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Artificial Transcription Factors which Mediate Double-Strand DNA Cleavage

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Abstract: A new family of artificial transcription factor (ATF)-based conjugates have been designed and synthesized as potent chemical nucleases. Polyamides as the important and efficient ATFs were used to modify and activate several anchor compounds. The results demonstrate that the resulting conjugates remarkably promote the rate accelerations and non-random double-strand DNA cleavage activity. Interestingly, the cleavage activity of both the hydrolytic and oxidative agents was promoted efficiently through the modification of the ATFs.

Keywords: artificial transcription factors · DNA binding · DNA cleavage · double-strand cleavage · polyamide

Introduction

The recent impressive progress in the field of genomic and biotechnology has stimulated the search for effective tools to manipulate DNA.[1] There has been much interest in the development of synthetic small molecules as nucleic acid cleavage agents. Among them, double-strand break (dsb) agents are thought to be more biologically significant than single-strand breaks (ssb) as a source of cell lethality, because they apparently are less readily repaired by DNA repair mechanisms.[2] In the past few years, agents that facilitate dsDNA cleavage were reported, such as the bleomycins,[3] the calicheamicin,[4] prodigiosin[5] and Ce^{II}-based complexes.[6]

Particular interest has been paid to the field of artificial transcription factors (ATFs), where the essential function of some transcription factors is to recruit and promote the assembly of a larger transcription complex, leading to the expression of a gene of interest.[7–10] In so-called artificial transcription factors (ATFs), natural DNA-binding domains are replaced by synthetic variants, such as triplex-forming peptide nucleic acids (PNAs) and hairpin polyamides.[11] Polyamides[12] are an important class of artificial transcription factors derived from nonproteinogenic amino acids, which contain *N*-methylpyrrole (Py), *N*-methylimidazole (Im), or *N*-methylhydroxypyrrole (Hp) groups that can bind in a hairpin motif to the minor groove of double-stranded DNA.[13] Because of their wide sequence recognition ability and sequence specificity, polyamides have an enhanced capability to function as regulators of transcription in directed applications.[14–17]

To further develop highly efficient dsb's chemical nucleases of practical value, we sought to explore new families of ligands that were readily available, of lower toxicity and facile to activate under physiologically relevant conditions. In this paper, our efforts focus on the modification of less reactive and random dsb's small compounds by Py/Im polyamide as artificial transcription factors. Through connection of the two parts with simple linker, we obtained a new family of ATFs-based DNA dsb chemical nucleases. In detailed experiments, we will show that remarkable rate accelerations and non-random dsb activity of ATFs-based conjugates can be achieved based on the powerful affinity of the

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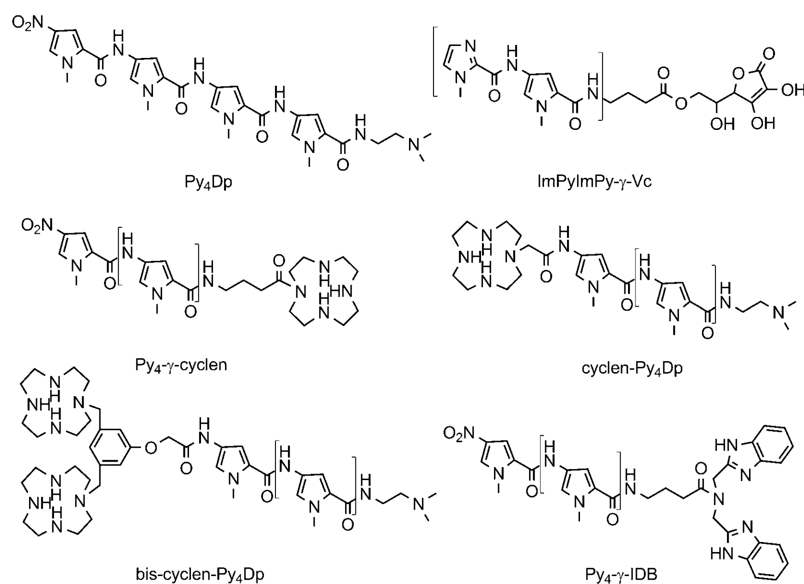
ATFs moiety to double-stranded DNA compared with free compounds.

Results and Discussion

Design and synthesis of ATFs-based conjugates: To confirm whether the modification of ATFs moiety for improving the ability of double strand cleavage is generally applicable, diverse small cleavage molecules were conjugated to ATFs, including polyamines (cyclen) and bis(2-benzimidazolylmethyl)amine (IDB) for a hydrolytic and ascorbic acid (Vc) for a oxidative mechanism. In addition, we attempted to conjugate these molecules to C-terminus or N-terminus of oligopolyamide. The design and structure of the ATFs-based conjugates are shown in Scheme 1. In all cases, the ligands were prepared starting from a common precursor, that is, the oligopolyamide backbone. Py$_4$-γ-cyclen, cyclen-Py$_4$Dp and Py$_4$-γ-IDB have been prepared as previously described.[18–20] A detailed synthesis route of bis-cyclen-Py$_4$Dp and ImPyImPy-γ-Vc is given in the Supporting Information.



Scheme 1. Structures of ATFs-based conjugates studied.

DNA cleavage with oxidative agents: As for Vc, incubation of supercoiled pUC18 DNA with free Vc at 37 °C results in DNA cleavage at lower concentrations. Subsequently, all supercoiled DNAs (form I) were converted to nicked DNA (Form II) by increasing the Vc concentration while the linear DNA (form III) was detected. Further, linear DNA was gradually degraded into rogressively smaller fragments when excessive Vc was added to the reaction mixture (Figure 1 a). However, the catalytic activity was enhanced remarkably due to the introduction of the ATF moiety. The linear DNA was detected when the concentration of ImPyImPy-γ-Vc was about 0.1 mM. The strong binding affinity of polyamide to DNA leads to an increase of the effective molarity of the catalytic group in the cleavage reaction and as a consequence the minimum of cleavage concentration lowered apparently. The appearance of a well-defined electrophoresis band for the linear DNA by both Vc and ImPyImPy-γ-Vc suggests a double-strand cleavage process, although it is not clear whether single-strand cleavage can be compared with double-strand cleavage.

In order to assess whether the observed linearization represents a non-random linearization process, the statistical test of double-strand cleavage was applied.[21,22] Such analysis assumes a Poisson distribution of the strands cuts and calculates the average number of single (n_1) and double (n_2) strands cuts from the fraction of supercoiled and linear DNA present after the reaction. The Freifelder–Trumbo re-

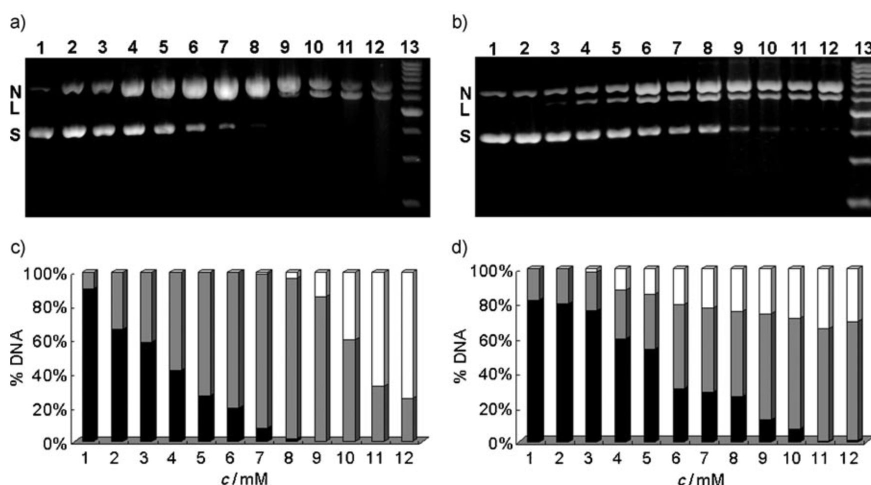


Figure 1. Concentration dependence of pUC18 plasmid DNA cleavage by a) Vc and b) ImPyImPy-γ-Vc in 40 mM pH 7.7 Tris-HCl buffer for 12 h at 37 °C. Lane 1, DNA control; lanes 2–12, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mM cleavage agents in the reaction, respectively; lane 13, 500 bp DNA ladder (S: supercoiled DNA, N: nicked DNA, L: linear DNA). c) and d) Quantitation of % various DNA forms per lane by c) Vc and d) ImPyImPy-γ-Vc.

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lation suggests that approximately 100 ssb are required per dsb if the process is completely random.[21] Thus, the smaller the n_1/n_2 value, the higher the linearization activity of an agent in question.[23] Table 1 shows the statistical results of

Table 1. Comparison of DNA linearization (n_1/n_2) activities of Vc and ImPyImPy-γ-Vc as function of reaction conditions.[a]

Complex	c [mM]	T [h]	n_1	n_2	n_1/n_2
Vc	1.0	12	4.0290	0.0420	95.9
	0.3	20	2.0103	0.0299	67.2
ImPyImPy-γ-Vc	0.2	12	0.3690	0.1485	2.5
	1.0	12	0.9933	0.3576	2.8
	2.0	12	1.6947	0.5126	3.3
	0.3	0.5	0.1391	0.0246	5.7
	0.3	7	0.4293	0.2208	1.9
	0.3	12	0.5781	0.3508	1.6
	0.3	20	0.8708	0.6710	1.3

[a] pUC18 DNA, 40 mM Tris-HCl, pH 7.4, 37 °C.

DNA linearization experiments of Vc and ImPyImPy-γ-Vc performed under a variety of reaction conditions. Vc led to DNA cleavage at 12 h (1.0 mM) and 20 h (0.3 mM) and afford n_1/n_2 ratios 95.9 and 67.2, respectively. Linearization derived from dsb, which is observed only when two cuts randomly, occur sufficiently close on the two strands.[24] We can thus demonstrate that Vc is not a true dsb agent in a sense that a dsb is not formed as a result of a single excitation event. However, the values of n_1/n_2 in the range 1–6, under all reaction conditions for ImPyImPy-γ-Vc, suggested a non-random process for all combinations of reactions conditions. In other words, dsb with ImPyImPy-γ-Vc are caused by a single event or interdependent events and not by a coincidence of random ss breaks on opposite strands. We propose that DNA affinity of the ATF moiety play an important role in promoting activity of the true dsb. Due to the introduction of ATFs moiety, one DNA strand is cleaved, and then the cleavage agent remains close to the first strand break to carry out strand breaks elsewhere on the DNA. In addition, the steady values under various conditions for ImPyImPy-γ-Vc suggest that the changes both in concentration and reaction time hardly affect non-random cleavage path to efficiently form linear DNA.

Possible process of non-random and random dsb: The different cleavage models of plasmid DNA by cleavage agents in the absence and presence of ATFs moiety are depicted in Figure 2. Upon addition of free ligands to the cleavage reaction, many sites of the supercoiled DNA were attacked non-selectively to form ssb products (nicked DNA observed). Subsequently, dsb (linear DNA observed) is observed only when two cuts randomly occur sufficiently close on the two strands. Thus, the observed linearization arose from a random cleavage for free ligands. However, on account of the strong affinity of the ATF to DNA, cleavage agents modified were attached tightly to certain DNA sequences and the effective molarity of the catalytic group might increase to a sufficiently high level to allow facile cleavage of

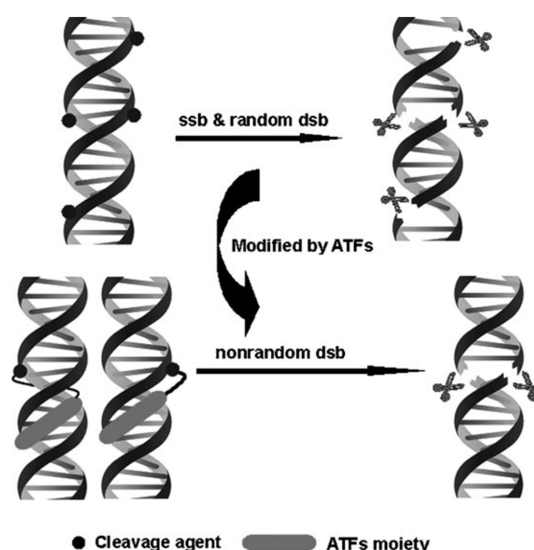

Figure 2. ATFs moiety mediate plasmid DNA dsb's model.

the double-strand. Further, since the cleavage agents were restrained in its movements, a random cleavage was controlled effectively. In this process, dsb in the presence of ATFs moiety are caused by a single event or interdependent events and not by the coincidence of random ssb on opposite strands. In addition, there is a strong evidence of non-random cleavage as no smaller fragments were obtained at higher concentrations and longer reaction time (Figure 1b).

DNA cleavage with hydrolytic agents: The advantage of ATF-based conjugates was demonstrated further through the comparison of azacrown compounds. Figure 3a shows the DNA cleavage behaviour of bis-cyclen-2Zn^{II} under physiological conditions. The cleavage agent converted the supercoiled plasmid to a mixture of supercoiled and nicked DNA with non-selective ssb and random dsb reactions (see Supporting Information for statistical data). As shown in Figure 3b, however, the appearance of 1500–2000 bp linear fragment provided strong evidence that the complex modified by ATFs (bis-cyclen-2Zn^{II}-Py_4Dp) works via a non-random dsb event, since random cutting was expected to produce fragments while increasing the cleavage agent concentration.[2] The similar results, using other ATFs-based conjugates studied, were observed (see Supporting Information for details).

In order to better demonstrate whether the ATF-based conjugates possess a better affinity to DNA compared with free small compounds and free ATFs, the CD spectra of Py_4Dp, cyclen, cyclen-Py_4Dp and Py_4-γ-cyclen were recorded as shown in Figure 4. None of the ligands used showed CD signals in the CT DNA absorption region. The absence of any detectable ICD in the case of cyclen is indicative of the absence of interaction between this small molecule and DNA (Figure 4a). However, upon addition of cyclen-Py_4Dp and Py_4-γ-cyclen to CT DNA substantially induced CD signals (ICD) arise in 310–400 nm spectral region (Fig-

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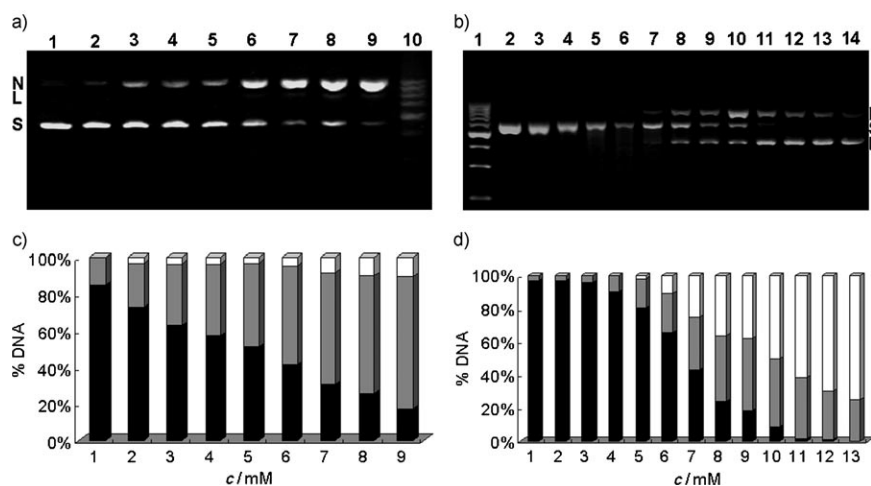

Figure 3. a) Concentration dependence of pUC18 plasmid DNA cleavage by Bis-Cyclen-2Zn^{II} in 40 mM pH 7.7 Tris-HCl buffer for 12 h at 37 °C. Lane 1, DNA control; lanes 2–9, 0.1, 1.0, 10, 20, 40, 60, 80, 100 μM cleavage agents in the reaction, respectively; lane 10, 500 bp DNA ladder (S: supercoiled DNA, N: nicked DNA, L: linear DNA). b) pUC18 plasmid DNA cleavage by Bis-Cyclen-2Zn^{II}-Py$_4$Dp in the same conditions to Bis-Cyclen-2Zn^{II}. Lane 1, 500 bp DNA ladder; lane 2, DNA control; lanes 3–14, 0.05, 0.1, 0.5, 1.0, 10, 25, 30, 40, 50, 60, 80, 100 μM cleavage agents in the reaction, respectively (S: supercoiled DNA, N: nicked DNA, L: small fragment). c) and d) Quantitation of % various DNA forms per lane by c) bis-cyclen-2Zn^{II} and d) bis-cyclen-2Zn^{II}-Py$_4$Dp.

ure 4c,d). The intensity of the induced Contton effect observed upon DNA binding of ATF-based conjugates was markedly higher than that observed when the same number of free Py$_4$Dp (without linked cyclen) was bound to CT DNA.

Furthermore, various important characteristic interactions between ATF-based conjugates studied and duplex DNA were studied, including CD spectra, fluorescence quenching, T_m measurements and UV spectrophotometric titrations. All experiments on the binding affinity are given in Table 2 (see Supporting Information for details). The results were consistent with DNA cleavage ability of smaller molecules in the absence and presence of ATF, which were obtained by gel electrophoresis experiments.

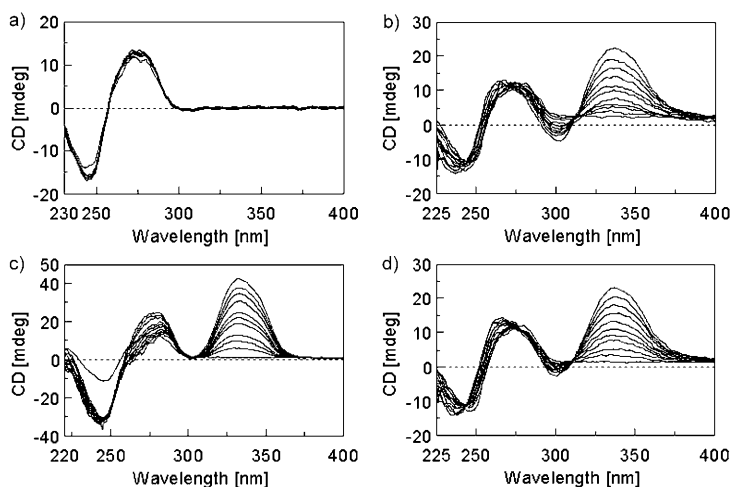

Figure 4. CD spectra of CT DNA upon addition of increasing amount of ligands. a) Cyclen. b) Py$_4$Dp. c) Cyclen-Py$_4$Dp. d) Py$_4$-γ-Cyclen. All experiments were carried out at pH 8.0 (10 mM Tris-HCl buffer) with 10 mM NaCl. Spectra were recorded at the following r values: 0, 0.001, 0.002 to 0.01 (from bottom to top at 339 nm).

Table 2. Summary of melting temperature measurements and apparent binding constant measurement.

Compound	ΔT_m [°C]	c_{50} [mM]	K_{app} [M^{-1}]
distamycin[a]			7.7×10^{6}
Py$_4$Dp	8.4	9.1×10^{-3}	1.4×10^{8}
cyclen	2.2	3700	3.4×10^{3}
Py$_4$-cyclen	11.5	8.8×10^{-3}	1.7×10^{8}
cyclen-Py$_4$Dp	13.8	7.2×10^{-3}	2.6×10^{8}
bis-cyclen		2500	6.4×10^{6}
bis-cyclen-Py$_4$Dp		8.2×10^{-2}	3.6×10^{7}
IDB			7.8×10^{3}
Py$_4$-IDB		1×10^{-1}	5.0×10^{4}
Py$_4$-IDB-Zn^{II}		9×10^{-2}	1.3×10^{5}

[a] See refs. [25,26].

Conclusion

In summary, we have shown herein that a new family of ATFs-based conjugates as effective double-strand DNA cleavage agents were designed and evaluated. Polyamide, a prevalent kind of ATFs, was chosen to modify some lower capable and random dsb small molecule agents. The preliminary gel electrophoresis suggested that the conjugates promoted remarkably rate accelerations and non-random dsb activity. Due to the introduction of ATFs, small molecules were effectively fixed on the certain site in double-strand DNA and it resulted in effective molarity of the catalytic group was enhanced. The probability of both strands cleavage is higher than single strand of that, since cleavage agents are restricted within narrow limits. Owing to the significant sequence selectivity of hairpin polyamides, small cleavage agents conjugated to hairpin polyamide have been an ongoing area of study in our group, and they could regulate efficiently double-strand cleavage with well-defined sequence selectivity.

Experimental Section

General information: Details MS (ESI) mass spectral data were recorded on a Finnigan LCQDECA mass spectrometer. ^{1}H NMR spectra were measured on a Bruker AV600 spectrometer and chemical shifts in ppm are reported relative to internal Me$_4$Si (CDCl$_3$) or (D$_2$O). Electrophoresis apparatus was using a Biomeans Stack II-Electrophoresis system, PPSV-010. Bands were visualized by UV light and photographed, recorded on an Olympus Grab-IT2.0 Annotating Image Computer System. All

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other chemicals and reagents were obtained commercially and used without further purification. Electrophoresis grade agarose, plasmid DNA (pUC18), poly(dA–dT)·poly(dA–dT) and poly(dC–dG)·poly(dC–dG) were purchased from Promega Corporation. Calf thymus DNA (CT DNA) was purchased from Sigma (USA) Company and used as received.

Synthesis of bis-cyclen-Py_4Dp: Detailed compound structures and synthesis route of Bis-Cyclen-Py_4Dp are given in the Supporting Information (Scheme S1).

Compound 2: Compound **1** was dissolved in DMF (30 mL) containing anhydrous K_2CO_3 (1.38 g, 10.0 mmol) with stirring at room temperature. Then, BnBr (1.78 mL, 15 mmol) was dropwise to the solution above for 30 min. After 4 h, the reaction was quenched by cold water (100 mL) and solution was extracted with $CHCl_3$ (50 mL×6). The combined organic phases were washed with saturated aqueous NaCl and dried over Na_2SO_4. Filtration and evaporation of the solvent yield compound **2** (2.34 g, 78%). MS (ESI$^+$): m/z: 323 [M+Na]$^+$.

Compound 3: Compound **2** (1.20 g) dissolved in anhydrous THF (70 mL), which was dropwise to suspension THF (20 mL) containing $LiAlH_4$ (0.77 g, 20 mmol) for 30 min at 0°C under an N_2 atmosphere. After 2 h, CH_3OH (5 mL), H_2O (5 mL) and NaOH (10 mL, 10%) were added in the solution above, respectively, and the mixture was filtrated with celatom. Evaporation of solution gave compound **3** (0.97 g, 99%) as yellow solid. MS (ESI$^+$): m/z: 251 [M+Li]$^+$.

Compound 4: Compound **3** (0.73 g, 3.0 mmol) was dissolved in anhydrous CH_2Cl_2. PBr_3 (0.66 mL, 6.9 mmol) was added in the solution with stirring 3 h at 0°C under an N_2 atmosphere. After warming to room temperature, the mixture was poured in cold water (70 mL), and its pH was adjusted to pH 7.0–7.2 using saturated aqueous $NaHCO_3$. The solution was extracted with Et_2O (50 mL×5). The combined organic phases were dried over Na_2SO_4. Filtration and evaporation of the solvent yield compound **4** (0.68 g, 61%). MS (ESI$^-$): m/z: 372 [M]$^-$.

Compound 5: Under an N_2 atmosphere, 3Boc-Cyclen (1.39 g, 2.95 mmol) was dissolved in CH_3CN (70 mL) and compound **4** (0.55 g, 1.48 mmol) and anhydrous Na_2CO_3 (0.38 g, 3.57 mmol) was added in the solution. The remaining reaction was refluxed at 85°C for 20 h. Filtration and evaporation of the solvent yield yellow solid. Crude product was purified by a silica gel column with ethyl acetate/petroleum ether (50:50) as eluent. ^{1}H NMR (600 MHz, $CDCl_3$): δ=1.40, 1.44 (br, 54H, $OC(CH_3)_3$), 2.60 (m, 8H, CH_2NCH_2), 3.2–3.29 (m, 16H, NCH_2CH_2), 3.56 (m, 8H, NCH_2CH_2), 3.68 (s, 4H, NCH_2Ar), 4.97 (s, 2H, OCH_2Ar), 6.61 (s, 1H, $OArH_4$), 6.75 (s, 2H, $OArH_2$, $OArH_6$), 7.25–7.28 (br, 1H, ArH_4), 7.33–7.36 ppm (br, 4H, ArH_2, ArH_3, ArH_5, ArH_6); ^{13}C NMR (100 MHz, $CDCl_3$): δ=155.73, 137.45, 128.53, 127.93, 127.43, 115.94, 79.41, 69.92, 56.04, 55.47, 54.15, 49.86, 47.95, 28.47 ppm; MS (ESI$^+$): m/z: 1176 [M+Na]$^+$.

Compound 6: Compound **5** (230.8 mg, 0.2 mmol) and Pd/C (5%) (22.7 mg) were added to anhydrous CH_3CH_2OH. The solution was stirred at 1 atm under an H_2 atmosphere. After 3 h, filtration and evaporation of the residue gave brown solid (161.7 mg, 76%). ^{1}H NMR (600 MHz, $CDCl_3$): δ=1.24–1.71 (br, 54H, $OC(CH_3)_3$), 2.11–2.61 (m, 8H, CH_2NCH_2), 3.35–3.70 (m, 24H, NCH_2CH_2), 4.11–4.17 (s, 4H, NCH_2Ar), 6.85 (s, 1H, ArH_4), 7.38, 7.73 ppm (s, 2H, ArH_2, ArH_6); MS (ESI$^+$): m/z: 1064 [M+H]$^+$.

The above-mentioned product (424.8 mg, 0.40 mmol), anhydrous K_2CO_3 (55.4 mg, 0.40 mmol) and $BrCH_2CH_2COOEt$ (134 μL, 1.20 mmol) were added to acetone (25 mL). The solution was extracted with ethyl acetate (40 mL×4) and dried over Na_2SO_4 after refluxed for 10 h. Filtration and evaporation of the solution yield yellow solid. Subsequently, it was hydrolyzed to obtain the corresponding acid compound **6** (391.6 mg, 95%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.23–1.47 (br, 54H, $OC(CH_3)_3$), 2.86 (br, 8H, CH_2NCH_2), 3.40–3.89 (m, 28H, NCH_2CH_2, NCH_2Ar), 4.62 (br, 2H, $ArOCH_2$), 6.96 ppm (br, 3H, ArH_2, ArH_4, ArH_6); MS (ESI$^+$): m/z: 1122 [M+H]$^+$.

Bis-cyclen-Py_4Dp: Compound **6** (657.2 mg, 0.59 mmol) was converted into activated esters with HOBt (135.1 mg, 1 mmol) and DCC (206.5 mg, 1 mmol) in CH_2Cl_2 (20 mL). NH_2Py_4Dp was added to the stirred solution in room temperature and the reaction mixture was stirred overnight. Filtration and evaporation of the mixture yield yellow solid. Subsequently, the Boc groups were removed with TFA (0.4 mL) in CH_2Cl_2 (2 mL). The crude product was purified by a silica gel column with $CHCl_3/CH_3OH/NH_3{\cdot}H_2O$ (66:33:1) as eluent to obtain the title compound (347.5 mg, 35%). ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ=1.85 (m, 2H), 2.73 (m, 8H), 2.79, 2.80 (s, 6H), 2.84 (m, 8H), 3.05–3.12 (br, 18H), 3.25 (q, 2H, J=6.0), 3.74 (s, 4H), 3.82–3.86 (br, 12H), 6.79 (s, 1H), 6.93 (s, 2H), 6.96–7.10 (br, 4H), 7.15–7.20 (br, 4H), 8.14 (s, 1H), 9.46–9.93 (br, 4H), 10.16 ppm (s, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=165.56, 162.24, 159.34, 159.11, 158.45, 136.84, 125.16, 123.18, 122.92, 122.25, 121.29, 119.16, 118.04, 115.98, 105.30, 105.08, 67.49, 55.62, 55.23, 47.41, 44.81, 42.69, 42.35, 42.22, 39.99, 39.86, 39.72, 39.59, 39.45, 39.31, 39.18, 36.44, 35.82, 25.01 ppm; MS (ESI$^+$): m/z: 1094 [M+H−7TFA]$^+$; HRMS (ESI$^+$): m/z: calcd for $C_{57}H_{87}N_{18}O_8F_3$: 604.3454; found: 604.3453 [M+2H]$^{2+}$.

ImPyImPy-γ-Vc: The detailed structure and synthesis route of ImPyImPy-γ-Vc are given in the Supporting Information (Scheme S1). Treatment of L-ascorbic acid with acetyl chloride in acetone afforded the 5,6-ketal of L-ascorbic acid. Benzylation of the C-2′ and C-3′ hydroxy groups of the lactone ring in compound **7** was accomplished by using K_2CO_3 and benzyl bromide in DMF to provide compound **8**. Deblocking of the 5,6-O,O-protected derivative of L-ascorbic acid **8** with acetic acid in methanol gave 2,3-O,O-dibenzyl-L-ascorbic acid (**9**). The flexible γ-aminobutyric acid linker was conjugated to compound **9** by BOP reagent and DIEA to obtain compound **10**. Then the polyamide backbone ImPyImPyCOOH and compound **10** were conjugated by BOP reagent and DIEA in DMF/dichloromethane. Finally, the hydrogenation reaction catalyzed by Pd/C removed the benzyl group. The desired product was purified by silica gel column chromatography with $CHCl_3/CH_3OH$ 60:40 as eluent. ^{1}H NMR (600 MHz, [D$_4$]CD_3OD): δ=7.46 (s, 2H), 7.39 (d, 1H), 7.28 (s, 2H), 7.04–7.08 (d, 1H), 6.85 (s, 1H), 4.75 (d, 1H), 4.26 (m, 1H), 4.25 (m, 1H), 4.24 (s, 1H), 4.23 (s, 6H), 4.08 (s, 3H), 3.97 (s, 3H), 2.49 (t, 2H), 1.94 (t, 2H), 1.55 ppm (t, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=174.0, 173.1, 162.6, 160.7, 157.9, 157.7, 156.3, 154.4, 154.2, 146.0, 134.7, 131.4, 131.2, 130.8, 130.5, 126.0, 125.9, 123.5, 120.3, 120.1, 118.5, 77.3, 65.7, 65.6, 39.8, 37.3, 36.9, 34.4, 34.7, 31.0, 23.7 ppm; MS (ESI$^-$): m/z: 734.5 [M]$^-$; HRMS (ESI$^+$): m/z: calcd for $C_{32}H_{36}O_{11}N_{10}$: 737.2643; found: 737.2638 [M+H]$^+$.

Circular dichroism measurements: All experiments were performed under a continuous flow of nitrogen using a Jasco-720 spectropolarimeter. A path length cell of 1 cm was used and all experiments were performed at room temperature. A 0.5 μM solution of cyclen, Py_4Dp and cyclen-Py_4Dp was titrated into the DNA solutions (at pH 8.0, 10 mM Tris-HCl buffer with 10 mM NaCl). The standard scan parameters for all experiments were as follows: The wavelengths were scanned from 400 to 220 nm. The sensitivity was set at 100 mdeg and the scan speed was set at 200 nm per minute. Three scans were accumulated and averaged by the computer.

Plasmid DNA cleavage: Electrophoresis experiments were performed with plasmid DNA (pUC18). In a typical experiment, supercoiled pUC18 DNA (5 μL, 0.08 μg μL^{-1}) in Tris-HCl buffer (40 mM) was treated with different concentration ligands (dissolved in dimethyl sulfoxide, Vc system in water) at different pH value, followed by dilution with the Tris-HCl buffer to a total volume of 80 μL. The samples were then incubated at different temperature and time intervals, and loaded on a 1% agarose gel containing ethidium bromide (1.0 μg mL^{-1}). Electrophoresis was carried out at 85 V for 1 h in TAE buffer. Bands were visualized by UV light and photographed followed by the estimation of the intensity of the DNA bands using a Gel Documentation System.

Fluorescence quenching assay: Fluorescence spectra were recorded on Hitachi model F-4500 spectrofluorimeter, with excitation and emission band bass: 10 nm (λ_{ex}=520, λ_{em}=620 nm). 0.5 μM solution of Py_4Dp, bis-cyclen and bis-cyclen-Py_4Dp were titrated into the DNA-EB solution at pH 8.0 10 mM Tris-HCl buffer with 10 mM NaCl. Apparent binding constants (K_{app}) of the ligands with CT DNA were estimated and compared by measuring the loss of EtBr fluorescence as a function of added ligand. The K_{app} values were calculated from: $K_{EtBr}{\cdot}[EtBr]=K_{app}{\cdot}[ligand]$, where [EtBr] and K_{EtBr} are the concentrations and binding constants of EtBr, respectively, and [ligand] is the concentration of ligand at 50% of maxi-

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mal EtBr fluorescence. The binding constant of EtBr was taken to be 1×10^7.

***T_m* measurements**: T_m measurements were performed by following the changes in the optical densities at 260 nm as a function of temperature. Experiments were performed in Quartz cuvettes sealed with Teflon caps on a Beckman model 640 spectrophotometer fitted with a temperature controller. The samples were heated at a rate of $1\,°C\,min^{-1}$ and the absorbencies were recorded for every 0.1 °C increase in temperature. Experiments were carried out at pH 8.0 (40 mM Tris-HCl buffer) with 10 mM NaCl.

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