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Polyhydroxylated Sapphyrins: Multisite Non-metallic Catalysts for Activated Phosphodiester Hydrolysis

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Abstract: Enhanced hydrolysis rates for the cleavage of bis(4-nitrophenyl)phosphate (BNPP), a model phosphodiester, may be achieved by using appropriately designed ditopic receptors containing the known phosphate-binding nucleus, sapphyrin, attached covalently to suitably oriented polyhydroxyl subunits. Evidence for the interaction between sapphyrin and BNPP comes from solid-state X-ray diffraction analysis of a diprotonated dihydroxylated sapphyrin−BNPP complex and from solution-phase ^{31}P NMR spectroscopic binding studies. The sapphyrins described in this paper may have a role to play as oligonucleotide cleavage agents.

Introduction

Phosphate diester hydrolysis is a reaction of continuing interest since such processes are of fundamental biological importance. Under normal, uncatalyzed conditions, phosphate diesters are extremely resistant to hydrolysis with DNA and bis(4-nitrophenyl)phosphate (BNPP) displaying half-lives of 2×10^8 and 2×10^3 years, respectively, at pH 7.0 and 25 °C.[1] On the other hand, several phosphodiesterases, including staphylococcal nuclease,[2] alkaline phosphatase,[3] and DNase I,[4] are known that are very efficient catalysts for the hydrolysis of their phosphodiester substrates. This efficiency often derives from the cooperation of various phosphate binding center(s), such as metal ions[5] (e.g., Zn^{2+}, Mg^{2+}, or Mn^{2+}) and guanidinium with a general base provided from the side chains of His, Lys, Arg, Asn, or Glu.[2,5a,6] The activation of a water molecule, generating an efficient nucleophilic species, is also implicated as playing a vital role in the proposed mechanism of staphylococcal nuclease.[2] Likewise, a serine OH group, together with three metal cations and Arg, has been suggested as being important in the functioning of alkaline phosphatase.[3]

Mimicking the myriad activities of nucleases presents a formidable challenge to those who wish to design artificial catalytic systems capable of promoting efficient hydrolysis of phosphodiester bonds, including those of RNA and, perhaps, even DNA. Yet meeting this challenge is considered important in terms of advancing areas such as supramolecular chemistry and antisense technology. Not surprisingly, therefore, considerable effort has been devoted to the problem, and currently a number of metal-containing systems are known that are designed to enhance catalytically phosphodiester hydrolysis. These include systems wherein two metals are used in a bifunctional catalytic sense,[7] systems wherein a single metal center in conjunction with some other group acts to augment hydrolysis efficiency,[8−12] and metal-free systems,[13,14] including ones

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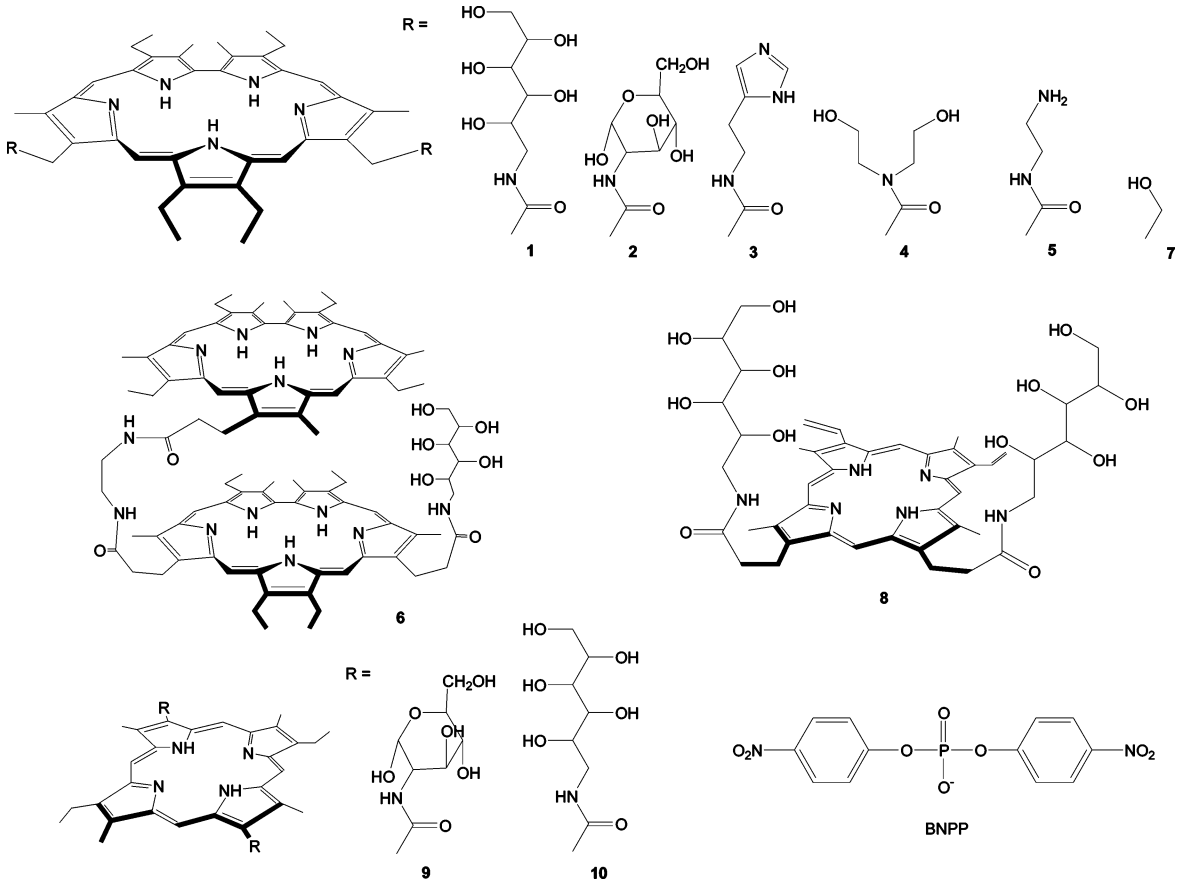
Scheme 1. Molecular Structures of BNPP and Macrocycles Tested as Hydrolysis Catalysts



wherein one or more guanidinium or guanidinium-like binding sites[15−18] are allowed to cooperate with other groups so as to enhance the rate-limiting step of phosphodiester hydrolysis. Systems based on the use of two rather different metal centers (e.g., Zn(II) and Fe(III)) have been also reported.[7f,19] A large number of cyclodextrin-based inclusion complexes with functionalized sidearms, including ones that do not rely on catalytic metal centers, have also been shown to act as phosphodiester hydrolysis catalysts.[20] Nonetheless, the number of functioning, non-metal-based catalyst systems remains rather limited.

In this paper we report an approach to achieving metal-free catalytic phosphodiester hydrolysis that is based on the use of sapphyrins, pentapyrrolic porphyrin-like macrocycles, as the key phosphodiester binding entity. Earlier, we established that certain expanded porphyrins, namely, sapphyrin and rubyrin, act as efficient and selective phosphate binding receptors at neutral pH.[21] We have also described that appropriately designed sapphyrin-based systems may be used as carriers for the through-model-membrane transport of biologically important phosphorylated species, such as nucleotides.[21] Therefore, we rationalized that a combination of a sapphyrin phosphate binding unit and a properly positioned "internal" nucleophile (i.e., appended to the macrocyclic core) might provide a functioning metal-free phosphodiester hydrolysis system. Here, we report that the polyhydroxysapphyrin derivatives **1** and **2** (Scheme 1) act to effect the catalytic hydrolysis of bis(4-nitrophenyl)phosphate (BNPP) at near-neutral pH. We also report the results of hydrolysis experiments carried out using the control porphyrins **8**, **9,** and **10**, as well as the dimeric sapphyrin **6**.

Experimental Section

Materials and Methods. BNPP hydrolysis rates were monitored by following the increase in absorbance at 400 nm, reflecting the

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Table 1. Rate Constants for the Hydrolysis of BNPP by Sapphyrins and Porphyrins[a]

catalyst	rate constant (10^{-5} h^{-1})	catalyst	rate constant (10^{-5} h^{-1})
1	38	**6**	9.5
2	37	**8**	4.3
3	23	**9**	6.7
4	17	**10**	7.0
5	11		

[a] Conditions: pH 7.5, 1×10^{-5} M catalyst, 1×10^{-4} M BNPP, 37 °C. For further details, see: Experimental Section.

production of 4-nitrophenolate anion, as a function of time. All reactions were carried out in doubly distilled water, which was boiled prior to use. Appropriate amounts of $NaNO_3$ and HEPES buffer were introduced in order to make up test solutions with total concentrations of 0.1 and 0.01 M in these two entities, respectively. These solutions were then adjusted to pH 7.5 and, in both cases, filtered through a Nylon 66 (0.45 μm) Millipore filter. Typically, the kinetic experiments were carried out at 37 °C using solutions that were 0.1 M in $NaNO_3$, 0.01 M in HEPES buffer, 1×10^{-5} M in the studied sapphyrin or porphyrin derivative, and 1×10^{-4} M in BNPP. In the sapphyrin- and porphyrin-free control experiments, the concentrations of glucamine, glucosamine, and imidazole were 2×10^{-5} M, with the concentration of BNPP being 1×10^{-4} M at pH 7.5. The rate constants shown in Table 1 were estimated from kinetic runs monitored over a 9 or 10 day period and were determined from the method of initial rates. The concentration of the 4-nitrophenolate anion produced was obtained from absorption at 400 nm ($\epsilon_{400} = 18\,500$ M^{-1} cm^{-1}) in aliquots whose pH was adjusted to pH 10. The concentration of BNPP at each time period was calculated by subtracting the observed 4-nitrophenolate anion concentration from that of the BNPP initially present. The listed rate constants were then determined by dividing the slopes of the linear traces, obtained by plotting this derived BNPP concentration versus time, by the initial BNPP concentration. All rate constants reported in Table 1 were then corrected for the rate of hydrolysis observed in a "blank" run consisting of a mixture of BNPP (1×10^{-4} M) and $NaNO_3$ (0.1 M) in HEPES buffer (0.01 M) at pH 7.5. All kinetic experiments were run at least three times and were found to give rate constant values reproducible to within 10%, a margin of error considered acceptable for the purposes of the present study.

Synthesis of Catalysts. The sapphyrin compounds were prepared in accord with literature procedures reported earlier.[22] The sapphyrins and control porphyrins were prepared by introducing the desired functionality onto the macrocycle periphery via the formation of amide bonds. This was done by reacting the activated bis-acid form of the appropriate macrocycle with a suitable, functionality-bearing amino component. As the amino components, we have used histamine for the preparation of **3**, 1-amino-1-deoxy-D-glucitol (D-glucamide) for the preparation of **1**, **6**, **8**, and **10**, and 1,2,3,4-tetra-*O*-acetyl-2-amino-2-deoxy-D-glucopyranose for the preparation of the precursors to **2** and **9**. In the case of the latter species, deprotection of the acetylated forms was achieved using standard procedures,[23] giving **2** and **9** in good yields. This approach to preparing **2** and **9** is based on the use of an O-protected starting compound and thus represents a different strategy than that used earlier to prepare analogous protoporphyrin derivatives.[24] The reactions were performed in water−DMF (1:1; DMF = dimethylformamide) for histamine and D-glucamide, and in dichloromethane or a 1:1 mixture of dichloromethane and DMF for 1,2,3,4-tetra-*O*-acetyl-2-amino-2-deoxy-D-glucopyranose. As the coupling reagent, either 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) or diisopropylcarbodiimide was used. Final purification of the various water-soluble macrocyclic compounds was achieved using reverse-phase column chromatography.

X-ray Diffraction Analysis. Experimental procedure for $(C_{42}H_{55}N_5O_2)$-$[((C_6H_4NO_2)O)_2PO_2]_2$: Crystals were grown as dark plates from 1:1 chloroform−methanol of sapphyrin **7** and BNPP (1:2 molar ratio) by allowing diethyl ether to diffuse into it over the course of 3 weeks. The data crystal was a plate of approximate dimensions; $0.13 \times 0.56 \times 0.56$ mm. The data were collected at 188 K on a Siemens P3 diffractometer, equipped with a Nicolet LT-2 low-temperature device and using a graphite monochromator with Mo Kα radiation ($\lambda =$ 0.710 73 Å). Four reflections (−1,2,4; 2,3,0; 2,−1,−5; 2,0,2) were remeasured every 96 reflections to monitor instrument and crystal stability. A smoothed curve of the intensities of these check reflections was used to scale the data. The scaling factor ranged from 0.970 to 1.01. The data were corrected for Lp effects but not absorption. Data reduction and decay correction were performed using the SHELXTL/PC software package.[25] The structure was solved by direct methods and refined by full-matrix least- squares on F^2 with anisotropic thermal parameters for the non-H atoms.[25] The hydrogen atoms were calculated in idealized positions (C−H, 0.96 Å; N−H, 0.90 Å) with isotropic temperature factors riding at 1.2 or 1.5 × *U*eq of the attached atom. The higher factor is used for all methyl hydrogens. The function, $\sum w(|F_o|^2 - |F_c|^2)^2$, was minimized, where $w = 1/[(\sigma(F_o))^2 + (0.04P)^2]$ and $P = (|F_o|^2 + 2|F_c|^2)/3$. The absolute configuration was determined by internal comparison. Neutral atom scattering factors and values used to calculate the linear absorption coefficient are from the International Tables for X-ray Crystallography (1990).[26] Other computer programs used in this work are listed elsewhere.[27] All figures were generated using SHELXTL/PC.[25] Tables of positional and thermal parameters, bond lengths, angles and torsion angles, figures, and lists of observed and calculated structure factors are located in the Supporting Information.

Results and Discussion

Design of the Catalysts. The functionalized sapphyrins were designed in consideration of the mechanism of phosphodiester hydrolysis; this is believed to be of an A_ND_N type wherein the transition state resembles a pentavalent phosphorane or is close in energy to a stable pentavalent phosphorane intermediate.[20b] In our design we also took into consideration previous studies showing that nucleophilic displacement plays an important role in mediating phosphodiester hydrolysis.[28] Therefore, we combined the known phosphate ester binding species, sapphyrin, with additional groups that could possibly function as "internal" or "tethered" nucleophiles. Here, our thoughts were that if the latter entities could position themselves near the phosphodiester bond of the putative substrate, good phosphodiester cleavage rates might be obtained. We also appreciated that the fast hydrolysis rates seen for RNA relative to DNA are generally ascribed to the 2′ hydroxyl group that is present in RNA but absent from DNA. In any event, the use and utility of pendant hydroxyl groups in phosphate ester hydrolysis has been demonstrated previously in the case of zinc(II) complexes bearing

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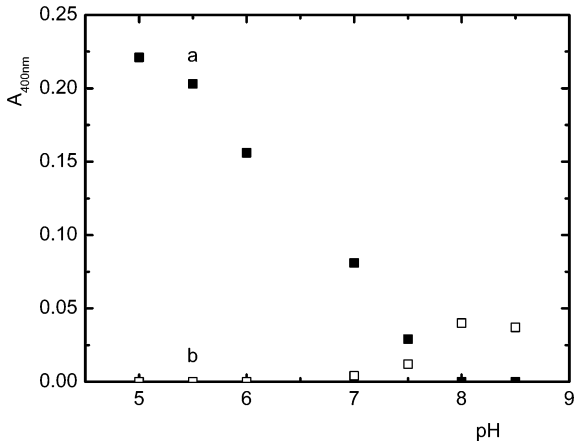

Figure 1. Absorbance changes monitored after 2 days for the production of *p*-nitrophenolate anion from BNPP in the presence (a) and absence (b) of **1** at differing pH values. For further details pertaining to these measurements, see Experimental Section.

an alcohol group[8] and in the case of guanidinium derivatives bearing attached OH groups.[16] In our case, the use of such groups was found to lead not only to apparent catalytic efficiency as described below but also to acceptable water solubility.

Hydrolysis of BNPP. For the present study, we have chosen to use bis(4-nitrophenyl)phosphate (BNPP) as a model phosphodiester substrate and to study the efficacy of all 10 systems given in Scheme 1 at pH 7.5.[29] From Table 1, it is evident that the polyhydroxylated sapphyrins, **1** and **2**, are the most efficient BNPP hydrolysis catalysts within the present sapphyrin- and porphyrin-based set. Not surprisingly, compounds **1** and **2** are far less effective than most of the metal-containing systems reported in the literature. However, in contrast to at least some of these latter systems, **1** and **2** appear to be true catalysts, with a turnover of 1.2 being obtained for both sapphyrins over the course of 10 days. Further, at 37 °C and pH 7.5, K_m, V_{max}, and k_{cat} values of 2.0×10^{-3} M (500 M^{-1}), 6.4×10^{-7} M·h^{-1}, and 3.2×10^{-4} h^{-1}, respectively, could be derived for sapphyrin **1** on the basis of a Michaelis−Menten plot. Explanations for these findings now follow. In the case of **1**, we also studied the effect of pH on the rate of hydrolysis. The rate increases considerably at pH values below 7.5 (Figure 1).

First we felt it appropriate to confirm that BNPP can bind to sapphyrin and can thus serve as a potential substrate for a putative sapphyrin-based catalysis process. The interaction between sapphyrin and this model phosphodiester was established in the solid state on the basis of a single-crystal X-ray diffraction analysis of the 1:2 complex formed between diprotonated dihydroxylated sapphyrin **7** and BNPP. This complex,[30] which was obtained following extraction experiments carried out at pH 3.4, is characterized by two different kinds of phosphate-to-protonated sapphyrin interactions (Figure 2). The first of these is analogous to what was seen in earlier sapphyrin phenyl phosphate and diphenyl phosphate structures,[21c,e,31] in that it involves a single phosphate oxygen atom hydrogen bound to two of the sapphyrin NH sites (the oxygen-to-H hydrogen bonding distances are 1.83 and 2.18 Å, respectively). In contrast, the second type of binding mode is new in our experience and involves not only "normal" oxyanion-to-NH hydrogen bonding interactions but also a set of hydrogen bonding interactions between the bipyrrole subunit and another oxygen of the BNPP substrate (oxygen-to-NH distance, 2.20 Å). This observed interaction provides a possible "hint" of how the suggested sapphyrin-derived phosphodiester activation process could take place, namely, that the macrocycle-derived binding of these two oxygen atoms renders the central P atom more susceptible to nucleophilic attack.

To complement the above analyses, we have also studied the interactions between BNPP and sapphyrins **1** and **2** in methanol-d_4 using ^{31}P NMR spectroscopy. For equimolar 1:1 mixtures of these sapphyrins and BNPP we observed that the chemical shifts of the phosphorus atom were displaced to higher field by ca. 8 ppm compared to what is observed for BNPP alone (for which $\delta = -12.62$ ppm); this, presumably, is the result of the induced ring current associated with the aromatic sapphyrin nucleus. Similar upfield shifts were seen when analogous experiments were performed using sapphyrin **7** in D_2O−methanol-d_4 but not when the water-soluble porphyrin **10** was used instead under the same experimental conditions. These findings, in conjunction with the solid-state results presented immediately above, thus provide support for our previously postulated contention,[21] namely, that the protonated forms of sapphyrin can act as specific and efficient receptors for different phosphates. In the context of the present study, these results are also important in that they are consistent with the suggestion that appropriately functionalized sapphyrins can act to enhance BNPP hydrolysis as the result of, in part, phosphate binding. This postulate is discussed further below.

While ^{31}P NMR spectroscopic measurements in pure water were complicated by precipitation, UV−vis titrations of sapphyrin **2** and porphyrin **9** with BNPP could be carried out at pH 7.5 (0.01 M HEPES and 0.1 M $NaNO_3$). From these, K_a values of ca. 400 and ≤100 M^{-1} could be calculated for the formation of the 1:1 sapphyrin−BNPP and porphyrin−BNPP complexes, respectively, through use of a standard curve fitting program.[32] The sapphyrin **2**−BNPP binding constant, K_a, for the formation of complex **2**−BNPP is at least four times higher than that for the formation of the corresponding porphyrin complex (i.e. **9**−BNPP). This factor of 4 (or more) thus correlates well with the differences in BNPP hydrolysis rates observed for these two putative catalysts (factor of 5; see Table 1). This observation, together with the finding that the rate of hydrolysis increases with decreasing pH (Figure 1), conditions under which the sapphyrin-to-phosphate binding affinity also increases,[21] further supports our contention that phosphate binding plays an important role in inducing phosphodiester hydrolysis. Consistent with this conclusion is the finding that various putative nucleophiles (hydroxyls, imidazole, D-glucosoamine, D-glucamine) fail to effect much in the way of BNPP hydrolysis rate enhancement when tested on their own (data not shown).

(29) Kinetics studies were not performed with **7** as it is insoluble in water.
(30) High-resolution FAB MS and combustion analyses are consistent with the formation of a 1:1 monoprotonated sapphyrin−BNPP complex following extraction experiments carried out at pH 6.0. HRMS. Calcd for $C_{54}H_{63}N_7O_{10}P$ $[M + H]^+$: 1000.437. Found: 1000.440. Anal. Calcd for $C_{54}H_{62}N_7O_{10}P$ (1000.11): 64.86% C; 6.25% H; 9.80% N. Found: 64.61% C; 6.31% H; 9.65% N.
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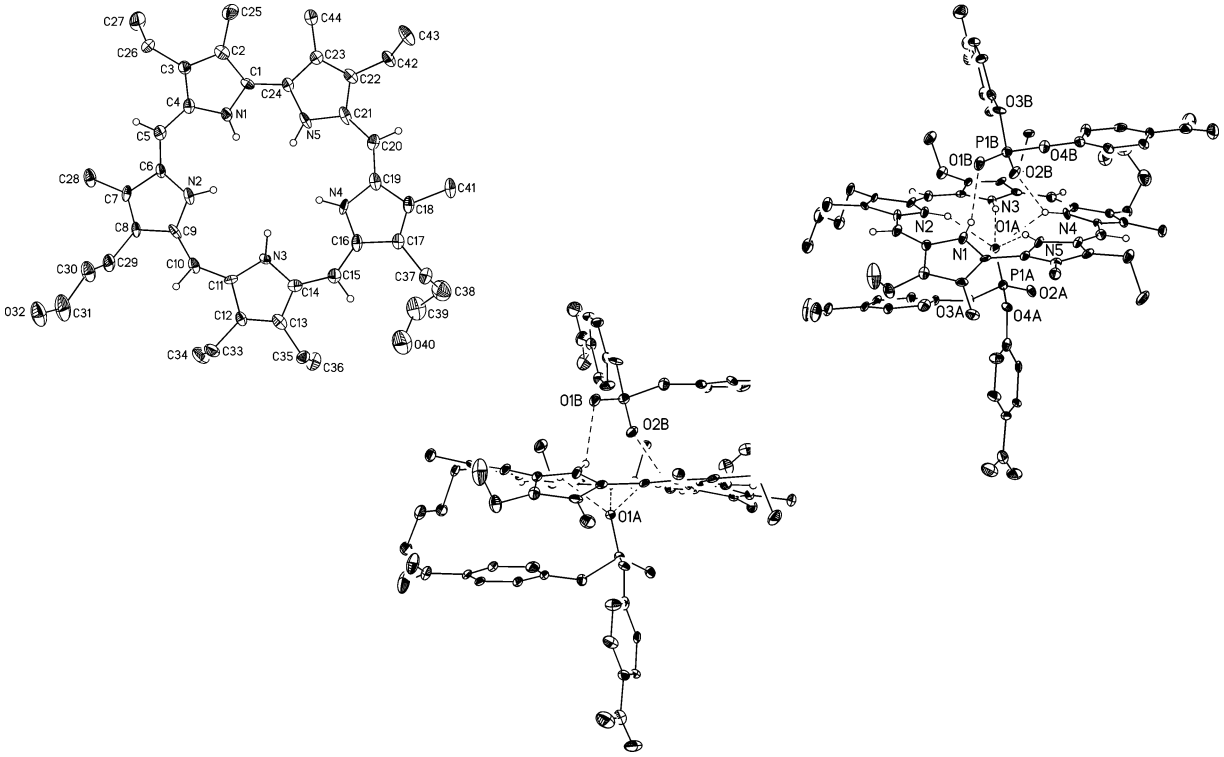

Figure 2. Two views of the H-bound complex, **7**-$(BNPP)_2$, showing partial atom labeling scheme. Also shown is the sapphyrin macrocycle with the BNNP anions removed for clarity. Thermal ellipsoids are scaled to the 30% probability level. The mode of coordination is different for the two anions. One of the bis(*p*-nitrophenyl)phosphate (BNPP) anions is coordinated by a single oxygen to four imine NH groups to one side of the macrocycle. The second BNPP anion is coordinated by two oxygen atoms. For both anions, one of the nitrophenyl groups lies approximately parallel to macrocycle mean plane. The relevant hydrogen bonding interactions are as follows: (N2−H2···O1a) N···O, 3.02(1) Å; N−H···O, 147.3(8)°. (N3−H3···O1a) N···O, 2.73(1) Å; N−H···O, 150.4(8)°. (N4−H4···O1a) N···O, 3.09(1) Å; N−H···O, 138.5(8)°. (N5−H5···O1a) N···O, 2.97(1) Å; N−H···O, 148.9(8)°. (N1−H1···O1b) N···O, 2.76(1) Å; (N−H···O) 119.1(8)°. (N4−H4···O2b) N···O, 2.76(1) Å; N−H···O, 134.4(8)°. The phosphorus to oxygen distances are as follows: O1A···P1A, 1.506(6) Å; O2A···P1A, 1.480(6) Å; O3A···P1A, 1.626(7) Å; O4A···P1A, 1.568(8) Å; O1B···P1B, 1.431(7) Å; O2B···P1B, 1.483(7) Å; O3B···P1B, 1.569(7) Å; O4B···P1B, 1.630(8) Å.

It is also important to appreciate that the presence of a sapphyrin phosphate binding element alone (i.e., without the cooperation of a suitable nucleophilic group) does not, in and of itself, suffice to obtain an efficient BNPP hydrolysis catalyst. Support for this conclusion comes from a consideration of sapphyrins **5** and **6**. The amino groups in **5** are protonated at neutral pH and are thus not expected to be able to function as nucleophiles. Compound **6**, on the other hand, is a bis-sapphyrin conjugate bearing a polyhydroxyl substituent on only one side of the compound. The size of this group and the obvious bulk of the second sapphyrin subunit are not expected (on the basis of Corey−Pauling−Koltun (CPK) models) to allow the side-chain hydroxyls to orient themselves in such a way as to interact favorably with the phosphodiester (i.e., in a manner conducive to bond cleavage).

We found that sapphyrin **3**, containing somewhat more tightly bound imidazole substituents, gave lower (but still appreciable) hydrolysis rates than **1** and **2**. This, we believe, further underscores the importance of establishing appropriate geometric orientations. Consistent with this supposition, sapphyrin **4**, containing four hydroxyl substituents, was also found to be less effective than **1** or **2** in terms of promoting BNPP hydrolysis. Again, this finding is rationalized in terms of steric effects. The crystal structure of **7** (Figure 2), a sapphyrin bearing two *n*-hydroxylpropyl substituents, revealed no hydrogen bonding between the hydroxyl groups and the phosphate group of the phosphodiester. In this particular case, the length of the tethering

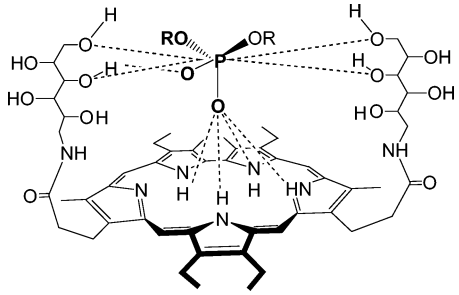

Figure 3. Proposed structure of the **1**−BNPP complex (R = *p*-nitrophenyl).

"arms" does not allow these hydroxyl groups to come within reasonable reach of the targeted phosphate ester. The same kind of argument can be invoked in the case of **4**. There is not sufficient sidearm length and flexibility in **4** to allow for effective OH-to-P orbital overlap. In the case of **1** and **2**, on the other hand, the longer arms should allow precisely these kinds of hydrolysis-promoting interactions, as suggested schematically in Figure 3.

Conclusions

In summary, we have shown that appropriately functionalized sapphyrins can act as efficient catalysts for activated phosphodiester bond hydrolysis. From the mechanistic point of view, our results are consistent with phosphate binding to a sapphyrin macrocycle serving to enhance the efficacy of appropriately

bound nucleophilic (e.g., OH) groups. While the relative importance of these two limiting factors is still being assessed in semiquantitative terms, it is nonetheless clear that phosphate binding plays an important role, as evidenced by the faster hydrolysis rates seen for the sapphyrin-based systems as compared to those for the porphyrin controls. These findings lead us to suggest that the present ditopic approach could prove useful in the construction of catalysts capable of enhancing the hydrolysis of other less-active phosphodiester substrates including oligonucleotides. In fact, in preliminary work, it was found that incubation of a dA−dT mixed 10-mer with sapphyrins **1**−**3** at pH 7.5 for 2−48 h yielded fragments that were, at least in part, phosphorylated (cf. Supporting Information), as would be expected for a cleavage mechanism where hydrolysis plays an important role. Current work is thus designed to explore further this intriguing possibility.

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Supporting Information Available: Description of X-ray data analysis of the 1:2 complex formed between diprotonated dihydroxylated sapphyrin **7** and BNPP. Mass spectrometric analysis of fragments produced upon incubation of dODNs. This material is available free of charge via the Internet at http://pubs.acs.org.

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