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Synthesis and DNA Cleavage Activity of Artificial Receptor 1,4,7-Triazacyclononane Containing Guanidinoethyl and Hydroxyethyl Side Arms

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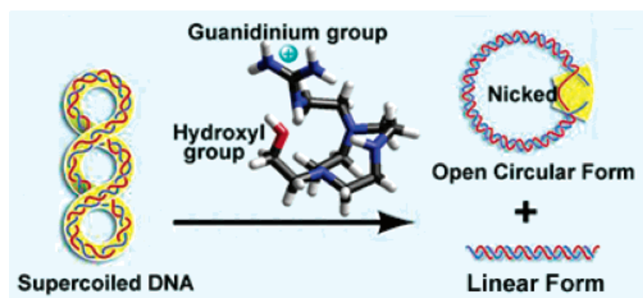
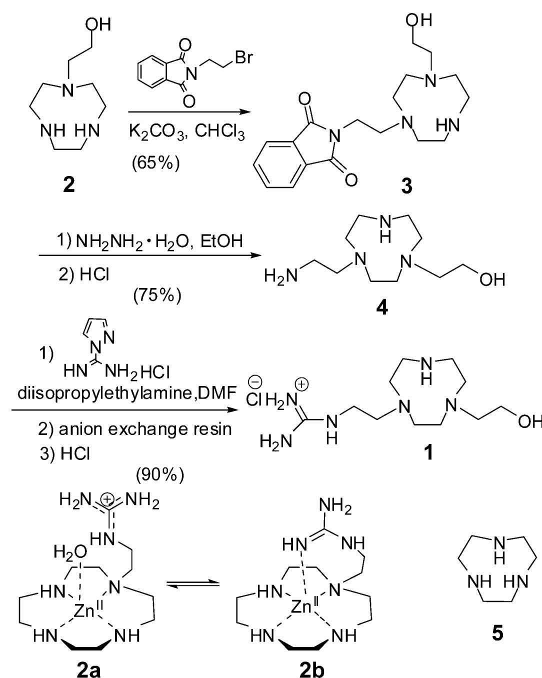


A novel phosphodiester receptor 1-(2-guanidinoethyl)-4-(2-hydroxyethyl)-1,4,7-triazacyclononane hydrochloride **1** was synthesized. DNA cleavage efficiency of **1** exhibits remarkable increases compared with its Zn^{II} complex and corresponding nonguanidinium compound *N*-(2-hydroxyethyl)-1,4,7-triazacyclononane and parent 1,4,7-triazacyclononane. Kinetic data of DNA cleavage promoted by **1** fit to a Michaelis–Menten-type equation with k_{max} of 0.160 h^{-1} giving 10^7-fold rate acceleration over uncatalyzed DNA. The acceleration is driven by the spatial proximity of the nucleophilic hydroxyl group and the electrophilic activation for the phosphodiester by the guanidinium group.

The artificial nucleic acid cleaving agents have attracted extensive attention due to their potential applications in the fields of molecular biological technology and drug development.[1] Metal complexes as cleaving agents of nucleic acids have been widely investigated and found to be quite efficient,[2] but their pharmic use is hampered by concerns over the lability and toxicity due to free-radical generation during the redox processes of some transition metal,[3] such as Cu. Recently, metal-free cleaving reagents have been put forward by Göbel and coworkers;[4] they are considered safer for their hydrolytic pathway of cleaving the P–O bond of phosphodiester in nucleic acids

SCHEME 1



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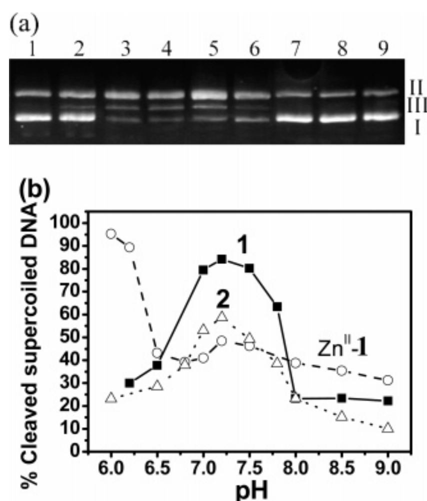

FIGURE 1. (a) Agarose gel (1%) of pUC 19 DNA (0.05 mM bp) incubated for 18 h at 37 °C with 0.130 mM **1** in different pH buffer: lanes 1−9, pH 6.2, 6.5, 7.0, 7.2, 7.5, 7.8, 8.0, 8.5 and 9.0, respectively. (Agarose gel of **2** and Zn^{II}−**1** see Figure S13, Supporting Information.) (b) pH-dependent profile for DNA cleavage promoted by **1** (■), **2** (△), and Zn^{II}−**1** (○).

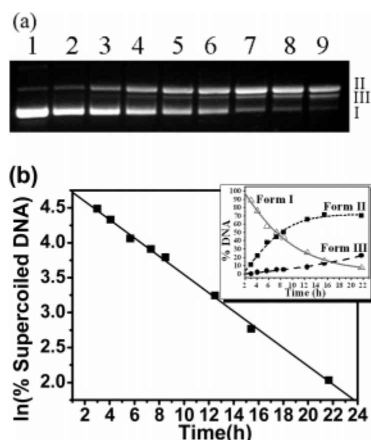

FIGURE 2. Time course of pUC 19 DNA (0.05 mM bp) cleavage promoted by **1**. (a) Agarose gel (1%) of the time-variable reaction products: lane 1, DNA control; lanes 2−9, reaction time of 3.00, 4.08, 5.67, 7.33, 8.50, 12.50, 15.42, and 21.67 h, respectively. (b) Plot of ln(% supercoiled DNA) vs reaction time. Inset of Figure 3b is the plot of % DNA vs time.

and have shown clinical potential. Such small organic molecules as guanidinium derivatives,[4,5] cyclodextrin derivatives,[6] macrocyclic polyamines,[7] and dipeptides[8] have been used to cleave

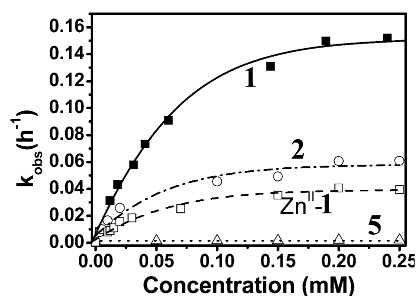

FIGURE 3. Saturation kinetics plot of k_{obs} versus various concentrations of **1** (■), **2** (○), **5**(△), and Zn^{II}−**1** (□).

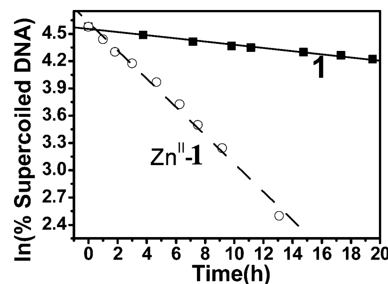

FIGURE 4. Time course of pUC 19 DNA (0.05 mM bp) cleavage promoted by **1** (0.10 mM) and Zn^{II}−**1** (0.10 mM) at pH 6.0 (37 °C) in 50 mM Tris−HCl/10 mM NaCl buffer.

phosphodiester. It is known that guanidinium is the arginine residue and the key functionality at the active site in staphylococcal nuclease (SNase) which imparts 10^{16}-fold rate enhancement for DNA hydrolysis.[9] Some guanidinium receptors as nuclease mimics for cleavage of active phosphodiester, such as bis(*p*-nitrophenyl) phosphate (BNPP) and bis(2,4-dinitrophenyl) phosphate (BDNPP), have been reported by several laboratories (e.g., Anslyn,[1j,3a,5b,c] Göbel,[4,5d] Hamilton,[5e,f] and their coworkers), and a few of them were identified as powerful cleavers of RNA.[3a,4b,c] We report here, for the first time, the design and synthesis of a novel phosphodiester receptor 1-(2-guanidinoethyl)-4-(2-hydroxyethyl)-1,4,7-triazacyclononane hydrochloride **1** and the studies of its DNA cleavage activity. As comparison, the DNA cleavage activities of *N*-(2-hydroxyethyl)-1,4,7-triazacyclononane **2**, 1,4,7-triazacyclononane (TACN) **5**, and Zn^{II}−**1** complex are also studied.

In compound **1**, the guanidinium group serves to recognize, bind, and electrophilically activate the anionic phosphodiester through hydrogen bonding and electrostatic interaction.[10] The hydroxyl group works as a nucleophilic group[11] in the transphosphorylation reaction, which is expected to be highly efficient because of the proximity effect.[4a,5d] A "couple hardness with

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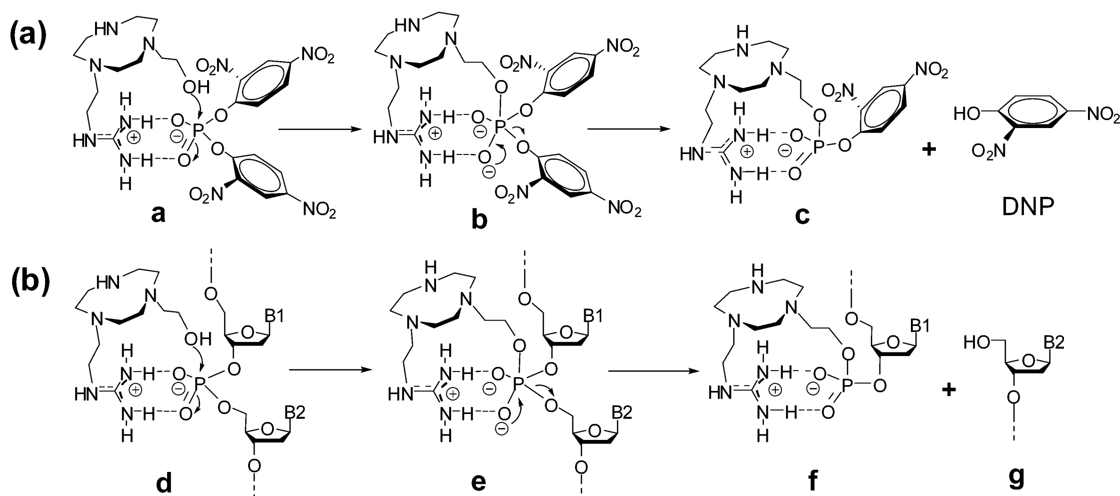
SCHEME 2



softness" TACN is designed to connect these two groups. **1** was synthesized via a three-step reaction (nucleophilic substitution, hydrazinolysis, and guanylation) starting from **2**[12] (Scheme 1). Guanylation of the primary amino group was carried out by using the guanylating reagent 1*H*-pyrazole-1-carboxamidine hydrochloride.[13] The crude product was purified by column chromatographic separation on strong base anion exchange resin followed neutralization by 10% hydrochloride acid to give **1** in good yield. All new compounds are characterized by ^{1}H NMR, ^{13}C NMR, and ESI-MS spectra (see the Supporting Information).

Figure 1a is the agarose gel of pH-dependence assays which indicates that the supercoiled DNA (form I) relaxes to form a nicked circular DNA (form II) in the presence of **1** and then the linear DNA (form III) forms when the pH is in the range of 6.5 to 7.8. pH dependence data of **1** (Figure 1b) presents a bell-shaped profile which indicates that pH 7.2 is the optimal pH for DNA cleavage in the presence of **1**. The pH dependence profile of **2** is similar to **1** (Figure 1b). A relatively low NaCl concentration (10 mM) was selected to control the ionic strength in all experiments. (For the ionic strength assays, see the Supporting Information.)

Figure 2a shows the agarose gel of supercoiled plasmid DNA cleavage promoted by **1** (0.144 mM) in Tris−HCl/NaCl buffer (pH 7.2) at 37 °C into nicked and linear forms. The kinetic plot indicates that the extension of supercoiled DNA cleavage varies exponentially with the reaction time, giving a pseudo-first-order kinetics with an apparent first-order rate constant of 0.131 ± 0.002 h^{-1} (Figure 2b). The apparent first-order rate constants of DNA cleavage reactions promoted by a series of various concentrations of **1**, **2** and **5** under the same conditions as described above are summarized in Tables S1 and S2 (Supporting Information). Data of **1** and **2** were fit to a curve based on a Michaelis−Menten-type equation ($k_{obs} = k_{max}$[enzyme]/(K_d + [enzyme])), where k_{obs} is the apparent first-order rate constant at various concentrations, the k_{max} is the maximum rate constant at saturation concentration, and K_d is the apparent dissociation constant for the enzyme−substrate complex.[14] The saturation kinetics profiles of the supercoiled DNA cleavage at various concentrations of **1** and **2** are shown in Figure 3. The kinetic data are linearized through a double-reciprocal plot of $1/k_{obs}$ vs 1/[enzyme] ([enzyme] is the concentrations of **1** or **2**), giving the first-order rate constant k_{max} of 0.160 h^{-1}, 0.063 h^{-1} and the association constant K_d of 0.052 mM, 0.038 mM, respectively (Figure S16, Supporting Information). The non-substitutive **5** shows very low cleavage activity (Figure 3), and the cleavage efficiency of **2** has a 30-fold enhancement than **5**, which indicates that the pendent hydroxyl group effectually increases the DNA cleavage activity. Furthermore, **1** has remarkable rate acceleration over non-guanidinium compound **2**, which is due to the binding and electrophilic activation for the phosphodiester of DNA by the guanidinium group of **1**.

As comparison, the DNA-cleaving behavior of Zn^{II}−**1** (1:1) was also investigated under the same conditions and was expected to be higher DNA cleavage activity than **1**. It is surprising that the k_{max} of Zn^{II}−**1** is only 0.039 h^{-1} (Figure S17, Supporting Information); that is, the cleavage activity reduces instead. Why does the activity reduce? Kimura and co-workers reported that the pendent guanidine of (2-guanidinyl)ethylcyclen is a good Zn^{II}-binding ligand (Scheme 1, **2b**) at neutral pH in aqueous solution, so that **2b** did not catalyze the hydrolysis of 4-nitrophenyl acetate.[15] The structure of **1** is similar to (2-guanidinyl)ethylcyclen except for the 2-hydroxyethyl arm. Therefore, the inhibition of DNA cleavage activity can be ascribed to the formation of Zn^{II}-binding complex with the pendent guanidine group. However, the pH dependence of Zn^{II}−**1** is quite different from **1** (Figure 1), Zn^{II}−**1** shows extraordinarily high DNA cleavage activity at the weakly acidic conditions. We compared the first-order rate constants of **1** and Zn^{II}−**1** (1:1) at pH 6.0 under the same conditions (Figure 4).

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The rate constants of Zn^{II}−**1** (0.155 h^{-1}) is 10-fold than **1** (0.015 h^{-1}). According to Kimura's reports,[15] guanidinium group does not bind with Zn^{II} at weakly acidic conditions; thus, the guanidinium group of Zn^{II}−**1** could promote the phosphodiester cleavage freely by electrostatic activation. Moreover, the hydroxyl group can be a better nucleophile because of the deprotonation by the nearby Zn^{II} trapped in the macrocyclic ring at pH 6.0.[11b]

To study the cleavage mechanism, BDNPP was used as the DNA mimics. BDNPP and **1** were dissolved in DMF/H_2O, and after 0.5 h equilibration time at room temperature, ESI-MS analysis was carried out. In ESI-MS spectrum, the peaks at *m*/*z* 504.87 and 184.82 show the $[M + H]^+$ signals of **c** (calcd 505.18) and 2,4-dinitrophenol (DNP) (calcd 185.01), respectively (Figure S15, Supporting Information). The generation of **c** indicates that the phosphodiester bond of BDNPP would be cleaved by **1** via a transphosphorylation pathway (Scheme 2a).[5] Thus, similar to BDNPP, transphosphorylation is one of the possible mechanisms for the DNA cleavage promoted by **1** at pH 7.2, which is schematically depicted in Scheme 2b.

In conclusion, design and synthesis of a novel phosphodiester receptor **1** containing guanidinoethyl and hydroxyethyl side arms was achieved successfully. Kinetic data of DNA cleavage promoted by **1** are fit to a Michaelis−Menten-type equation with a k_{max} of 0.160 h^{-1} which gives 10^7-fold rate acceleration over uncatalyzed double-stranded DNA (10^{-8} h^{-1}).[16] This substantial acceleration of cleavage reaction is due not only to the spatial proximity of the nucleophilic hydroxyl group but also the electrophilic activation for the phosphodiester of DNA by the binding guanidinium group.

Experimental Section

Plasmid DNA Cleavage. DNA cleavage experiments were performed using 500 ng per reaction of pUC 19 derived plasmid of 2686 bp length. The DNA fragments after cleavage assays were separated and monitored by agarose gel electrophoresis. The supercoiled DNA in 50 mM Tris−HCl buffer containing 10 mM NaCl was treated with different compound concentrations followed by dilution with the buffer to a total volume of 15 μL. The sample was incubated at 37 °C. The loading buffer (30 mM EDTA, 0.05% (w/V) glycerol, 36% (V/V) bromophenol blue) 3 μL was added to end the reactions and the mixture was loaded on 1% agarose gel containing 1.0 μg dm^{-3} EB. Electrophoresis was carried out at 80 V for 1.5 h in 0.5 M Tris-acetate EDTA (TAE) buffer. Bands were visualized by UV light and photographed. The proportion of DNA in the supercoiled, nicked, and linear forms after electrophoresis was estimated quantitatively from the intensities of the bands using TotalLab analysis software.

Synthesis: 1-(2-Guanidinoethyl)-4-(2-hydroxyethyl)-1,4,7-triazacyclononane Hydrochloride (1). A mixture of compound **4** (0.09 g, 0.41 mmol), 1*H*-pyrazole-1-carboxamidine hydrochloride (0.06 g, 0.41 mmol), and DIEA (diisopropylethylamine) (0.06 g, 0.46 mmol) in DMF (10 mL) was stirred for 8 h at room temperature under a dry nitrogen atmosphere. After anhydrous ether (25 mL) was added, the brown oil deposition appeared immediately. The crude product was dissolved in deionized water and chromatographed on a strong base anion-exchange resin column (eluted with deionized water). Subsequently, the eluent was evaporated under reduced pressure to remove water, and the residue was washed with ether (3 × 30 mL) to eliminate the unreacted reactant **4** and other organic impurities. The residue was then dissolved in deionized water (15 mL) and neutralized to pH 7.0 with 10% hydrochloric acid. Water was removed in vacuum to give compound **1** as strong hygroscopic brown solid (0.11 g, 0.37 mmol). Yield: 90%. (For NMR and ESI-MS data, see the Supporting Information.)

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Supporting Information Available: Detailed descriptions of experimental procedures, spectra for all new compounds, photographs of the agarose gels, and kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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