACN) ligand, and the role of general base was played by the hydroxo form of a ligated metal ion (Zn^{II}, Cu^{II}), Figure 1.

These bifunctional catalysts showed from modest (Zn^{II}) to fairly high (Cu^{II}) degrees of cooperation between catalytic units in the transesterification of HPNP (eq 1) in 4:1 (v/v) DMSO−



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water (heretofore referred to as 80% DMSO), and rate enhancements relative to background hydrolysis of 1×10^4-fold and 4×10^4-fold, respectively, at 5 mM catalyst concentration.[4]

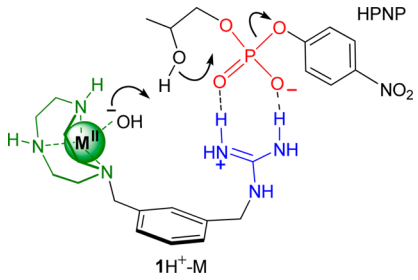

Figure 1. Suggested mechanism of HPNP cleavage catalyzed by the metal complexes of $\mathbf{1}H^+$ (M^{II} = Cu^{II}, Zn^{II}).

Catalytic efficiency requires that in the transition state, substrate and catalyst form a well-matched pair in terms of size and geometrical features. Consequently, the catalytic performance of a bifunctional catalyst critically depends on the nature of the scaffold on which the catalytic units are implanted.

The use of the upper rim of *cone*-calix[4]arenes as a convenient platform for the introduction of catalytic units is well documented.[6] The high catalytic efficiency achieved in a number of cases has been ascribed, inter alia, to a high level of adaptability resulting from the conformational mobility of the calix[4]arene scaffold itself.[6e] It seemed, therefore, worthwhile to synthesize

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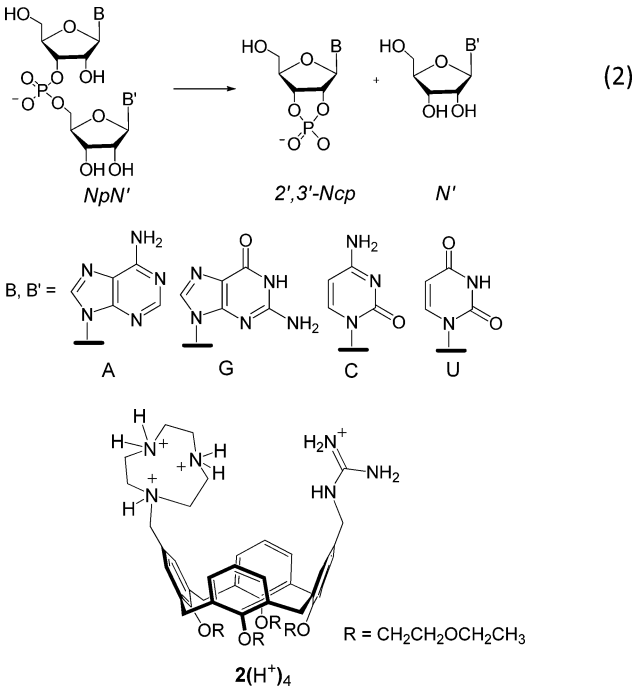
compound **2**, and to compare the phosphodiesterase activity of its Zn^{II} and Cu^{II} complexes to that of the corresponding complexes of **1**.

Calix[4]arenes functionalized with two or more different units at the upper rim are quite rare,[7] and to the best of our knowledge, there are no important examples where calixarene catalysts or receptors can benefit from the cooperation of these different functional groups. The exploitation of such an unexplored arrangement of functions is, moreover, also hampered by the paucity of synthetic methodologies able to differentiate between the groups present at the upper rim of a calixarene scaffold.

In this paper, we report on the synthesis of the bifunctional compound **2** and a kinetic investigation of the cleavage of HPNP in the presence of the Cu^{II} complex of $\mathbf{2}H^+$. The finding that the catalyst turned out to cleave HPNP much more effectively than the Cu^{II} complex of $\mathbf{1}H^+$ encouraged an extension of the catalytic study to the cleavage of a number of diribonucleoside monophosphates (eq 2).



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RESULTS AND DISCUSSION

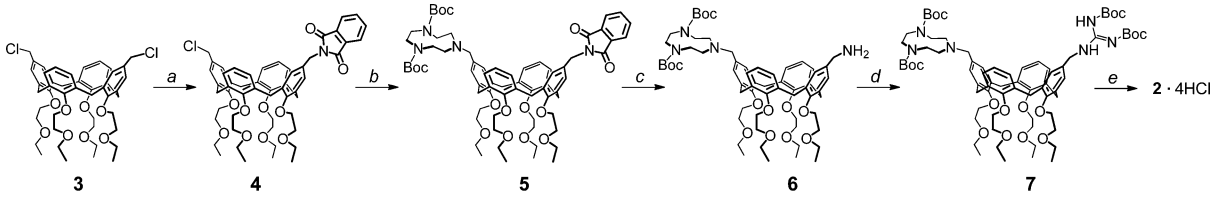
Synthesis of the Ligand. Compound **2** was synthesized as described in Scheme 1, starting from 5,17-bis(chloromethyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (**3**).[8]

The first step resulted to be the crucial one in the desymmetrization of the two functional groups present at the upper rim of the calixarene scaffold. With the use of a stoichiometric amount of potassium phthalimide and dichloromethyl calixarene **3**, a nearly statistical distribution of isolated products could be found. Besides the monochloromethyl-monophthalimidomethyl-derivative **4** as main-product (38%), a mixture of the diphthalimidomethyl-calixarene (16%) and of unreacted **3** (23%) was obtained. The following synthetic steps proceeded smoothly. The chloromethyl group in **4** was reacted with *N*,*N*′-bis(Boc)-1,4,7-triazacyclononane to obtain **5** (70% yield), which was quantitatively deprotected from phthalimide to **6**. Guanidinylation of the CH_2NH_2 group with [*N*,*N*′-bis(Boc)]-*N*′′-triflylguanidine[9] afforded **7** (68% yield) and final Boc removal with trifluoroacetic acid, followed by trifluoroacetate anion exchange with chloride, gave compound **2**·4HCl (98% yield). All the newly synthesized compounds **4**−**7** and **2** show NMR and mass spectra consistent with their structures. ^{1}H- and ^{13}C-NMR spectra of compounds **5**−**7** show patterns of signals compatible with the presence of different rotamers as a consequence of the presence of the bis(Boc)-protected triazacyclononane at their upper rim and in agreement with what found with other bis(Boc)-TACN functionalized derivatives.[10] These rotamers and their sets of signals completely disappear upon removal of the Boc groups, and the NMR spectra of **2** become perfectly compatible with the presence of a single chemical species.

Potentiometric Titrations. Preliminary to the kinetics, potentiometric titrations of **2**·4HCl in the absence and presence of $ZnCl_2$ or $CuCl_2$ were carried out in 80% DMSO at 25 °C. In this medium, the autoprotolysis of water is strongly suppressed, pK_w = 18.4 at 25 °C,[11] and this implies that pH 9.2 corresponds to a neutral solution. The results of the potentiometric titrations (Figures 2 and 3) are summarized in Table 1. Titration of **2**·4HCl with Me_4NOH (Figure 2a) showed, as expected, four titratable protons. The least acidic proton (pK_a = 13.8) belongs to the guanidinium unit, whereas the three most acidic protons are those of the fully protonated nitrogen ligand. Addition of 1 mol equiv of $ZnCl_2$ strongly modified the shape of the titration curve (Figure 2b) and caused an increase to five of the number of titratable protons. The three most acidic protons were again assigned to the fully protonated azamacrocycle, whose increase in apparent acidity was ascribed to the strong binding of the ligand to the metal ion (log K_{Zn} = 7.6). The pK_a value of 9.9 was assigned to a Zn^{II}-coordinated water molecule.

Titration of **2**·4HCl in the presence of 1 mol equiv of $CuCl_2$ gave similar results (Figure 3). The affinity of Cu^{II} for the triazacyclononane ligand (log K_{Cu} = 9.2) is larger than that of

Scheme 1. Synthesis of 2[a]



[a] *a*: Potassium phthalimide; toluene, 110 °C. *b*: *N*,*N*′-Bis(*tert*-butoxycarbonyl)-1,4,7-triazacyclononane, K_2CO_3; CH_3CN, rt. *c*: $N_2H_4{\cdot}H_2O$; MeOH, 60 °C. *d*: *N*,*N*′-Bis(*tert*-butoxycarbonyl)-*N*″-triflylguanidine, NEt_3; CH_2Cl_2, rt. *e*: (1) CF_3COOH (TFA), Et_3SiH (TES); CH_2Cl_2, rt; and then (2) 1 M aqueous HCl; EtOH, rt.

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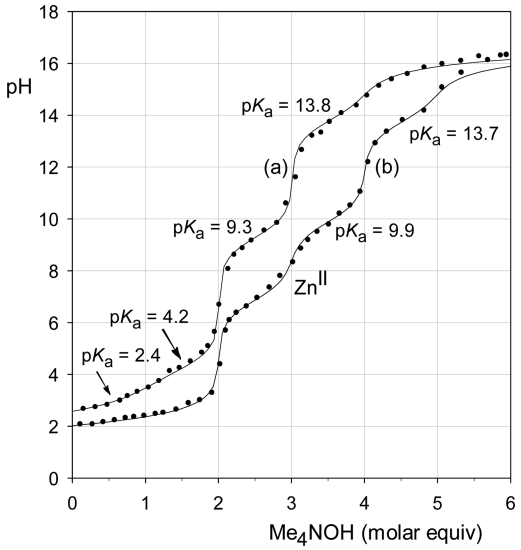

Figure 2. Potentiometric titration of 1.0 mM $\mathbf{2}(H^+)_4$ with Me_4NOH in 80% DMSO in the absence (a) and presence (b) of 1 molar equiv of Zn^{II}. Data points are experimental and the lines are calculated.

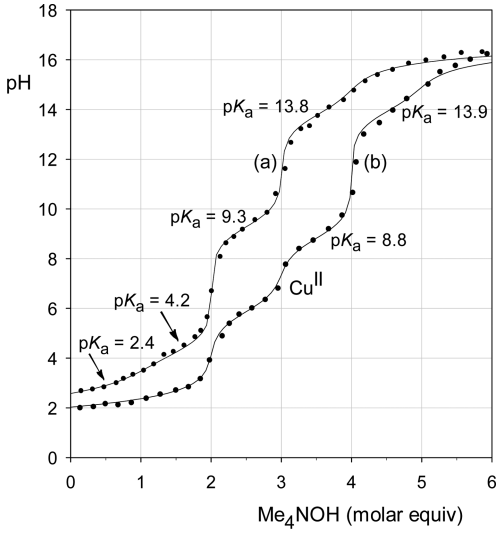

Figure 3. Potentiometric titration of 1.0 mM $\mathbf{2}(H^+)_4$ with Me_4NOH in 80% DMSO in the absence (a) and presence (b) of 1 molar equiv of Cu^{II}. Data points are experimental and the lines are calculated.

Table 1. Acidity Constants (pK_a) of 2·4HCl, in the Absence and Presence of 1 Molar Equiv of Zn^{II} or Cu^{II}, (80% DMSO, 25 °C)[a]

entry	additive	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}	pK_{a5}
1	none	2.4	4.2	9.3	13.8[b]	
2	Zn^{II} [c]	<2	<2	6.9	9.9	13.7
3	Cu^{II} [d]	<2	<2	5.9	8.8	13.9

[a] pK_a data from potentiometric titration with Me_4NOH of 1.0 mM **2**·4HCl solutions in the presence of 10 mM Me_4NClO_4. Experimental uncertainty = ±0.1 pK units or less. [b] Under the same conditions, the pK_a of guanidine·HCl is 13.7 (see ref 4). [c] From the potentiometric titration: log K_{Zn} = 7.6 ± 0.4, for the binding of Zn^{II} to the triazacyclononane moiety in $\mathbf{2}H^+$. [d] From the potentiometric titration: log K_{Cu} = 9.2 ± 0.4, for the binding of Cu^{II} to the triazacyclononane moiety in $\mathbf{2}H^+$.

Zn^{II}, and the metal-coordinated water molecule is significantly more acidic (pK_a = 8.8).

Cleavage of HPNP. In a first set of catalytic runs, the pH was equal to the pK_a of the metal-coordinated water molecule, namely, pH 9.9 for the Zn^{II} complex and pH 8.8 for the Cu^{II} complex. At the given pH values, the guanidine unit is fully protonated, the triazacyclononane ligand is fully bound to the metal ion, and the latter is 50% in the hydroxo form. The results of the kinetic experiments are listed in Table 2 as pseudo-first-order specific rates $k_{obs} = v_0/[HPNP]$, where v_0 is the spectrophotometrically determined initial rate of *p*-nitrophenol release.

Table 2. Pseudo-First-Order Rate Constants and Acceleration Factors Relative to Background for the HPNP Cleavage in the Presence of Additives (80% DMSO, 25 °C)[a]

entry	conditions	additives	k_{obs} (s^{-1})[b]	k_{obs}/k_{bg} [c]
1	pH 9.9	TACN + $ZnCl_2$	2.0×10^{-5}	400
2		$\mathbf{2}H^+$ + $ZnCl_2$	1.1×10^{-4}	2200
3	pH 8.8	TACN + $CuCl_2$	1.4×10^{-6}	350
4		$\mathbf{2}H^+$ + $CuCl_2$	1.3×10^{-3}	320000

[a] 0.20 mM HPNP; 0.50 mM additive; 0.10 M *N*,*N*-diisopropyl ethanolamine buffer. [b] k_{obs} calculated as $v_o/[HPNP]$; error limits on the order of ±10%. [c] From ref 4: $k_{bg} = 5.0 \times 10^{-8}$ s^{-1} at pH 9.9, and $k_{bg} = 4.0 \times 10^{-9}$ s^{-1} at pH 8.8.

The metal complexes of the bifunctional compound $\mathbf{2}H^+$ are catalytically more effective than their monofunctional controls (compare entries 1 and 3 with entries 2 and 4, respectively),[12] which indicates the existence of cooperation of the hydroxo complexes of the ligated metal ions with the neighboring guanidinium. However, the Zn^{II} complex of $\mathbf{2}H^+$ is only 5 times more effective than the corresponding complex of TACN. This disappointingly moderate degree of synergism between catalytic units is very similar to that observed for the Zn^{II} complex of $\mathbf{1}H^+$, showing that as long as the Zn^{II} complexes are involved, replacement of a *m*-phenylene spacer with an upper rim 1,3-distal calix[4]arene does not improve catalytic efficiency.

Excellent results, on the other hand, were obtained with the Cu^{II} complex. The catalytic efficiency of $\mathbf{2}H^+$-Cu is 10^3 times higher than that of the monofunctional control TACN-Cu, and 25 times higher than that of the corresponding complex of $\mathbf{1}H^+$–Cu,[4] showing that the remarkably high catalytic performance arises from a very large degree of cooperation between catalytic units. Notably, the catalytic rate enhancement relative to background hydrolysis (k_{obs}/k_{bg}) is as high as 3.2×10^5.

The strictly linear dependence of initial rate on substrate concentration (Figure 4) shows that association of the catalyst to HPNP, if any, is too weak to affect the kinetics in the investigated concentration range, as previously found to be the case with catalyst $\mathbf{1}H^+$–Cu.[4] In other words, there is no significant binding in the reactant state that may be ascribed to two-point hydrogen bonding interaction of the guanidinium with the anionic phosphate group. All of the available binding energy arising from the above interaction is utilized in transition state stabilization and, consequently, is fully translated into catalysis.

Investigation of the catalytic efficiency of $\mathbf{2}H^+$–Cu as a function of pH gave a sigmoid-shaped pH-rate profile (Figure 5). The catalyst is inactive at pH < 7, but becomes increasingly more active at higher pH values until saturation is reached at pH > 11. Data points were fitted to eq 3, where

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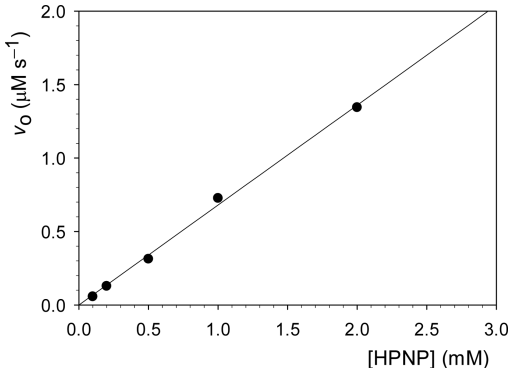

Figure 4. Plot of initial rates for the cleavage of HPNP in 80% DMSO catalyzed by 0.50 mM **2**H$^+$-Cu vs substrate concentration, pH 8.8, 25 °C. From the slope of the straight line, $k_{obs} = (1.36 \pm 0.06) \times 10^{-3}$ s^{-1}.

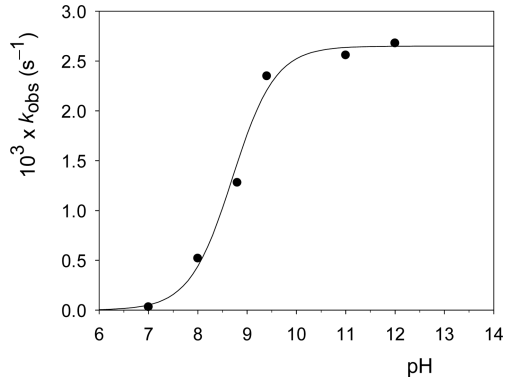

Figure 5. pH-rate profile for the cleavage of 0.20 mM HPNP catalyzed by 0.50 mM **2**H$^+$-Cu in 80% DMSO, 25 °C. Data points are experimental and the line is the plot of eq 3.

$$k_{obs} = \frac{kC_{cat}}{1 + [H^+]/K_a} \quad (3)$$

where k is the second-order rate constant for reaction of the fully deprotonated form of a species whose acidity constant is K_a and stoichiometric concentration is C_{cat}. A least-squares fitting procedure gave pK_a = 8.7 ± 0.1 and k = 5.3 ± 0.2 M^{-1} s^{-1}. The nice fit of data points to eq 3 and the good agreement of the kinetically determined pK_a value with the potentiometric value of 8.8 ± 0.1 are clearly consistent with the idea that the hydroxo form of the ligated Cu^{II} ion is the sole catalytically active species. The limiting value of $(2.65 \pm 0.10) \times 10^{-3}$ s^{-1} approached by the quantity $k_{obs} = kC_{cat}$ in the high pH domain (i.e., when $[H^+]/K_a \ll 1$) is twice as large as the k_{obs} value measured at pH 8.8 (Table 2, entry 4 and caption to Figure 4), where only half of the catalyst is in its active form.

Cleavage of Diribonucleoside Monophosphates. Independent of whether the cleavage of phosphate diesters occurs via a two-step ($A_N + D_N$) or a concerted (A_ND_N) mechanism,[1b] there seems to be little doubt that upon replacement of a good leaving group with a poor one the transition state bears a closer resemblance to a pentavalent phosphorane dianion. Consequently, larger rate enhancements are expected for the reactions of phosphodiesters less activated than HPNP, arising from a stronger electrostatic/electrophilic interaction with the neighboring guanidinium of the bifunctional catalyst. Accordingly, the high catalytic performance of **2**H$^+$−Cu in the cleavage of HPNP prompted us to expand the study to the cleavage of diribonucleoside-3′,5′-monophosphates *Np*N′ (eq 2). A set of four *Np*N′ substrates, namely GpA, GpU, UpU, and CpA, was subjected to catalytic cleavage under the same conditions adopted for HPNP, with the sole difference that the temperature was raised from 25.0 to 50.0 °C because diribonucleoside monophosphates are much more reluctant than HPNP to undergo hydrolytic cleavage. The reaction progress was monitored as before[13] by HPLC analysis of periodically withdrawn samples of the reaction mixtures. Pseudo-first-order specific rates k_{obs} listed in Table 3 were calculated as

Table 3. Cleavage of Diribonucleoside 3′,5′-Monophosphates *Np*N′ by 2H$^+$-Cu[a]

entry	*Np*N′	$10^6 \times k_{obs}$ (s^{-1})[b]	$10^{11} \times k_{bg}$ (s^{-1})	k_{obs}/k_{bg}
1	GpA	160	1.2[c]	1.3×10^7
2	GpU	85	2.5[d]	3.4×10^6
3	UpU	26	2.0[e]	1.3×10^6
4	CpA	1.2	1.4[d]	8.6×10^4

[a]0.50 mM precatalyst **2**·4HCl; 0.50 mM $CuCl_2$; 0.10 mM *Np*N′; 0.10 M *N*,*N*-diisopropyl ethanolamine buffer; 80% DMSO, pH 8.8, 50.0 °C. [b]Error limits on the order of ±10%. [c]Extrapolated to pH 8.8, from data measured in the presence of 1.0 mM Me_4NOH in 80% DMSO, 50.0 °C. [d]Calculated at pH 8.8 from data in ref 13a. [e]Calculated at pH 8.8 from data in ref 13b.

$k_{obs} = v_0/[NpN']$, where v_0 is the initial rate of nucleoside N′ formation (eq 2). Table 3 shows that **2**H$^+$−Cu effectively cleaves the investigated substrates, with catalytic rates decreasing in the order GpA > GpU > UpU ≫ CpA. Because of the different temperatures at which the kinetics were carried out, namely, 50 vs 25 °C, a convenient way to compare catalytic efficiencies in the cleavage of diribonucleoside monophosphates vs HPNP is to resort to catalytic rates relative to background (k_{obs}/k_{bg}), under the assumption that the temperature coefficients of the k_{obs}/k_{bg} ratios are not too different. In line with expectations, comparison of the k_{obs}/k_{bg} values in Table 3 with the corresponding value for HPNP ($k_{obs}/k_{bg} = 3.2 \times 10^5$ in Table 2) shows that **2**H$^+$−Cu catalyzes the cleavage of GpA, GpU, and UpU much more efficiently than the cleavage of HPNP, but the reverse holds for CpA.[14] Since the value of k_{bg} of the latter is similar to that of the other diribonucleoside monophosphates, the low rate of catalytic cleavage cannot be ascribed to the inherently low reactivity of CpA, but presumably to unfavorable interactions between the catalyst and the altered substrate in the transition state, that are either absent or less important for the more reactive diribonucleoside monophosphates.

Attempts at a quantitative comparison of the catalytic efficiency of artificial phosphodiesterases in the cleavage of diribonucleoside monophosphates are hampered by the fact that available data often consist of erratic examples, related to different substrates and scattered over diverse experimental conditions.[15] Another difficulty arises from the paucity of reliable k_{bg} data required for the calculation of k_{obs}/k_{bg} values. The only sets of catalytic data that are homogeneous enough in terms of solvent and temperature for a meaningful comparison with data from the present work are listed in Table 4. All these systems are based on the guanidine−guanidinium catalytic dyad, held in close proximity by means of different strategies, i.e., using a *cone*-calix[4]arene spacer or nanostructured supports, see footnotes *c*−*e* to Table 4. It is apparent that catalytic rates critically depend on the identity of the catalyst-substrate pair. For example, CpA is the worst substrate for catalyst **2**H$^+$−Cu, but turns out to be the most reactive with catalytic system IV in Table 4. In spite of these limitations, the data point to a marked superiority of catalyst

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Table 4. Phosphodiesterase Activity of Guanidino-Based Catalytic Systems (I–IV) in 80% DMSO; $10^6 \times k_{obs}$ (s^{-1}) and k_{obs}/k_{bg} Values (In Parentheses) Normalized to 1 mM Catalyst Concentration

entry	substrate	I[a]		II[b]		III[c]		IV[d]	
1	GpA[e]	320	(2.7×10^7)	0.50	(1.0×10^3)	*n.d.*[f]		*n.d.*[f]	
2	GpU[e]	170	(6.8×10^6)	33	(3.4×10^4)	14	(2.3×10^4)	8.0	(2.6×10^4)
3	UpU[e]	52	(2.6×10^6)	7.0	(8.6×10^3)	22	(4.3×10^4)	12	(4.6×10^4)
4	CpA[e]	2.4	(1.7×10^5)	0.37	(6.5×10^2)	0.92	(2.6×10^3)	30	(1.7×10^5)
5	HPNP[g]		(6.4×10^5)		(70)		(9.2×10^2)		(5.7×10^3)

[a]Catalyst: $2H^+$-Cu; pH 8.8 (this work). [b]Catalyst: 1,2-vicinal diguanidinocalix[4]arene; pH 10.4 (data from ref 13c). [c]Catalyst: guanidine based self-assembled monolayers on Au nanoparticles; pH 10.2 (data from ref 13b). [d]Catalyst: guanidine-based polymer brushes grafted onto silica nanoparticles; pH 9.9 (data from ref 13a). [e]Data at 50.0 °C. [f]Not determined. [g]Data at 25.0 °C.

$2H^+$–Cu, both in terms of absolute catalytic rates and rates relative to background hydrolysis, at 1 mM catalyst concentration.

To sum up, we have found that catalyst $2H^+$–Cu is 40-fold more active than $1H^+$–Cu in the transesterification of HPNP, which indicates that the calix[4]arene linker in $2H^+$–Cu strongly enhances the extent of cooperation between guanidinium and the ligated Cu^{II} ion. Catalyst $2H^+$–Cu promotes the cleavage of GpA, GpU, and UpU much more effectively than that of HPNP, but the reverse holds for CpA. The rate enhancement relative to the background hydroxide reaction is 10^5 for the latter substrate, but unprecedented values as high as 10^7 are found in the reactions of the best substrates.

Combination of catalytic data from the present work with analogous data from previous works reinforces the view that the upper rim of *cone*-calix[4]arenes is a very convenient platform for the construction of bifunctional catalysts. Inspection of CPK molecular models suggests that the strength of the interaction between the conformationally mobile substrates and catalyst critically depends on a large number of attractive and repulsive secondary interactions that are difficult to rationalize with a computational approach. Thus, the question of catalytic effectiveness does not resolve itself into a mere question of "distance" between catalytic groups. As a matter of fact, the novel arrangement obtained through the implantation of two different functionalities at the upper rim of a calix[4]arene, now easily achievable via the synthetic strategy herein reported, can give rise to remarkable cooperation factors and might therefore play a strategic role in the preparation of further catalysts and receptors with increased molecular diversity.

EXPERIMENTAL SECTION

Instruments. NMR spectra were recorded on either a 400- or 300-MHz spectrometer. 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 (ESI) 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.[3c]

Spectrophotometric measurements were carried out at 400 nm on either a double beam or on a diode array spectrophotometer. HPLC analyses were performed on a liquid chromatograph fitted with a UV–vis detector operating at 254 nm.

Materials and General Procedures. All reactions, with the sole exception of the deprotection of **7**, were carried out under a nitrogen atmosphere. Flash chromatography was carried out on 230–240 mesh silica gel. Anhydrous CH_2Cl_2 (DCM) 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 potentiometric experiments. HPNP[16] and 5,17-bis(chloromethyl)-25,26,27,28-tetrakis-(2-ethoxyethoxy)calix[4]arene **3**[8] were prepared according to literature procedures. All other solvents and reagents were commercial samples and used as such. Commercial samples of *NpN′*[13] and their aqueous solutions were stored at −20 °C.

Warning! Care was taken when handling tetramethylammonium perchlorate because it is potentially explosive.[17] No accident occurred in the course of the present work.

*5-(N-Phthalimidomethyl)-17-(chloromethyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (**4**).* To a solution of **3** (0.242 g, 0.30 mmol) in dry toluene (20 mL) were added potassium phthalimide (0.061 g, 0.30 mmol) and dicyclohexyl-18-crown-6 (0.11 g, 0.30 mmol). The reaction mixture was heated to 110 °C and stirred for 6 h, then was quenched by adding a 1 M HCl solution (25 mL) and vigorously stirred for additional 30 min at room temperature. The organic phase was separated, washed with distilled water (2 × 20 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The crude was purified by flash chromatography (hexane/AcOEt 6:4) to give **4** as a white solid foam (0.105 g, 0.114 mmol; 38% yield): mp 68–72 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.87–7.82 (m, 2H), 7.70–7.69 (m, 2H), 6.80 (s, 2H), 6.77 (s, 2H), 6.51 (m, 6H), 4.54 (s, 2H), 4.51 (d, 2H, J = 13.4 Hz), 4.44 (d, 2H, J = 13.4 Hz), 4.40 (s, 4H), 4.15 (t, 4H, J = 5.6 Hz), 4.12–4.03 (m, 4H), 3.85–3.78 (m, 8H), 3.56–3.45 (m, 8H), 3.16 (d, 2H, J = 13.4 Hz), 3.11 (d, 2H, J = 13.4 Hz), 1.28–1.11 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 168.1, 157.1, 156.5, 155.8, 135.9, 135.6, 134.4, 134.2, 133.9, 132.2, 130.9, 129.7, 128.7, 128.2, 128.0, 123.2, 122.5, 73.3, 73.1, 72.9, 69.7, 69.6, 66.4, 66.3, 46.8, 41.3, 30.8, 30.7, 15.3. HR ES-MS: m/z Calcd for $C_{54}H_{62}O_{10}ClNNa$ [(**4** + Na)$^+$] 942.39599, found 942.39545; m/z Calcd for $C_{54}H_{66}ClN_2O_{10}$, [(**4** + NH_4)$^+$] 937.44060, found 937.44005.

*5-(N-Phthalimidomethyl)-17-[N,N′-bis(tert-butoxycarbonyl)-1,4,7-triazacyclo-non-1-yl]methyl-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (**5**).* To a solution of **4** (0.105 g, 0.114 mmol) in dry CH_3CN (4 mL) were added 1,4-bis(*tert*-butoxycarbonyl)-1,4,7-triazacyclononane (0.055 g, 0.17 mmol) and K_2CO_3 (0.021 g, 0.15 mmol). The reaction mixture was stirred for 4 days at room temperature, during which additional 1,4-bis(*tert*-butoxycarbonyl)-1,4,7-triazacyclononane (2 × 0.015 g, 0.046 mmol) was added. The reaction was then quenched by evaporating the solvent under reduced pressure. The residue was taken with DCM (20 mL) and the organic phase was washed with a saturated solution of $NaHCO_3$ (20 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude was purified by flash chromatography (hexane/AcOEt 6:4; AcOEt/MeOH 7:3) to give **5** as a colorless oil (0.11 g; 0.080 mmol; 70% yield) and recover unreacted 1,4-bis(*tert*-butoxycarbonyl)-1,4,7-triazacyclononane (0.066 g, 0.055 mmol; 48% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.83–7.82 (m, 2H), 7.70–7.67 (m, 2H), 7.08, 6.99, 6.97 (3 s, 2H), 6.89, 6.85–6.75 (3 s, 2H), 6.33–6.30 (2 m, 6H), 4.68 (4 s, 2H), 4.55–4.40 (m, 4H), 4.18–3.92 (m, 8H), 3.86–3.76 (m, 8H), 3.57–3.45 (m, 14H), 3.23–3.08 (m, 8H), 2.36 (m, 4H), 1.47–1.44 (m, 18H), 1.28–1.11 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 168.1, 157.6, 155.8–155.3, 136.2–135.7, 133.9, 132.5–132.0, 129.8, 129.2, 127.8, 123.3, 122.4, 73.7, 73.3, 72.7, 69.6, 66.4, 60.7, 52.6, 52.4, 50.3, 49.9, 49.4, 41.6, 30.8, 28.6, 15.3. HR ES-MS: m/z Calcd for $C_{70}H_{93}N_4O_{14}$ [(**5** + H)$^+$] 1213.66883, found 1213.66828; m/z Calcd for $C_{70}H_{92}N_4O_{14}Na$, [(**5** + Na)$^+$] 1235.65078, found 1235.65453.

5-(Aminomethyl)-17-[N,N′-bis(tert-butoxycarbonyl)-1,4,7-triaza-cyclonon-1-yl]-methyl-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]-

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*arene (**6**)*. To a solution of **5** (0.043 g, 0.035 mmol) in dry MeOH (4 mL) was added $N_2H_4{\cdot}H_2O$ (44 μL, 1.42 mmol). The reaction mixture was heated to 60 °C and stirred for 3 h, then was quenched by evaporating the solvent under reduced pressure. The residue was taken with DCM (20 mL) and the organic phase was washed with a solution of NaOH 1 M (20 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. **6** was obtained as a colorless oil (0.038 g, 0.035 mmol; quantitative yield), pure enough to avoid further purifications. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.83, 6.75, 6.67 (3 s, 2H), 6.79, 6.69, 6.64 (3 s, 2H), 6.53−6.33 (3 m, 6H, Ar*H*), 4.48−4.43 (m, 4H), 4.17−4.01 (m, 8H), 3.86−3.81 (m, 8H), 3.69−3.41 (m, 16H), 3.13−3.08 (m, 8H), 2.60−2.46 (m, 4H), 1.47−1.44 (m, 18H), 1.28−1.17 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 157.1, 155.9, 155.8, 155.7, 155.6, 155.5, 155.3, 136.7, 135.9−134.0, 133.2, 133.0, 132.8, 129.2, 128.9, 128.5, 128.1, 127.9, 127.8, 127.8, 127.1, 127.0, 122.3, 79.4, 79.3, 79.3, 73.5−72.6, 69.7, 69.6, 66.4−66.2, 60.5, 53.6, 53.4, 50.3, 49.9, 49.6, 49.3, 46.1, 30.9, 28.6, 15.3. HR ES-MS: *m*/*z* Calcd for $C_{62}H_{91}N_4O_{12}$ [(**6** + H)$^+$] 1083.66335, found 1083.66638; *m*/*z* Calcd for $C_{62}H_{90}N_4O_{12}Na$, [(**6** + Na)$^+$] 1105.64530, found 1105.64758.

*5-N-[N′,N″-bis(tert-butoxycarbonyl)guanidine]methyl-17-[4,7-bis(tert-butoxycarbonyl)-1,4,7-triazacyclonon-1-yl]methyl-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (**7**)*. To a solution of **6** (0.040 g, 0.037 mmol) in dry DCM (4 mL) were added *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-triflylguanidine (0.017 g, 0.044 mmol) and triethylamine (5 μL, 0.037 mmol). The reaction mixture was stirred overnight at room temperature, then was quenched by adding distilled water (20 mL) and was vigorously stirred for an additional 30 min. The aqueous phase was separated and washed with DCM (2 × 20 mL), then the combined organic phases were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude was purified by preparative TLC plates (hexane/AcOEt 6.5:3.5) to give **7** as a light yellow oil (0.033 g, 0.025 mmol; 68% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 11.52 (s, 1H), 8.50 (t, 1H, *J* = 4.8 Hz), 6.96, 6.87, 6.79 (3 s, 2H), 6.93, 6.85, 6.78 (3 s, 2H), 6.40−6.20 (m, 6H), 4.52−4.39 (m, 6H), 4.21−4.10 (m, 4H), 4.03−3.94 (m, 4H), 3.87−3.81 (m, 8H), 3.56−3.46 (m, 14H), 3.23−3.09 (m, 8H), 2.65−2.55 (m, 4H), 1.51−1.44 (m, 36H), 1.26−1.22 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 163.6, 157.7, 156.9, 156.5, 155.9, 155.7, 155.5, 155.1, 154.8, 136.7−135.3, 133.9−133.0, 130.5, 129.6, 129.3, 128.8, 128.0, 127.8, 122.3, 83.0, 79.5−79.2, 73.5−72.3, 69.7, 69.6, 66.4−66.2, 60.5, 53.9−53.2, 51.0−49.0, 44.2, 30.9, 28.6, 28.3, 28.1, 15.3. HR ES-MS: *m*/*z* Calcd for $C_{73}H_{109}N_6O_{16}$ [(7 + H)$^+$] 1325.79001, found 1325.78946; *m*/*z* Calcd for $C_{73}H_{108}N_6O_{16}Na$ [(7 + Na)$^+$] 1347.77195, found 1347.77140.

*5-N-Guanidinomethyl-17-N-[1,4,7-triazacyclonon-1-yl]methyl-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene Tetrahydrochloride (**2**·4HCl)*. In a mixture of DCM/TFA/TES 95:2.5:2.5 (10 mL), **7** (0.040 g, 0.030 mmol) was dissolved. The reaction mixture was stirred overnight at room temperature and quenched by evaporating the solvent under reduced pressure. The residue was first taken in a solution of HCl 1 M in EtOH (3 mL) and vigorously stirred for 30 min three times, to exchange the TFA anion, then was recrystallized from DCM with hexane to give **2**·4HCl as a light gray solid (0.032 g, 0.030 mmol; 98% yield): mp 123−130 °C. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.56 (t, 1H, *J* = 5.0 Hz), 7.08 (s, 1H), 6.89 (s, 2H), 6.80 (s, 2H), 6.53 (d, 4H, *J* = 6.6 Hz), 6.49 (t, 4H, *J* = 6.6 Hz), 4.59 (d, 4H), 4.19−4.15 (m, 6H), 4.08−4.05 (m, 4H), 3.92−3.85 (m, 8H), 3.70 (s, 2H), 3.60−3.54 (m, 12H), 3.22−2.97 (m, 8H), 2.69 (m, 4H), 1.23−1.16 (m, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 157.1, 156.9, 156.8, 155.8, 136.1, 135.8, 134.2, 134.1, 130.2, 129.4, 129.4, 127.9, 127.8, 127.4, 122.0, 73.3, 72.1, 69.9, 69.7, 69.6, 66.0, 58.8, 47.4, 44.8, 43.3, 42.1, 30.5, 30.4, 14.3. ES-MS: *m*/*z* 925.9 [(**2** + H)$^+$]. HR ES-MS: *m*/*z* Calcd for $C_{53}H_{77}N_6O_8$ [(**2** + H)$^+$] 925.58029, found 925.58091.

Potentiometric Titrations. Potentiometric titrations were carried out as previously reported.[3c,13a,b] A solution of 70 mM Me_4NOH in 80% DMSO was added in small increments under a nitrogen atmosphere to 6 mL of a 1 mM solution (80% DMSO) of $\mathbf{2}(H^+)_4$, 10 mM Me_4NClO_4, in the absence or presence of stoichiometric 1 mM $ZnCl_2$ or $CuCl_2$ (80% DMSO, 25 °C). Analysis of the titration plots was carried out by using the programm HYPERQUAD 2000.[18]

Kinetic Measurements. Metal complexes were formed in situ by addition of the calculated stoichiometric amount of the aqueous solution of the metal salt ($ZnCl_2$ or $CuCl_2$) to the buffered reaction mixture. The solutions were incubated for 1 h before the start of the kinetic run by fast addition of a small volume of the substrate solution.

Cleavage of HPNP. Rate constants were obtained by UV−vis monitoring of *p*-nitrophenol liberation at 400 nm (initial rate method). Experiments were carried out in the presence of 0.50 mM precatalyst **2**·4HCl, 0.50 mM $ZnCl_2$ or $CuCl_2$, and 0.10 M *N*,*N*-diisopropyl ethanolamine buffer (80% DMSO, 25.0 °C).

Cleavage of Diribonucleoside Monophosphates. HPLC monitoring of nucleoside liberation (initial rate method) in the $\mathbf{2}H^+$−Cu promoted *NpN*′ cleavage was carried out on 0.50 mM solution of precatalyst **2**·4HCl, 0.50 mM $CuCl_2$, 0.10 mM *NpN*′, 0.10 M *N*,*N*-diisopropyl ethanolamine buffer (80% DMSO, pH 8.8, 50.0 °C). Aliquots of the reaction mixture (80 μL) were withdrawn at appropriate time intervals and quenched with 80 μL of a 10 mM solution of $HClO_4$ in 80% DMSO. After addition of *p*-hydroxybenzoic acid (internal standard) in 80% DMSO, the solution was filtered and subjected to HPLC analysis on a Supelcosil C-18 DB column (25 cm × 4.6 mm i.d., particle size 5 μm) by elution with H_2O (0.1% trifluoroacetic acid)/MeCN, linear gradient from 100:0 to 85:15 in 25 min, flow 0.9 mL min^{-1}.

The pseudo-first-order specific rate for background reaction (k_{bg}) of GpA was extrapolated at pH 8.8, from the initial rate of hydroxide catalyzed nucleoside liberation in the presence of 1.0 mM Me_4NOH (pH 15.4), 10 mM Me_4NClO_4, 80% DMSO, 50.0 °C.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra for the products. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b00965.

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Notes

The authors declare no competing financial interest.

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