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Phosphate diesters and DNA hydrolysis by dinuclear Zn(II) complexes featuring a disulfide bridge and H-bond donors

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ABSTRACT

The dinuclear ligand **1** based on the bis-(2-amino-pyridinyl-6-methyl)amine (BAPA) metal binding unit and featuring a two-atom disulfide bridge was synthesized and studied as hydrolytic catalysts for phosphate diesters. The Zn(II) complexes of BAPA are known to elicit the cooperation between the metal ion and the hydrogen-bond donating amino groups to greatly increase the rate of cleavage of phosphate diesters. The reactivity of the dinuclear complex **1**·$Zn(II)_2$ toward bis-*p*-nitrophenyl phosphate and plasmid DNA was investigated and compared with that of reference complexes devoid of the disulfide bridge or of the hydrogen-bond donating amino groups. The dimetallic Zn(II) complex produces remarkable accelerations of the rate of cleavage of both the substrates accompanied by significant differences. In the case of BNP, the presence of the disulfide bridge does not lead to the improvement of the cooperative action of the two metal ions expected as the result of better preorganization. On the other hand, in the case of DNA the complex **1**·$Zn(II)_2$ is much more reactive that the corresponding reference devoid of the disulfide bridge. Hence, different requisites must be fulfilled by a good catalyst for the cleavage of the two substrates. Moreover, binding studies with DNA indicated that the presence of two metal ions in the complex or of the pyridine amino groups, but not of the disulfide bridge, results into an enhanced affinity of the complexes toward this substrate.

1. Introduction

DNA phosphodiester bonds are hardly hydrolyzed under neutral conditions and still represent a challenge for chemists.[1] However, enzymes can reduce the half-life time of this reaction from 30 million years to some milliseconds, bringing about a 1×10^{17}-fold rate acceleration.[2] Such astonishing reactivity is the result of a perfect organization of metal ions and organic groups in the active sites of the enzymes.[3] Research on artificial hydrolytic agents for DNA cleavage has attracted a considerable effort over the last two decades both for the intellectual challenge represented to design a catalyst capable to rival enzymes' reactivity but also for the important potential applications of such systems, which span from detoxification of pesticides and chemical weapons, to the realization of artificial restriction enzymes for molecular biology and of anti-DNA drugs.[1]

Synthetic catalysts with good activity have been obtained by using complexes of transition metal ions with high Lewis acidity such as Ce(IV), Co(III), Cu(II), and Fe(III),[1a,e] up to Komiyama's ARCUT system, based on the Ce(IV)/EDTA complex and pseudo-complementary PNA strands, which recently allowed the first reports of DNA manipulation with man-made restriction agents.[4] In these systems, the metal ions can play several roles, mainly acting as a Lewis acid: they provide the nucleophile for the reaction by facilitating the deprotonation of a coordinated water molecule, activate the phosphate group toward nucleophilic attack, stabilize the developing negative charge on the transition state, and, in principle, may help the departure of the leaving group.

Still, the reactivity of enzymes is unmatched. First examples of hydrolytic agents were monometallic complexes of transition metal ions with high Lewis acidity.[1a] In these systems, the role of the ligand is more or less to keep the hydroxide form of the complex in solution. Several studies have been devoted to elucidate the effect of the ligand structure in modulating and possibly enhancing the reactivity of the metal center[5] but it is quite clear that the desired acceleration cannot be reached with simple mononuclear complexes.[6] The following step to increase the reactivity of artificial hydrolytic agents has been the use of bimetallic complexes, as occurs in several phosphatases and nucleases. In this case, the ligand must keep the two metal centers at the right distance to obtain the maximum cooperation.[7] Model studies performed with kinetically inert Co(III) complexes have demonstrated that accelerations up to 10^{12}-fold are achievable with this strategy.[7] However, when complexes of labile metal ions are used, complications arise from the formation of μ-hydroxo bridges between the two metal centers, which are poorly reactive and saturate the free binding sites on the

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metal disfavoring the interaction with the substrate.[8–10] This is particularly true in the case of Zn(II), which is one of the metal ions preferred by hydrolytic enzymes. Some Zn(II) features, such as low toxicity, bioavailability, and absence of relevant redox chemistry, make it a very attractive candidate for the development of artificial agents.[1c] However, bimetallic Zn(II) complexes have so far shown poor performances both with model substrates and DNA.

Another approach, again inspired by Nature, is the use of organic groups appropriately located in the structure of the ligand, as co-factors to increase the reactivity of the metal complex.[11] Such groups can act as general bases, general acids, nucleophiles, or hydrogen-bond donors similar to the aminoacid residues present in the active site of nuclease enzymes. Several examples demonstrate that high activity can be achieved by such strategy in the cleavage of model substrates and RNA oligonucleotides, but reactivity on DNA is still not reported.

Recently, we[12] and the groups of Williams and Mareque-Rivas[13] reported that Zn(II) complexes based on the bis-(2-amino-pyridinyl-6-methyl)amine unit (BAPA, Chart 1), are very active in promoting the cleavage of activated phosphate diesters, such as bis-*p*-nitrophenyl phosphate (BNP, Chart 1) and hydroxypropyl-*p*-nitrophenyl phosphate (HPNP, Chart 1). In the ultimate example proposed by Williams and Mareque-Rivas, the bimetallic complex **A** (Chart 1) produces million-fold accelerations of the cleavage of both HPNP and RNA dinucleotides at pH 7.[13b] The source of this high reactivity is the ability of the complex to activate the substrate toward the nucleophilic attack using both double metal activation and four hydrogen bonds. Transposition of such high reactivity to DNA would be highly desirable. With this in mind, we explored, and here report, a new way to assemble binuclear Zn(II) complexes containing the BAPA units and investigated their reactivity toward model substrate BNP and plasmid DNA.

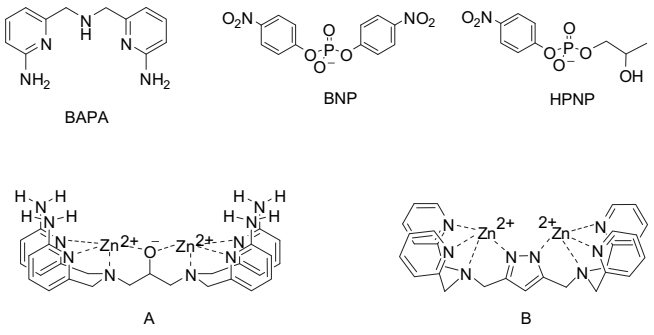


Chart 1. Activated phosphate esters and literature catalysts.

2. Results

2.1. Ligand design and synthesis

Spatial organization of the bimetallic structure has been shown to be crucial in determining the hydrolytic activity and the best results have been reported with rigid structures that employ bridging groups in the ligand structure to keep the metal ions in a precise arrangement.[1] Among these, one-atom bridging units such as phenolate and alkoxide (i.e., complex **A**, Chart 1) are very popular and found in many of the best performing RNA cleaving systems.[13,14] However, their efficiency in promoting the hydrolysis of DNA and its models is apparently lower, at least when Zn(II) is used as metal center. The studies of Meyer on di-zinc(II) complexes based on the pyrazolate linking unit (Chart 1) indicate that the greater intermetallic distance, allowed by this two-atom bridging group, disfavors the formation μ-hydroxo bridges leading to an increased reactivity of the hydrolytic complex toward BNP.[10]

On these bases, we found the disulfide group quite appealing as a new bridging unit for the preparation of bimetallic complexes.[15] The disulfide group, in fact, forms spontaneously when thiol derivatives are exposed to air in basic conditions. This reactivity, usually considered as a drawback in handling of thiols, reduces the problem of preparing binucleating ligands to the simpler synthesis of thiol containing mononuclear ligands (Chart 2).

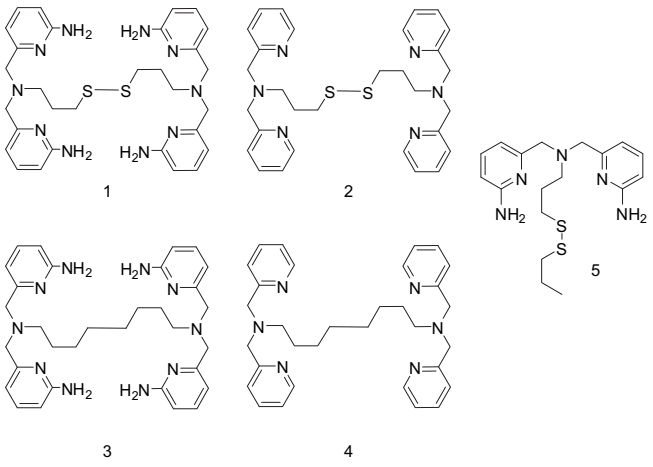
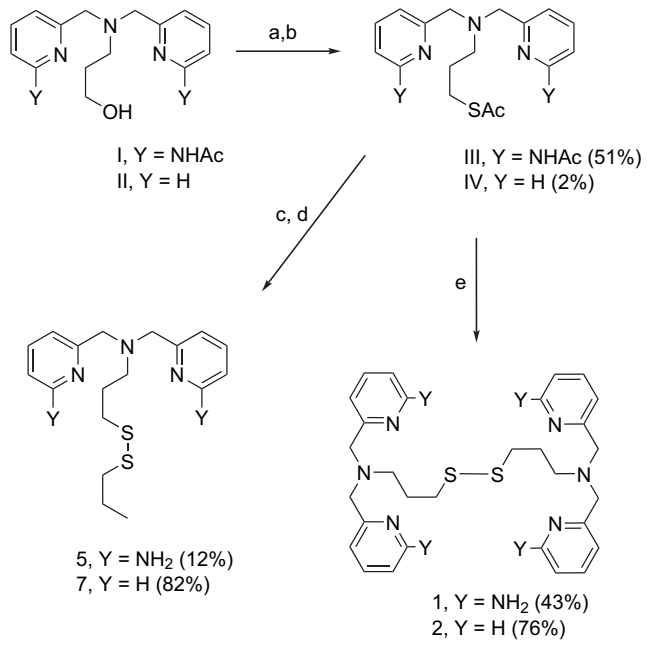
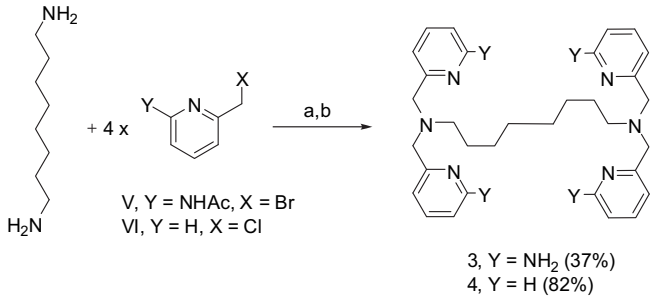


Chart 2. Ligands studied in this work.

With this in mind, compound **1** (Chart 1) was designed as binuclear ligand based on the BAPA structure and containing the disulfide group as two-atom bridging unit. Synthesis of **1** was performed (Scheme 1) from previously reported derivative **I**[12d] through tosylation of the hydroxyl group and substitution of the tosylate with thioacetate. Removal of the protecting acetyl form **II** in basic conditions provides **1**. Ligand **2**, devoid of the pyridine amino groups, was prepared in a similar way from previously described **III**.[12d] Ligands **3** and **4**, in which the disulfide bridging group is not present, were prepared (Scheme 2) by reaction of 1,8-diaminooctane, respectively, with compound **V** and picolyl chloride. Finally, the model mononuclear ligand **5** was prepared (Scheme 1) by deprotection of **II** in the presence of a large excess of propyl-1-thiol.



Scheme 1. Synthesis of ligands **1**, **2**, **5** and **7**: (a) tosyl chloride, triethylamine, CH_2Cl_2, rt, 24 h; (b) potassium thioacetate, acetone, reflux, 2 days; (c) HCl, EtOH/H_2O 1:1, rt, 16 h; (d) propanethiol, NaOH, EtOH/H_2O 1:1, reflux, 16 h; (e) NaOH, EtOH/H_2O 1:1, reflux, 16 h.



Scheme 2. Synthesis of ligands **3** and **4**: (a) For **3**, K_2CO_3, CH_3CN, reflux, 16 h; for **4**: NaOH, CTACl, water, rt, 20 h; (b) HCl, EtOH/H_2O 1:1, rt, 16 h (only in the case of **3**).

All the above ligands are sparingly soluble in water in their neutral form and this prevented the investigation of the Zn(II) binding through potentiometric titrations.

However, in the case of ligand **5**, we could study the formation of the Zn(II) complex through ^{1}H NMR investigation (Fig. 1). As usual for BAPA-based ligands,[12c] formation of the Zn(II) complex causes significant changes on the resonances of the ligand protons: pyridine H2 is downfield shifted while the resonances of H1 and H3 melt into a single signal. The methylene protons H4 of the pyridinylmethyl moieties experience the most important variation: their signal is downfield shifted and splits into an AB system, reasonably, as a consequence of the loss of conformational flexibility of this portion of the complex. Much more significant are the changes of the signal relative to the disulfide arm, which shows a significant modification of the shape and, to a minor extent, of the chemical shift of the signals relative to the methylene groups. Hence, also the disulphide group binds to the Zn(II) ion and **5** acts, at least at neutral pH, as a tetradentate ligand.

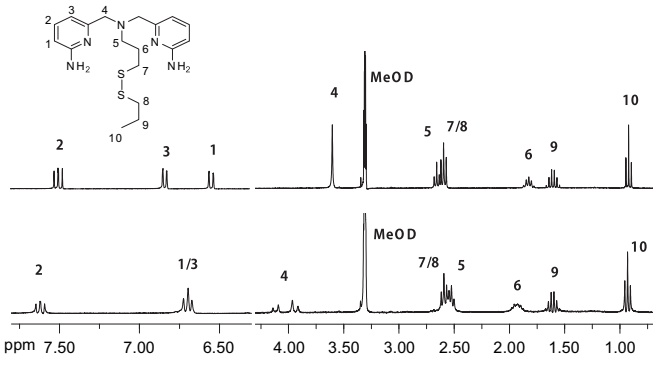


Figure 1. ^{1}H NMR spectra of **5** (upper trace) and **5**·Zn(II) (lower trace) at 1.0 mM in MeOD at 28 °C.

2.2. Reactivity toward BNP

Incubation of the Zn(II) complexes of **1**, **3**, **4**, **5** with BNP (Chart 1) in water at 40 °C results into substrate hydrolysis, while **2**·Zn(II)$_2$ was found to be completely unreactive. The kinetic studies were performed by monitoring the increase of the *p*-nitrophenoxide absorbance at 400 nm, using the initial rates method. Apparent second-order rate constants were obtained at each pH value by the linear fit of the pseudo-first order rate constants versus complex concentration data. The pH dependences of the apparent second-order rate constants are reported in Figure 2.

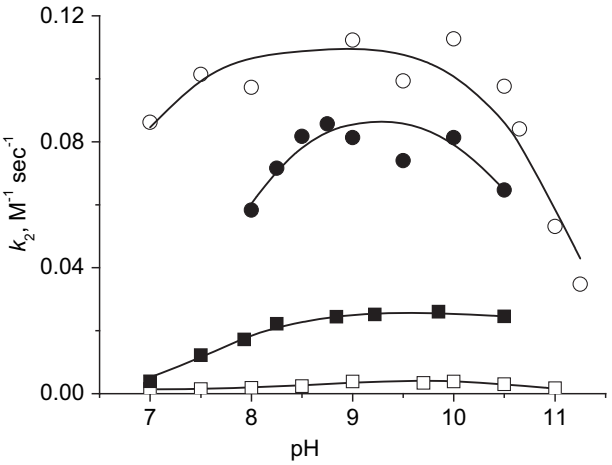


Figure 2. pH dependence of the apparent second-order rate constants (k_2') for the reaction between BNP and Zn(II) complexes of ligands **3** (○), **1** (●), **5** (■), **4** (□) at 40 °C ([ligand]=0.5–2.0×10^{-4} M, [BNP]=2.0×10^{-4} M, [buffer]=5.0×10^{-2} M). The lines represent the best fit of the experimental data as described in the text.

The reactivity of all the complexes increases with the pH up to a maximum around pH 8–9 followed by a decrease, above pH 10. This behavior is diagnostic of the deprotonation of two acidic functions of the complexes, the first deprotonation event leading to a reactivity increase and the second to the opposite effect. Fitting of the pH profiles with a kinetic model involving two deprotonation equilibria (see Experimental Part) allowed the determination of the pK_a values of the reacting species and of the second-order rate constants for the reactive monodeprotonated metal complex. Comparison of the second-order rate constants of Table 1 reveals the reactivity order of the complexes: **3**·Zn(II)$_2$≈**1**·Zn(II)$_2$>**5**·Zn(II)>**4**·Zn(II)$_2$.

Table 1
Metal coordinated water molecule acidity constants (pK_a) and second-order rate constants (k_2) for the reaction of BNP with the Zn(II) complexes of ligands **1**–**5** in water at 40 °C

Complex	pK^1_a	pK^2_a	k_2, M^{-1} s^{-1}
1·Zn(II)$_2$	7.7	10.9	0.09
3·Zn(II)$_2$	6.5	11.0	0.11
4·Zn(II)$_2$	8.7	10.7	0.005
5·Zn(II)	7.6	>11	0.026

However, if it is considered that the amount of Zn^{2+} is double in the case of bimetallic complex, the cooperativity between the two metal centers in **1**·Zn(II)$_2$ appears to be quite limited. This picture is confirmed by a kinetic experiment performed using a fixed concentration of **1** ligand and increasing amounts of metal ion (Fig. 3). The rate profile describes a smooth sigmoidal curve reaching a plateau for a ligand to metal ion ratio around 2. The reaction rate observed after the introduction of the second metal ion is approximately 2.9 times larger than that observed in the presence of 1 equiv of Zn(II), indicating again that the effect of the two complexed subunits is more than additive but the synergic action of the two metal ions is not very efficient. A different profile (not shown) was however observed with ligand **3**: in this case the kinetic data describe an almost linear curve up to 2 equiv of added metal ion then going to plateau reactivity.

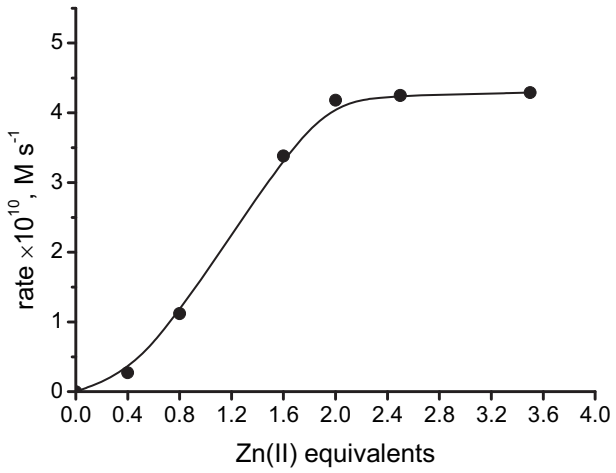


Figure 3. Kinetic profile for the BNP cleavage as a function of the Zn(II) to **1** ratio, in HEPES buffer 0.05 M, pH 9.0. [**1**]=2.0×10^{-5} M, [BNP]=4.0×10^{-4} M, 40 °C.

Finally, saturation kinetics were obtained measuring the reactivity of the complexes **1**·Zn(II)$_2$, **3**·Zn(II)$_2$, and **5**·Zn(II) in the presence of increasing amounts of BNP at pH 9.0. This behavior is in line with the accepted mechanism of the metal catalyzed hydrolysis of phosphate diesters, which involves a pre-complexation step of the substrate to the metal complex. Analysis of data with a Michaelis–Menten like equation allowed to estimate the binding constants of the substrate to the catalyst (K_b) of 2.7×10^3, 4.3×10^3, and 8.4×10^2 M^{-1}, respectively, **1**·Zn(II)$_2$, **3**·Zn(II)$_2$, and **5**·Zn(II).

2.3. Cleavage of plasmid DNA

Incubation of plasmid DNA pBR 322 with the Zn(II) complex of ligands **1**–**5** at pH 7 for 24 h at 37 °C results in different degrees of cleavage of DNA form I to form II (relaxed circular) depending on the structure of the ligand (Fig. 4). Surprisingly enough, the order of reactivity is quite different than that observed in the case of BNP cleavage. With plasmid DNA, in fact, the reactivity order is **1**·Zn(II)$_2$>**5**·Zn(II)≈**3**·Zn(II)$_2$, while **4**·Zn(II)$_2$ is substantially unreactive. A peculiar behavior is observed for **2**·Zn(II)$_2$, which produces a remarkable (about 20%) cleavage at low concentrations (10 μM) but becomes less reactive at higher concentrations.

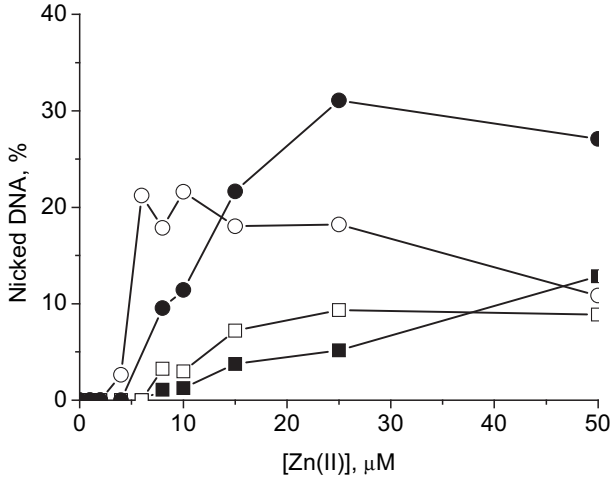


Figure 4. Extent of pBR 322 plasmid DNA form II (nicked) produced after cleavage with different concentrations Zn(II) complexes of ligands **1** (●), **2** (○), **3** (□), and **5** (■). [DNA$_{bp}$]=12 μM. [HEPES]=20 mM, pH 7, 37 °C, 24 h, complex concentration is expressed as total Zn(II) concentration (the lines connecting the data point are for a clearer inspection).

Control experiments performed in the presence of radical scavengers DMSO and isopropanol revealed that these additives have no effect on the extent of plasmid DNA cleavage by the Zn(II) complexes, ruling out the involvement of diffusible radical species. Moreover, the Ellmann test confirmed that the complexes containing the disulfide unit are not cleaved, in the conditions used for the DNA cleavage experiments, to form thiol containing species, while the presence of diethyl disulfide does not affect the reactivity of the complexes not containing the disulfide group.

Binding of the Zn(II) complexes to DNA was investigated by following the modification of the circular dichroism (CD) spectra of linear calf thymus DNA after the additions of increasing concentrations of metal complex. In all the cases Figure 5, a decrease of the dichroic signal, particularly of the 275 nm band, is observed, indicating that the interaction of the complexes with the DNA leads to a destabilization of the double-helix structure. Such effects are expected in the case of a predominant electrostatic interaction between the negatively charged DNA backbone and the positively charged complexes, which results into a partial charge neutralization of DNA. Unfortunately, precipitation of DNA in the presence of high metal complex concentrations and the overlap of the DNA dichroic bands with the ligand-induced dichroic bands hampered the quantitative determination of the affinity constants of the different complexes for DNA. However, if the amplitude of signal variation is taken as an approximate indication of the binding strengths, the DNA affinity order is: **1**·Zn(II)$_2$≈**3**·Zn(II)$_2$>**2**·Zn(II) $_2$>**4**·Zn(II)$_2$ (Fig. 5).

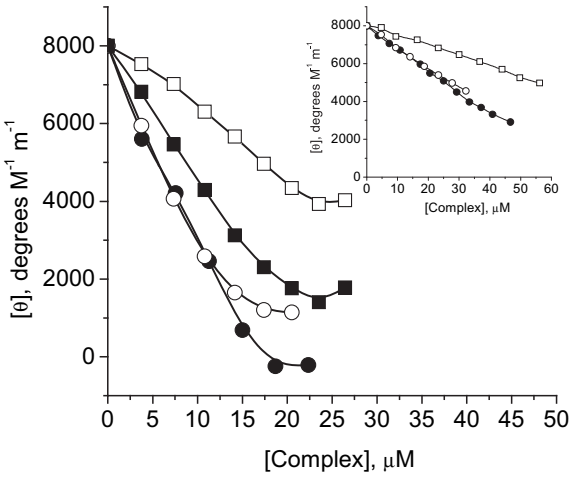


Figure 5. DNA molar ellipticity ([θ]) at 275 nm as a function of the Zn(II) complexes of ligands **1** (●), **2** (■), **3** (○), and **4** (□). Inset: DNA molar ellipticity ([θ]) at 275 nm as a function of the Zn(II) complexes of ligands **5** (●), **6** (○) and **7** (□). [ctDNA$_{bp}$]=180 μM. [HEPES]=20 mM, pH 7, 25 °C, 24 h, complex concentration is expressed as total Zn(II) concentration.

In order to obtain better insight into the reasons for the different affinities of the ligands for DNA, monometallic Zn(II) complexes **6**·Zn(II) and **7**·Zn(II) (Chart 3) were investigated and compared with **5**·Zn(II). When the comparison is restricted to mononuclear complexes, affinity order for DNA emerging from circular dichroism experiments is **5**·Zn(II)≈ **6**·Zn(II)>**7**·Zn(II).

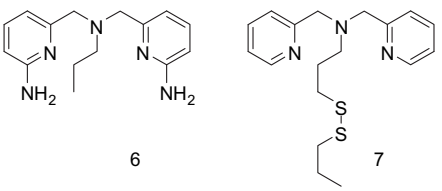


Chart 3. Reference ligands used in CD experiments.

3. Conclusions

When the literature on artificial hydrolytic catalysts for DNA and model phosphate diesters is examined, one is surprised by the lack of efficient bimetallic Zn(II)-based systems. This appears in striking contrast with natural systems, which often use two Zn(II) ions in the active sites of several nuclease enzymes. The studies of Bencini[9] and Meyer[10] have quite clearly pointed out that the source of such low efficiency lies in the formation of μ-hydroxo bridges that are poor nucleophiles and, on the other hand, block the available binding sites on the metal ions preventing the binding of the substrate.

The reactivity here reported for **1**·Zn(II)$_2$ and **3**·Zn(II)$_2$ toward the model substrate BNP confirms such a scenario. The two complexes are indeed highly reactive toward BNP, producing in the case of **3**·Zn(II)$_2$ a 40,000-fold rate acceleration over the background reaction at pH 7 and 40 °C,[16] and at a complex concentration as low as 50 μM. However, both the bimetallic complexes are only 3–4 fold more reactive that the monometallic reference **5**·Zn(II), indicating that cooperative action of the two metal centers is limited. This was expected for complex **3**·Zn(II)$_2$, where the non-bridging spacer produces very little pre-organization of the structure.[17] It is more surprising in the case **1**·Zn(II)$_2$, which is even slightly less reactive than **3**·Zn(II)$_2$.

Some insight into the causes of such low reactivity come from the analysis of the behavior of the other two dinuclear complexes studied, **2**·Zn(II)$_2$ and **4**·Zn(II)$_2$. Here the difference is dramatic: the reactivity of **2**·Zn(II)$_2$, which contains the disulfide bridge, is not only much lower than that of the bridging group devoid **4**·Zn(II)$_2$, but virtually absent. This indicates that the presence of the disulfide bridge is potentially detrimental for the reactivity of the system, probably because the arrangement of the two Zn(II) ions realized in the complex still allows for the formation of unreactive μ-hydroxo bridges. On these bases, the similar reactivity of complexes **1**·Zn(II)$_2$ and **3**·Zn(II)$_2$, accompanied by a similar affinity for the substrate as indicated by the BNP binding constants measured, could be attributed to the lack of or to a weak coordination of both the Zn(II) ions to the sulfur atoms of the bridge that would make **1** and **3** structurally equivalent.

Unfortunately, the solubility of the bimetallic complexes was too low to allow the investigation of their structures. In the case of monometallic **5**·Zn(II), coordination of sulfur to Zn(II) is supported by the NMR spectral changes, and the same occurs with similar ligands featuring a thioether arm studied by Berreau and coworkers.[18] Also the absence of reactivity observed with **2**·Zn(II)$_2$ supports the coordination of the Zn(II) ions to the sulfur atoms of the bridge. However, in the case of **1**·Zn(II)$_2$, steric hindrance between the pyridine amino groups, as emerges by inspection of molecular models, could disfavor the interaction between the metal ions and the disulfide bridge.

Differently from the behavior observed with the model substrate BNP, reactivity toward plasmid DNA is quite interesting. In fact, not only is the bimetallic complex **1**·Zn(II) substantially more reactive, at low concentrations, than the mononuclear counterpart but this time it is also much more reactive than complex **3**·Zn(II)$_2$ (and the same occurs if **2**·Zn(II)$_2$ and **4**·Zn(II)$_2$ are compared). With DNA, hence, the presence of the disulfide group produces an important effect on the reactivity. Binding experiments give some further hint: first, DNA affinity of bimetallic complexes is, as expected, generally higher than that of monometallic one; second, the presence, of the pyridine amines in the ligand substantially increases DNA affinity while insertion of the disulfide group has no sensible effects. Hence, the greater reactivity of **1**·Zn(II)$_2$ compared with **3**·Zn(II)$_2$ is not due to higher affinity but must be attributed to some effect of the disulphide bridge either on the geometry of the complex when bound to DNA or on the metal properties.

In conclusion, the use of disulfide bridges appears as an interesting strategy to the easy preparation and modification of bimetallic complexes. In perspective, mixed binuclear ligands featuring two different binding units or catalyst selection by the dynamic combinatorial approach can be envisaged using this structural element. Moreover, the complexes herein reported are quite reactive toward BNP and DNA, where they rank among the few Zn-based bimetallic complexes capable to promote DNA cleavage.[1] This confirms that the use of BAPA-based ligands is a promising strategy for the realization of efficient hydrolytic catalysts for phosphate esters and also for DNA. However, the results obtained indicate that the simple use of a two-atom bridge is not sufficient to overcome the difficulty to find the right spatial arrangements of the metal ions and achieve optimum cooperative action: a further design effort is needed to achieve the optimum geometry.

4. Experimental section

4.1. General

Solvents were purified by standard methods. All commercially available reagents and substrates were used as received. TLC analyses were performed using Merck 60 F_{254} precoated silica gel glass plates. Column chromatography was carried out on Macherey-Nagel silica gel 60 (70–230 mesh). NMR spectra were recorded using a Bruker AC250F spectrometer operating at 250 MHz for ^{1}H and 62.9 MHz for ^{13}C and a Bruker AV300 operating at 300 MHz for ^{1}H. Chemical shifts are reported relative to internal Me_4Si. Multiplicity is given as follows: s=singlet, d=doublet, t=triplet, q=quartet, qn=quintet, m=multiplet, br=broad peak. ESI MS mass spectra were obtained with an Agilent Technologies LC/MSD Trap SL mass spectrometer. UV–visible spectra and kinetic traces were recorded on Perkin–Elmer Lambda 16 and Lambda 45 spectrophotometers equipped with thermostated multiple cell holders. Zn(NO_3)$_2$ and Cu(NO_3)$_2$ were analytical grade products (Aldrich). Metal ion stock solutions were titrated against EDTA following standard procedures. The buffer components were used as supplied by the manufacturers: acetic acid (Aldrich), 2-morpholinoethanesulfonic acid (MES, Fluka), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPES, Sigma), 2-[cyclohexylamino]ethanesulfonic acid (CHES, Sigma), 3-[*N*-cyclohexylamino]-1-propanesulfonic acid (CAPS, Sigma). The substrate bis-*p*-nitrophenyl phosphate sodium salt (BNP) is an Aldrich product, used as received. Ligands **1**–**5** and **7** were prepared as described in the following paragraphs, synthesis of ligand **6** was previously reported.[12d] Concentrations of ligands **1**–**7** (prepared) stock solutions in methanol were determined by spectroscopic titrations with Cu(NO_3)$_2$ monitoring the increase of the Cu(II) absorption band (*d*–*d* transition) at 700 nm.

4.2. DNA cleavage experiments

DNA cleavage experiments were performed using pBR 322 (Fermentas) in 20 mM HEPES, pH 7.0. Reactions were performed incubating DNA (12 μM base pairs) at 37 °C in the presence/absence of increasing amount of Zn(II) complexes for 24 h and stopped by addition of EDTA and SDS to a final concentration of 0.06 M and 1%, respectively. Reaction products were resolved on a 1% agarose gel in TAE buffer (40 mM TRIS base, 20 mM, acetic acid, 1 mM EDTA) and visualized by ethidium bromide staining. The relative amounts of different plasmid structures were quantified using a Geliance 600 Imaging System (Perkin–Elmer) interfaced to a PC workstation.

4.3. CD experiments

CD measurements were performed using Jasco J-810 spectropolarimeter equipped with a thermostated cell holder. CD spectra were recorded in HEPES 20 mM pH 7.0 using a 1 cm pathlength quartz cells. Titrations were performed by addition of increasing complex concentrations to a calf thymus DNA solution 180 μM (base pairs) in HEPES 20 mM pH 7.0 at 25 °C. For each measurement two scans were run in the 220–350 nm range and recorded with 0.1 nm step resolution. Each measurement was repeated at least in triplicate in independent experiments. At the end of each measurement, observed ellipticities were converted to mean residue ellipticity $[\theta]=\text{deg}\times\text{cm}^2\times\text{dmol}^{-1}$.

4.4. Kinetic measurements

The kinetic traces were recorded on Perkin–Elmer Lambda 16 and Lambda 45 spectrophotometers equipped with a thermostated multiple cell holders. Reaction temperature was maintained at 25.0±0.1 °C or 40.0±0.1 °C. The reactions were started by adding 20 μL of a 2.0×10^{-3} M solution of substrate (BNP) to a 2 mL solution containing the appropriate buffer (0.05 M), $Zn(NO_3)_2$, and ligands **1**–**5** in the desired amount, and monitored by following the absorption of *p*-nitrophenoxide at 400 nm or *p*-nitrophenol at 340 nm. Reactions were followed up to about 5% of substrate hydrolysis. The pseudo-first order rate constants (k_ψ) were obtained from the slope of the absorbance versus time data (the fit error was always less than 1%) divided by the molar absorbivity of the *p*-nitrophenoxide and the concentration of substrate. Each experiment was performed in triplicate and the errors on the rate constants are always below 10%. Apparent pH dependent second-order rate constants (k_2') were obtained by linear regression fitting of the k_ψ versus metal complex concentration data. Kinetic K_a^n values and second-order rate constants (k_2) for the monodeprotonated complexes were obtained by non-linear regression analysis of the apparent second-order rate constants versus pH date according to the equation: $k_2'=k_2\cdot(K_a^1/[H^+]+1+[H^+]/K_a^2)$. BNP rate saturation experiments with Zn(II) complexes were fitted according to the Michealis–Menten equation: $rate=k_{max}\cdot[BNP]/(1/K_{BNP}+[BNP])$, where k_{max} is the limiting reaction rate in the experimental conditions and K_{BNP} is the apparent binding constant of the substrate to the catalyst.

*4.4.1. N-Bis(6-acetylamido-2-pyridylmethyl)-1-acetylsulfanyl-3-propylamine (**III**).* *N*-Bis(6-acetylamido-2-pyridylmethyl)-3-hydroxy-propylamine (**I**), was prepared as previously reported.[12d] To a stirred solution of compound **I** (0.675 g, 1.80 mmol) in dry pyridine (8 mL) were added *p*-toluenesulfonyl chloride (0.673 g, 3.50 mmol) and triethylamine (0.421 g, 4.16 mmol). The reaction mixture was stirred under N_2 at room temperature for 24 h. The pyridine was evaporated, the crude product was dissolved in chloroform, and washed with water (4×50 mL). The organic phase was dried over Na_2SO_4 and evaporated. 0.403 g (57%) of *N*-bis(6-acetylamido-2-pyridylmethyl)-3-chloro-propylamine were obtained as a white solid. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.05 (d, 2H, 8 Hz), 7.85 (br s, 2H), 7.68 (t, 2H, 8 Hz), 7.17 (d, 2H, 8 Hz), 3.70 (s, 4H), 3.58 (t, 2H, 7 Hz), 2.70 (t, 2H, 7 Hz), 2.21 (s, 6H), 1.99 (qn, 2H, 7 Hz). ESI MS (*m*/*z*): 390.8 [16%, M+H$^+$], 412.2 [100%, M+Na$^+$]. The above compound (0.403 g, 1.03 mmol) was dissolved in acetone (30 mL) and potassium thioacetate (0.240 g, 2.1 mmol) was added. The mixture was refluxed under nitrogen for 60 h, the solvent was evaporated and the solid residue dissolved in CH_2Cl_2 (20 mL). The organic solution was extracted with water (5×20 mL) and dried with Na_2SO_4. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH/NH_3 20:1:0.01). 0.386 g (90%) of **III** were obtained as a orange oil. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.08 (br s, 2H), 8.06 (d, 2H, 8 Hz), 7.67 (t, 2H, 8 Hz), 7.14 (d, 2H, 8 Hz), 3.68 (s, 4H), 2.89 (t, 2H, 7 Hz), 2.55 (t, 2H, 7 Hz), 2.32 (s, 3H), 2.22 (s, 6H), 1.86 (qn, 2H, 7 Hz).

*4.4.2. Bis(3-amino-N-bis(6-amino-2-pyridylmethyl)-propyl) disulfide (**1**).* Compound **III** (0.170 g, 0.40 mmol) was dissolved in a 1 M NaOH solution in H_2O/EtOH 1:1 (75 mL) and refluxed for 16 h under stirring. After this time, ethanol was evaporated and the resulting aqueous solution was extracted with chloroform. The organic phase was dried with Na_2SO_4 and the solvent evaporated. 0.103 g of **1** were obtained as a orange oil (43%).^{1}H NMR (CD_3OD, 250 MHz), δ: 7.39 (t, 4H, 8 Hz), 6.78 (d, 4H, 8 Hz), 6.41 (d, 4H, 8 Hz), 3.53 (s, 8H), 2.58 (t, 4H, 7 Hz), 2.54 (t, 4H, 7 Hz), 1.81 (qn, 4H, 7 Hz). ^{13}C NMR ($CDCl_3$, 62.9 MHz), δ: 160.3, 158.4, 139.5, 112.9, 108.2, 61.1, 53.8, 37.3, 27.7. APCI MS (*m*/*z*): 605.4 [M+H$^+$]. Elemental analysis, calcd for $C_{30}H_{40}N_{10}S_2$ (604.84): C 59.57, H 6.67, N 23.16, S 10.60; found: C 59.68, H 6.70, N 23.20, S 10.55%.

*4.4.3. Propyl-(3-amino-N-bis(6-amino-2-pyridylmethyl)-propyl) disulfide (**5**).* Compound **III** (0.386 g, 0.90 mmol) was dissolved in ethanol (20 mL) and concentrated HCl (1.8 mL) was added. The mixture was stirred at room temperature for 12 h and the solvent was evaporated to obtain the corresponding thiol as a brown solid (0.355 g, 95%). ^{1}H NMR (CD_3OD, 250 MHz), δ: 7.80 (t, 2H, 8 Hz), 6.87 (d, 2H, 8 Hz), 6.80 (d, 2H, 8 Hz), 3.83 (s, 4H), 2.61 (t, 2H, 7 Hz), 2.51 (t, 2H, 7 Hz), 1.74 (qn, 2H, 7 Hz). The above thiol (0.265 g, 0.88 mmol) was dissolved in a 0.1 M NaOH solution in H_2O/EtOH 1:1 (40 mL) and 1-propanethiol (1.00 g, 13.1 mmol) was added under stirring. The mixture was refluxed under nitrogen for 12 h, and then 50 mL of chloroform were added. The organic solution was separated, extracted with 10% $NaHCO_3$ (2×100 mL), and dried with Na_2SO_4. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH 20:1). 0.039 g (12%) of **5** were obtained as a colorless oil. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 7.38 (t, 2H, 8 Hz), 6.83 (d, 2H, 8 Hz), 6.34 (d, 2H, 8 Hz), 3.62 (s, 4H), 2.68 (t, 2H, 7 Hz), 2.60 (t, 4H, 7 Hz), 1.87 (qn, 2H, 7 Hz), 1.66 (m, 2H, 7 Hz), 0.95 (t, 3H, 7 Hz). ^{13}C NMR ($CDCl_3$, 62.9 MHz), δ: 158.1, 158.0, 112.9, 107.0, 60.4, 52.9, 41.2, 36.9, 26.9, 22.7, 13.3. ESI MS (*m*/*z*): 378.2 [M+H$^+$]. Elemental analysis, calcd for $C_{30}H_{40}N_{10}S_2$ (377.57): C 57.26, H 7.21, N 18.55, S 16.98; found: C 57.35, H 7.17, N 18.42, S 17.08%.

*4.4.4. N-Bis(2-pyridylmethyl)-1-acetylsulfanyl-3-propylamine (**IV**).* *N*-Bis(2-pyridylmethyl)-3-hydroxy-propylamine (**II**) was prepared as previously reported.[12d] Compound **II** (4.00 g, 15.7 mmol) was dissolved in CH_2Cl_2 (40 mL). Tosyl chloride (6.000 g, 31.4 mmol) and triethylamine (5.10 mL, 36.0 mmol) were added and the resulting mixture was refluxed for 3 days. During this time, the pH of the mixture was controlled with pH paper and adjusted to 9–10 with triethylamine. The reaction mixture was allowed to cool, diluted with CH_2Cl_2, and extracted with 5% $NaHCO_3$ (3×20 mL). The organic phase was dried with Na_2SO_4 and evaporated. The crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH 10:1). *N*-Bis(2-pyridylmethyl)-3-chloro-propylamine (0.440 g (7%)) was obtained as a yellow oil. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.41 (d, 2H, 8 Hz), 7.55 (t, 2H, 8 Hz), 7.40 (d, 2H, 8 Hz), 7.04 (t, 2H, 8 Hz), 3.72 (s, 4H), 3.46 (t, 2H, 7 Hz), 2.61 (t, 2H, 7 Hz), 1.87 (qn, 2H, 7 Hz). The above compound (0.472 g, 1.21 mmol) was dissolved in acetone (40 mL) and potassium thioacetate (0.420 g, 3.60 mmol) was added. The mixture was refluxed under nitrogen for 60 h, the solvent was evaporated and the solid residue dissolved in CH_2Cl_2 (20 mL). The organic solution was extracted with water (5×20 mL) and dried with Na_2SO_4. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH/NH_3 10:1:0.01). Compound **IV** (0.110 g (29%)) was obtained as a brown oil. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.43 (d, 2H, 8 Hz), 7.59 (t, 2H, 8 Hz), 7.43 (d, 2H,

8 Hz), 7.07 (t, 2H, 8 Hz), 3.72 (s, 4H), 2.78 (t, 2H, 7 Hz), 2.52 (t, 2H, 7 Hz), 2.19 (s, 3H), 1.72 (qn, 2H, 7 Hz). ESI MS (*m*/*z*): 315.0 [M+H$^+$].

*4.4.5. Propyl-(3-amino-N-bis(2-pyridylmethyl)-propyl) disulfide (**7**).* Compound **IV** (0.055 g, 0.17 mmol) was dissolved in ethanol (4 mL) and 6 M HCl (2 mL) was added. The mixture was stirred at room temperature for 3 h, and the solvent was evaporated to obtain the desired thiol as a brown solid (0.063 g, 97%). ^{1}H NMR (CD_3OD, 250 MHz), δ: 8.88 (d, 2H, 8 Hz), 8.61 (t, 2H, 8 Hz), 8.20 (d, 2H, 8 Hz), 8.05 (t, 2H, 8 Hz), 4.39 (s, 4H), 2.79 (t, 2H, 7 Hz), 2.46 (t, 2H, 7 Hz), 1.83 (qn, 2H, 7 Hz). ^{13}C NMR (CD_3OD, 62.9 MHz), δ: 153.2, 147.6, 142.1, 127.9, 126.8, 56.0, 53.4, 30.1, 21.67. The above compound (0.048 g, 0.126 mmol) was dissolved into a 0.3 M NaOH solution of H_2O/EtOH 1:1 (30 mL), 1-propanethiol (0.66 g, 8.7 mmol) was added and the mixture was refluxed for 16 h. After this time 50 mL of chloroform were added and the organic solution was extracted with 10% $NaHCO_3$ (2×100 mL) and dried with Na_2SO_4. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH 20:1). 0.036 g of **7** (82%) were obtained as a yellowish oil. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.52 (d, 2H, 8 Hz), 7.67 (t, 2H, 8 Hz), 7.51 (d, 2H, 8 Hz), 7.15 (t, 2H, 8 Hz), 3.82 (s, 4H), 2.64 (m, 6H), 1.92 (qn, 2H, 7 Hz), 1.65 (m, 2H), 0.96 (t, 3H, 7 Hz). ^{13}C NMR ($CDCl_3$, 62.9 MHz), δ: 159.8, 149.2, 136.6, 123.1, 122.2, 60.6, 53.0, 41.3, 36.9, 27.0, 22.7, 13.3. ESI MS (*m*/*z*): 348.2 [M+H$^+$]. Elemental analysis, calcd for $C_{18}H_{25}N_3S_2$ (357.54): C 62.21, H 7.25, N 12.09, S 18.45; found: C 62.35, H 7.17, N 12.38, S 18.23%.

*4.4.6. Bis(3-amino-N-bis(2-pyridylmethyl)-propyl) disulfide (**2**).* Compound **IV** (0.291 g, 0.92 mmol) was dissolved in a 1 M NaOH solution in H_2O/EtOH 1:1 (40 mL) and refluxed under stirring for 16 h. After this time ethanol was evaporated and the resulting aqueous solution was extracted with chloroform. The organic phase was dried with Na_2SO_4 and the solvent evaporated to obtain 0.199 g of product (**4**) as a brown solid (76%). ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.51 (d, 4H, 8 Hz), 7.66 (t, 4H, 8 Hz), 7.51 (d, 4H, 8 Hz), 7.15 (t, 4H, 8 Hz), 3.82 (s, 8H), 2.65 (t, 8H, 7 Hz), 1.89 (qn, 4H, 7 Hz). ^{13}C NMR ($CDCl_3$, 62.9 MHz), δ: 159.5, 148.8, 136.3, 122.8, 121.9, 60.3, 52.6, 36.4, 26.6. ESI MS (*m*/*z*): 567.9 [M+Na$^+$]. Elemental analysis, calcd for $C_{30}H_{36}N_6S_2$ (544.78): C 66.14, H 6.66, N 15.43, S 11.77; found: C 66.31, H 6.75, N 15.05, S 11.90%.

*4.4.7. N,N,N′,N′-Tetra(6-amino-2-pyridylmethyl)-1,8-dioctylamine (**3**).* Compound **V** (1.500 g, 6.55 mmol), prepared as previously reported,[12d] and K_2CO_3 (1.143 g, 8.27 mmol) were added to a solution of 1,8-diaminooctane (0.210 g, 1.46 mmol) in acetonitrile (20 mL). The mixture was stirred for 3 h under nitrogen at reflux, then insoluble salts were removed by filtration. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH/NH_3 10:1:0.01). 0.407 g (38%) of acetyl protected **3** were obtained as a yellow oil. ^{1}H NMR ($CDCl_3$ 250 MHz), δ: 8.93 (s, 4H), 8.05 (d, 4H, 8 Hz), 7.63 (t, 4H, 8 Hz), 7.19 (d, 4H, 8 Hz), 3.46 (s, 8H), 4.11 (t, 4H, 7 Hz), 2.18 (s, 12H), 1.45 (m, 4H), 1.15 (m, 8H). The above compound (0.407 g, 0.55 mmol) was dissolved in a 5 M NaOH in H_2O/EtOH 1:1 (45 mL). The reaction mixture was refluxed for 7 h. After this time, ethanol was evaporated and the resulting aqueous solution was extracted with chloroform. The organic phase was dried with Na_2SO_4 and the solvent evaporated. Compound **3** (0.302 g (97%)) was obtained as a orange oil (43%). ^{1}H NMR (CD_3OD, 250 MHz), δ: 7.38 (t, 4H, 8 Hz), 6.82 (d, 4H, 8 Hz), 6.40 (d, 4H, 8 Hz), 3.53 (s, 8H), 2.45 (t, 4H, 7 Hz), 1.47 (m, 4H), 1.17 (m, 8H). ^{13}C NMR ($CDCl_3$, 62.9 MHz), δ: 158.8, 158.2, 138.1, 112.6, 106.7, 60.5, 54.3, 29.4, 27.3, 26.9. Elemental analysis, calcd for $C_{32}H_{44}N_{10}$ (568.76): C 67.58, H 7.80, N 24.63; found: C 67.71, H 7.67, N 24.55.

*4.4.8. N,N,N′,N′-Tetra(2-pyridylmethyl)-1,8-dioctylamine (**4**).* To a stirred solution of 2-chloromethyl pyridine hydrochloride (4.55 g, 27.7 mmol) in NaOH 5 M (30 mL) were added 1,8-diaminooctane (1.00 g, 6.93 mmol) and hexadecyltrimethylammonium chloride (0.44 g, 0.14 mmol). The mixture was stirred for 20 h and the solution was extracted with CH_2Cl_2 (3×20 mL). The organic yellow solution was washed with brine (2×60 mL), water (2×60 mL), and dried with Na_2SO_4. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/MeOH 10:0.5). Compound **4** (2.902 g (82%)) was obtained as a white solid. ^{1}H NMR ($CDCl_3$, 250 MHz), δ: 8.33 (d, 4H, 8 Hz), 7.46 (t, 4H, 8 Hz), 7.38 (d, 4H, 8 Hz), 6.94 (t, 4H, 8 Hz), 3.65 (s, 8H), 2.36 (t, 4H, 7 Hz), 1.36 (m, 4H), 1.02 (m, 8H). ^{13}C NMR ($CDCl_3$, 62.9 MHz), δ: 159.9, 148.7, 136.1, 122.6, 121.6, 60.3, 54.2, 29.2, 27.0, 26.8. ESI MS (*m*/*z*): 509.4.0 [100%, M+H$^+$], 531.2 [59%, M+Na$^+$]. Elemental analysis, calcd for $C_{32}H_{40}N_6$ (508.70): C 75.55, H 7.93, N 16.52; found: C 75.76, H 7.70, N 16.58.

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