Selective and Efficient Recognition of Thymidylylthymidine (TpT) by Bis(Zn^{II}-cyclen) and Thymidylylthymidylylthymidine (TpTpT) by Tris(Zn^{II}-cyclen) at Neutral pH in Aqueous Solution

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Abstract: Following our previous reports of a Zn^{II}-cyclen (**1**) complex (cyclen = 1,4,7,10-tetraazacyclododecane) that selectively recognizes imide-deprotonated thymidine (T^-) to form a stable ternary complex, [T^--(Zn^{II}-cyclen)] (**2**), in aqueous solution, we now report on the interaction of thymidylylthymidine (TpT) with a dimeric Zn^{II}-cyclen complex, which contains a *p*-xylyl spacer, bis(Zn^{II}-cyclen) (**3**), and thymidylylthymidylylthymidine (TpTpT) with a trimeric Zn^{II}-cyclen complex, which is linked by two alternate *p*-xylyl spacers, tris(Zn^{II}-cyclen) (**4**). These interactions were studied by ^1H NMR spectroscopy, UV spectrophotometric titration, potentiometric pH titration, isothermal calorimetric titration, and FAB-MS measurements. In relation to the 1:1 [T^--(Zn^{II}-cyclen)] (**2**) complex (K_d = [uncomplexed T][uncomplexed **1**]/[**2**] = 7.9×10^{-4} M), far more stable 1:1 complexes of the dimeric and trimeric Zn^{II}-cyclen derivatives are formed with imide-deprotonated thymidine nucleotides: [T^-pT$^-$-bis(Zn^{II}-cyclen)] (**5**) (K_d = [uncomplexed TpT][uncomplexed **3**]/[**5**] = 6.3×10^{-7} M) and [T^-pT$^-$pT$^-$-tris(Zn^{II}-cyclen)] (**6**) (K_d = [uncomplexed TpTpT][uncomplexed **4**]/[**6**] = 8.0×10^{-10} M, determined by potentiometric pH titration) at pH 7.4 and 25 °C with $I = 0.10$ ($NaNO_3$). For comparison, we also determined K_d values for 1:1 2′-deoxyguanylylthymidine (GpT, $K_d = 1.3 \times 10^{-5}$ M), 2′-deoxycytidylylthymidine (CpT, $K_d > 10^{-4}$ M), and 2′-deoxyadenylylthymidine (ApT, $K_d > 10^{-4}$ M) complexes with the bis(Zn^{II}-cyclen) (**3**) (K_d = [uncomplexed XpT][uncomplexed **3**]/[1:1 complex], determined by isothermal calorimetric titration), and for the Zn^{II}-(1-benzyl-1,4,7,10-tetraazacyclododecane) complex (**16**) of (Zn^{II}-benzylcyclen) (**15**) with T^- (K_d = [uncomplexed T][uncomplexed **15**]/[**16**] = 5.0×10^{-4} M, determined by potentiometric pH titration) with $I = 0.10$ ($NaNO_3$) at pH 7.4 and 25 °C. This paper represents the first quantitative assessment of stoichiometric and reversible interactions of multinuclear metal complexes with oligonucleotides, whereby the selective and efficient recognition of TpT by **3** and TpTpT by **4** were discovered.

Keywords: macrocyclic ligands · molecular recognition · nucleotides · thymidine · zinc

Introduction

Small molecules that bind to nucleic acids have been attracting growing interest. The dynamic and biochemical functions of the resulting supramolecular complexes have been extensively studied.[1, 2] A number of clinically useful drugs belong to this type of complex; these were obtained mostly from natural products (e.g., bleomycin,[3] distamycin,[4] actinomycin D[5]). Their modes of interaction with nucleic acids are now fairly well understood. These molecules interact with DNA at multi-interaction sites (shape-selective recognition), mostly through hydrogen bonding and/or $\pi - \pi$ stacking interactions. Distamycin, for example, has four carbonyl oxygens and four amide hydrogens, which in a lipophilic DNA minor groove find appropriate hydrogen bonding partners on A-T-rich oligonucleotides.[2a, 4] Although each hydrogen-bonding interaction is weak (< 2 kcal mol^{-1}), such multiple bonding contributes to the formation of stable complexes with dissociation constants of less than 10^{-6} M at physiological pH.

Great efforts have been made to synthesize artificial molecules that work on DNA in a similar fashion. Conventional designs are mostly based on organic molecules that contain a hydrophobic linker for minor groove binding, aromatic rings for stacking, and/or protonated functional groups (e.g., amidine, guanidine, and quaternary amine)[6, 7] for electrostatic interactions with anionic phosphate or DNA bases.[8]

In the course of our continuing studies on the intrinsic properties of Zn^{II} in zinc enzymes with macrocyclic polyamine complexes,[9–11] we discovered that the [Zn^{II}-(1,4,7,10-tetraazacyclododecane)] complex (**1**; Zn^{II}-cyclen), *in aqueous*

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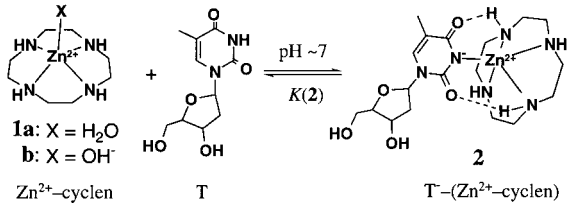
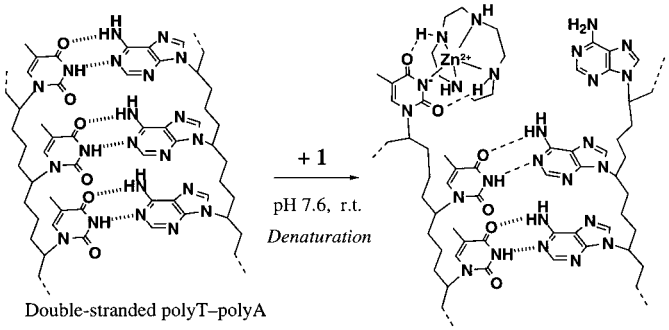
solution at physiological pH, selectively and efficiently interacts with thymidine (T), uridine (U), and their homologues that contain an "imide" functionality.[12–16] The recognition occurs through one coordination bond between Zn^{II} and the deprotonated imide N^- anion (represented for thymidine as T^- in Scheme 1)[13–16] and two complementary



Scheme 1.

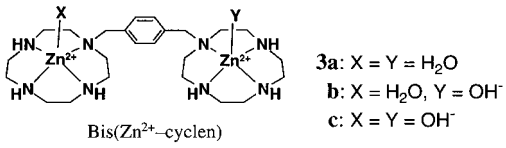
hydrogen bonds between the two imide oxygens and two NH groups of the cyclen (as established by X-ray crystal structures[14]); this yields a stable 1:1 T^--bound complex **2** with a binding constant, $\log K(\mathbf{2})$ $\{= \log([\mathbf{2}]a_{H^+}/[\mathbf{1a}][T])\}$, of -4.2, determined by potentiometric pH titration with ionic strength $I = 0.10$ ($NaNO_3$) at 25 °C.[13] The corresponding dissociation constant K_d (= [uncomplexed **1**][uncomplexed T]/[**2**]) at pH 7.4 is calculated, from the pK_a values for Zn^{II}-bound water (7.86 for $\mathbf{1a} \rightleftarrows \mathbf{1b} + H^+$)[11] and thymidine (9.76 for $T \rightleftarrows T^- + H^+$), to be 7.9×10^{-4} M.[13] Other nucleobases (A, G, and C) did not interact with **1** under the same conditions. Thus, complex **1** is a novel T(or U)-recognizing molecule. However, the dissociation constant of the order of 10^{-4} M at physiological pH may not be good enough for practical biochemical or medicinal applications.[3–5]

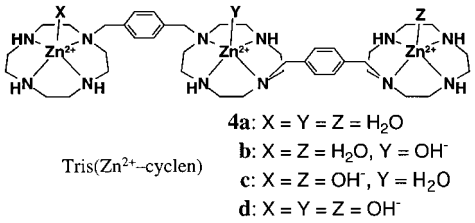
Recently we found that Zn^{II}-cyclen **1** disrupts double-stranded polyT-polyA at room temperature and pH 7.6 by invasion into the two complementary hydrogen-bonding sites in T–A pairs (see Scheme 2).[17–19] Such dissociative DNA recognition by Zn^{II}-cyclen **1** is remarkably different from the associative DNA recognition by distamycin[4] or other organic molecules.[1]

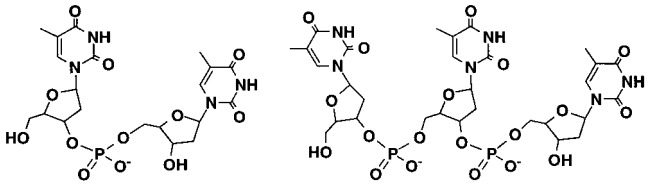


Scheme 2.

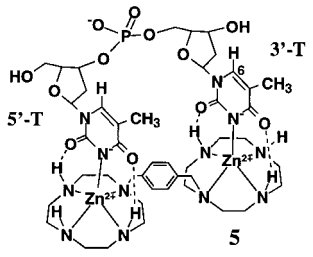
In order to develop host molecules that recognize multiple T(or U)-motif structures in DNA (or RNA), we studied a dimeric Zn^{II}-cyclen **3** {bis(Zn^{II}-cyclen)} with a *p*-xylyl spacer[20] and a trimeric Zn^{II}-cyclen **4** {tris(Zn^{II}-cyclen)} with two alternate *p*-xylyl spacers, which has been newly synthesized. It was hoped that **3** and **4** would form the stable 1:1

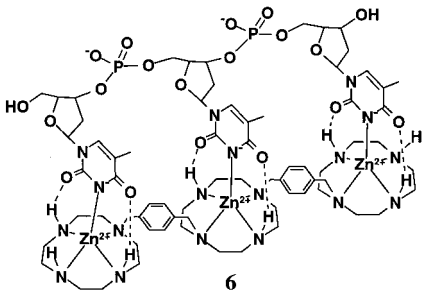






complexes **5** and **6** with thymidylylthymidine (TpT) and thymidylylthymidylylthymidine (TpTpT), respectively, at neutral pH in aqueous solution. Indeed the present qualitative and quantitative measurements have established the formation of complexes **5** and **6**, with much greater stability constants (i.e., much smaller dissociation constants) than that of complex **2**. These results set a new prototype of small molecules that bind to consecutive thymidine or uridine nucleotides at specific spots in DNA or RNA for controlling gene expression.





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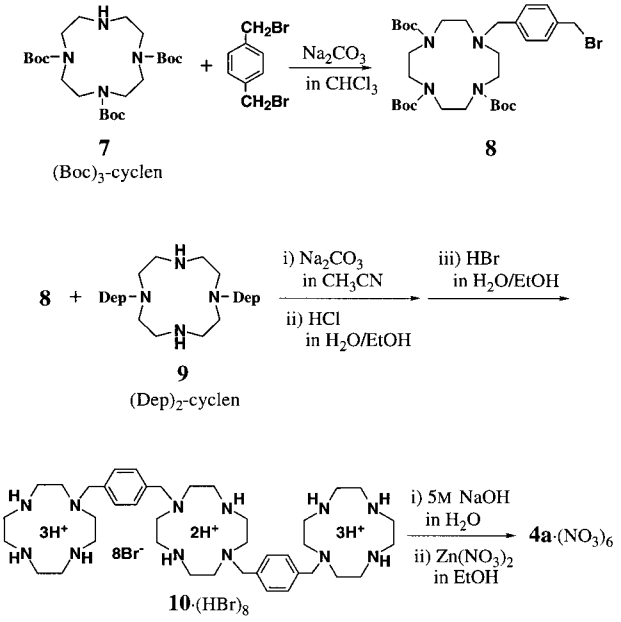
Results

Synthesis of tris(Zn^{II}-cyclen) (4): 1,4,7-Tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (**7**, $(Boc)_3$-cyclen)[21] was treated with 1,4-bis(bromomethyl)benzene in the presence of anhydrous Na_2CO_3 in $CHCl_3$ to obtain 1-bromomethyl-4-(4,7,10-tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-ylmethyl)benzene (**8**; Scheme 3). The monocyclic



Scheme 3.

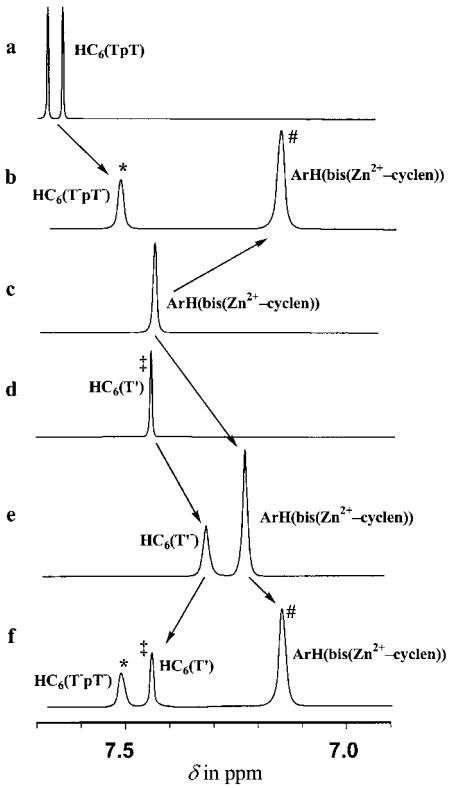


Figure 1. ^{1}H NMR spectra at pD 8.4 and 35 °C in D_2O with $I = 0.10$ ($NaNO_3$): a) 5 mM TpT; b) a + 5 mM bis(Zn^{II}-cyclen) (**3**); c) 5 mM **3**; d) 10 mM 1-methylthymine (T′); e) d + 5 mM **3** (= 5 mM **22**); f) e + 5 mM TpT.

compound **8** was treated with 1,7-bis(diethoxyphosphoryl)-1,4,7,10-tetraazacyclododecane (**9**, $(Dep)_2$-cyclen)[22] in the presence of anhydrous Na_2CO_3 in CH_3CN. The resulting product was deprotected by HCl in aqueous EtOH and then purified as the octahydrobromic acid salt of 1,7-bis(4-(1,4,7,10-tetraazacyclododecan-1-ylmethyl)benzyl)-1,4,7,10-tetraazacyclododecane (**10**). After the HBr salt had been neutralized with NaOH aqueous solution (5 M), the free ligand **10** was treated with $Zn(NO_3)_2$ in EtOH to obtain [**4a**][NO_3]$_6$

Stoichiometric interaction of bis(Zn^{II}-cyclen) (3) with thymidylylthymidine (TpT) and tris(Zn^{II}-cyclen) (4) with thymidylylthymidylylthymidine (TpTpT): In order to determine the stoichiometry of complex **5**, the complex formed from TpT and bis(Zn^{II}-cyclen) (**3**), in aqueous solution, we conducted a ^{1}H NMR titration experiment of TpT (5 mM) with varying concentrations of **3** (0–10 mM) in D_2O with $I = 0.10$ ($NaNO_3$) at 35 °C and pD 8.4 (see Figure 1). The chemical shifts of the aromatic HC(6) protons of TpT (for numbering, see structure of **5**) moved upfield from $\delta = 7.65$ and 7.68 (5′-T and 3′-T, respectively; for location of T, see structure of **5**) to $\delta = 7.51$ with peak broadening during the addition of one equivalent of **3** (see Figure 1a → b). The upfield shift is possibly due to the higher electron density of the deprotonated thymidine groups and/or the magnetic shielding effect of the phenylene group of **3**. Similar behavior was observed for a reverse titration of **3** (5 mM) with TpT (0–10 mM) under the same conditions; the chemical shifts of the aromatic protons (ArH) of complex **3** moved upfield from $\delta = 7.43$ to 7.14 (see Figure 1c → b). No further change in the chemical shifts was seen at higher concentration (> 5 mM) of the titrants; this suggests a 1:1 complexation to give **5**. Furthermore, the almost equivalent upfield shifts of the two HC(6) protons of TpT are consistent with the 1:1 structure of complex **5**, in which both of the thymine groups deprotonate to bind with Zn^{II} (see the following section on potentiometric pH titration study). In this context, it should be noted that Zn^{II}-cyclen (**1**) has a negligible interaction with the phosphodiester monoanion (each in mM concentration) in aqueous solution.[21] The ^{1}H NMR spectrum of TpTpT (5 mM) upon mixing with tris(Zn^{II}-cyclen) (**4**) in D_2O became too complex (due to peak broadening) to be analyzed.

The stoichiometric 1:1 complexation of **5** was independently established by UV-spectrophotometric titrations of TpT (0.5 mM, $\lambda_{max} = 267$ nm, $\varepsilon_d = 1.88 \times 10^4$ cm^{-1}M^{-1}, assigned to thymine group) with increasing concentrations of bis(Zn^{II}-cyclen) (**3**, 0–1.0 mM) with $I = 0.10$ ($NaNO_3$) at 25 °C and pH 7.4 (10 mM HEPES buffer; see Figure 2b). The results indicate a linear decrease in the UV absorption at 267 nm (due to gradual formation of the deprotonation of T)[13] up to the addition of equimolar amounts of **3** and a sharp break at equiv(titrant) = 1 ($\varepsilon_d' = 1.47 \times 10^4$), followed by a slight linear increase (due to the weak UV absorption of **3**, $\varepsilon = 2.5 \times 10^2$ at 267 nm). Under the same conditions, thymidine (T) and the T$^-$-bound Zn^{II}-cyclen complex **2** (prepared in situ with 1 mM T

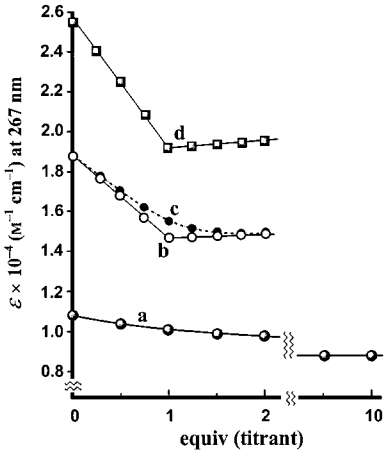


Figure 2. UV absorption changes of T, TpT, and TpTpT in the presence of cyclen derivatives at 25 °C and pH 7.4 (10 mM HEPES buffer) with $I = 0.10$ ($NaNO_3$): a) 1.0 mM T with Zn^{II}-cyclen (**1**) (0–10 mM); b) 0.50 mM TpT with bis(Zn^{II}-cyclen) (**3**) (0–1.0 mM); c) 50 μM TpT with **3** (0–0.10 mM); d) 50 μM TpTpT with tris(Zn^{II}-cyclen) (**4**) (0–0.10 mM). The ε values of the test solutions are based on the concentration of thymidine derivatives. Equiv(titrant) is the number of equivalents of titrant added.

and 10 mM **1**) exhibited molar absorption coefficients of 1.08×10^4 (ε_m) at $\lambda_{max} = 267$ nm and 8.8×10^3 $cm^{-1}M^{-1}$ (ε'_m) at 267 nm (see equiv(titrant) = 0 and 10 in Figure 2a), respectively. These ε values are almost half those of TpT in the absence and presence of equimolar amounts of **3** (Figure 2b at equiv = 0 and 1), respectively. Thus, the stoichiometric decrease in the UV absorbance of TpT upon mixing with **3** implies 1:1 complexation of **3** with doubly imido-deprotonated T^-pT^-; this lends support to the stoichiometric structure of [T^-pT^--bis(Zn^{II}-cyclen)] (**5**). The ε values per thymine base for TpT ($\varepsilon_d/2 = 0.94 \times 10^4$) and **5** ($\varepsilon'_d/2 = 0.74 \times 10^4$) are smaller than those of T and T^--(Zn^{II}-cyclen) (**2**), respectively. The hypochromic effect of **5** versus **2** (i.e., $2\varepsilon'_m/\varepsilon'_d = 1.20$) is larger than that of TpT versus T (i.e., $2\varepsilon_m/\varepsilon_d = 1.15$); this might originate from a face-to-face structure of T^-pT^- in **5**.

The 1:1 structure of complex **5** was finally proven by FAB-MS (positive mode) measurement of a 1:1 TpT/**3** mixture (30 mM) at pH 8 in aqueous solution. We observed a major peak at *m*/*z* 1119 with Zn isotopic peaks (1118, 1120 etc.) ascribed to the formation of [$(T^-pT^-)^{3-}$-$\mathbf{3}^{4+}$]$^+$, complex **5** (*m*/*z* 1120.84).

The stoichiometric 1:1 complexation of TpTpT with tris-(Zn^{II}-cyclen) (**4**) was established by a similar UV titration of TpTpT (50 μM) under the same conditions. The titration pattern shown in Figure 2d was similar to that for TpT with **3** (Figure 2b), that is, a linear decrease in the UV absorption of TpTpT ($\varepsilon_t = 2.56 \times 10^4$ $cm^{-1}M^{-1}$ at $\lambda_{max} = 267$ nm) until equiv(titrant) = 1 ($\varepsilon'_t = 1.92 \times 10^4$), followed by a slight increase (due to a weak UV absorption of **4**, $\varepsilon = 5.2 \times 10^2$ at 267 nm). This fact supports the formation of the 1:1 complex [$T^-pT^-pT^-$-tris(Zn^{II}-cyclen)] (**6**). The ε values per thymine base for TpTpT ($\varepsilon_t/3 = 0.85 \times 10^4$) and **6** ($\varepsilon'_t/3 = 0.64 \times 10^4$) are much smaller than those (ε_m and ε'_m) of T and T^--(Zn^{II}-cyclen) (**2**), respectively. In this case, too, the hypochromic effect of **6** versus **2** (i.e., $3\varepsilon'_m/\varepsilon'_t = 1.38$) is much larger than that of TpTpT versus T (i.e., $3\varepsilon_m/\varepsilon_t = 1.27$). The evidence for formula **6** was obtained by the FAB-MS data that revealed a major peak at *m*/*z* at 1763 with Zn isotopic peaks (1764, 1767 etc.) for [$(T^-pT^-pT^-)^{5-}$-$\mathbf{4}^{6+}$]$^+$, complex **6** (*m*/*z* 1762.81), prepared in situ with 1:1 TpTpT/**4** mixture (30 mM) at pH 8 in aqueous solution.

Quantitative measurement of the interaction of bis(Zn^{II}-cyclen) (3) with thymidine (T) and thymidylylthymidine (TpT): A more detailed study on the interaction of **3a** (1.0 mM) with two equivalents of thymidine (T; 2.0 mM) or equimolar amounts of dinucleotide TpT (1.0 mM) was conducted by means of potentiometric pH titrations with $I = 0.10$ ($NaNO_3$) at 25 °C in an identical manner as previously done for Zn^{II}-cyclen (**1**) with T.[13] Typical titration curves for **3a** with TpT and T are shown in Figure 3. The two imide

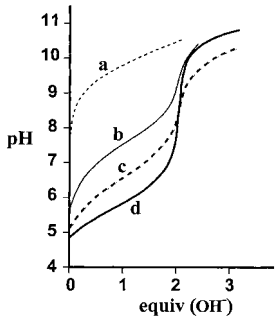


Figure 3. Potentiometric pH titration curves for TpT and **3a** at 25 °C with $I = 0.10$ ($NaNO_3$): a) 1 mM TpT; b) 1 mM **3a**; c) b + 2 mM T; d) b + 1 mM TpT. Equiv(OH^-) is the number of equivalents of base added.

deprotonation constants for dinucleotide TpT are estimated from the pH titration data (Figure 3a) to be 9.55 ± 0.02 for the first imide deprotonation (pK_1) and 10.25 ± 0.03 for the second (pK_2) [Eqs. (1) and (2)]. As before with complex **1** and T, the buffer region of the mixtures ($0 <$ equiv(OH^-) < 2), which corresponds to the deprotonation of the two Zn^{II}-bound waters in **3a** ($pK_a = 7.23$ and 7.88),[20] significantly dropped (see Figure 3c for T and Figure 3d for TpT); this implies the concomitant "two thymidyl imide" deprotonation for complex formation. The decrease in pH is more significant with TpT (1 mM) than with T (2 mM), which suggests that **3a** forms a more stable complex with one equivalent of TpT than with two equivalents of T.

$$TpT \rightleftarrows TpT^- + H^+ \qquad K_1 = [TpT^-]a_{H^+}/[TpT] \quad (1)$$

$$TpT^- \rightleftarrows T^-pT^- + H^+ \qquad K_2 = [T^-pT^-]a_{H^+}/[TpT^-] \quad (2)$$

$$\mathbf{3a} + TpT \rightleftarrows [TpT^-\text{-bis}(Zn^{II}\text{-cyclen})]\ (\mathbf{11}) + H^+ \qquad K(\mathbf{11}) = [\mathbf{11}]a_{H^+}/[\mathbf{3a}][TpT] \quad (3)$$

$$\mathbf{11} \rightleftarrows [T^-pT^-\text{-bis}(Zn^{II}\text{-cyclen})]\ (\mathbf{5}) + H^+ \qquad K(\mathbf{5}) = [\mathbf{5}]a_{H^+}/[\mathbf{11}] \quad (4)$$

The titration data (pH > 5) with equimolar amounts of **3a** and TpT (1 mM) were best treated for equilibria of [TpT^--bis(Zn^{II}-cyclen)] (**11**) [Eq. (3)] and [T^-pT^--bis(Zn^{II}-cyclen)] (**5**) [Eq. (4)], by use of the program "BEST"[23] for pH titration analysis (see Scheme 4). The obtained log K(**11**) and log K(**5**) values were -2.6 ± 0.1 and -5.5 ± 0.1, respectively.

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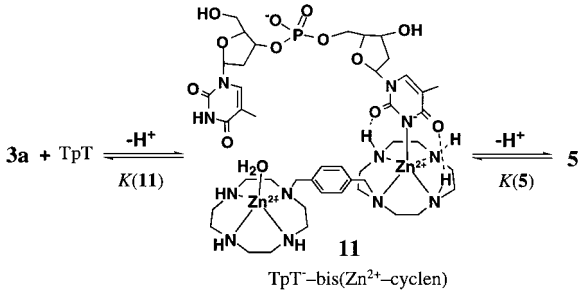


Scheme 4.

The species distribution diagram as a function of pH ($=-\log a_{H^+}$) for an aqueous solution of TpT (1 mM) in the presence of equimolar amounts of bis(Zn^{II}-cyclen) **3a** was calculated, and the result is shown in Figure 4. As expected by

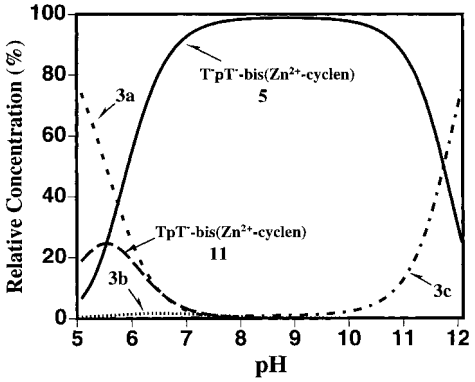


Figure 4. Species distribution that results from an aqueous solution of 1 mM **3a** in the presence of 1 mM TpT at 25 °C with $I = 0.10$ ($NaNO_3$).

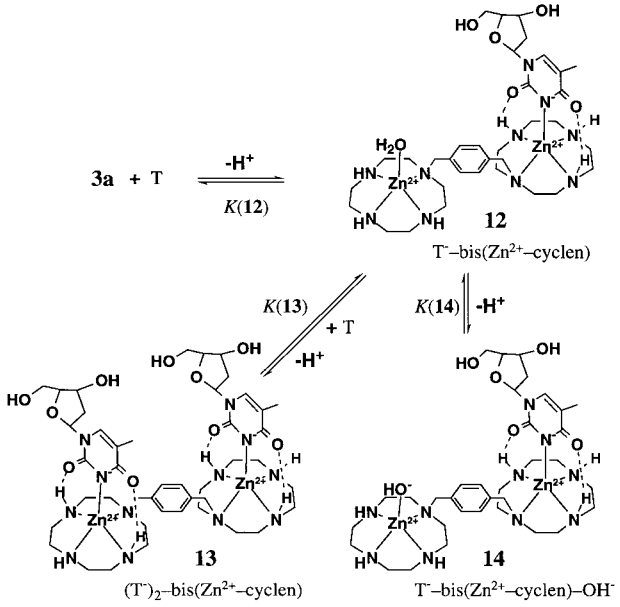
the initial ^{1}H NMR and UV titration studies, we see very little dissociation of **5** when **3a** and TpT (initially both 1 mM) are mixed at physiological pH (i.e., more than 95 % of **3a** and TpT are in the form of the 1:1 complex **5** in the pH range from 7 to 10). A dissociation constant K_d (= [uncomplexed TpT][uncomplexed **3a**]/[**5**]) at pH 7.4 and 25 °C is calculated to be 6.3×10^{-7} M. From the UV spectrophotometric titration curve (with diluted concentrations of TpT and **3a**) with $I = 0.10$ ($NaNO_3$) at pH 7.4 in HEPES buffer (Figure 2c), we could calculate a 1:1 complexation constant of $(1.0 \pm 0.2) \times 10^{-6}$ M, which is close to the value obtained by the potentiometric pH titration.

Complexation equilibria for a mixture of **3a** (1 mM) and T (2 mM) with $I = 0.10$ ($NaNO_3$) at 25 °C were determined from Figure 1c, see Scheme 5. The complexation constants for 1:1 and 2:1 thymidine complexes (**12** and **13**) and deprotonation constant for Zn^{II}-bound H_2O of **12** (to give **14**) are defined by Equations (5)–(7). The obtained $\log K(\mathbf{12})$, $\log K(\mathbf{13})$, and $\log K(\mathbf{14})$ values are -3.7 ± 0.1, -3.8 ± 0.1, and -7.5 ± 0.1, respectively. The complexation constants $K(\mathbf{12})$ and $K(\mathbf{13})$ are

$$\mathbf{3a} + \mathrm{T} \rightleftarrows [\mathrm{T^-}\text{-bis}(\mathrm{Zn^{II}}\text{-cyclen})]\ (\mathbf{12}) + \mathrm{H^+} \qquad K(\mathbf{12}) = [\mathbf{12}]a_{\mathrm{H^+}}/[\mathbf{3a}][\mathrm{T}] \qquad (5)$$

$$\mathbf{12} + \mathrm{T} \rightleftarrows [(\mathrm{T^-})_2\text{-bis}(\mathrm{Zn^{II}}\text{-cyclen})]\ (\mathbf{13}) + \mathrm{H^+} \qquad K(\mathbf{13}) = [\mathbf{13}]a_{\mathrm{H^+}}/[\mathbf{12}][\mathrm{T}] \qquad (6)$$

$$\mathbf{12} \rightleftarrows [\mathrm{T^-}\text{-bis}(\mathrm{Zn^{II}}\text{-cyclen})\text{-}\mathrm{OH^-}]\ (\mathbf{14}) + \mathrm{H^+} \qquad K(\mathbf{14}) = [\mathbf{14}]a_{\mathrm{H^+}}/[\mathbf{12}] \qquad (7)$$



Scheme 5.

almost the same, which indicates weak a interaction between the the two thymine anions in the 2:1 complex **13**. The species distribution diagram at varying pH is shown in Figure 5. In contrast to the above case with TpT, we see only about 50 % complexation to give $(T^-)_2$-bis(Zn^{II}-cyclen) (**13**) at pH 7.4 when **3a** (1 mM) and T (2 mM) are mixed.

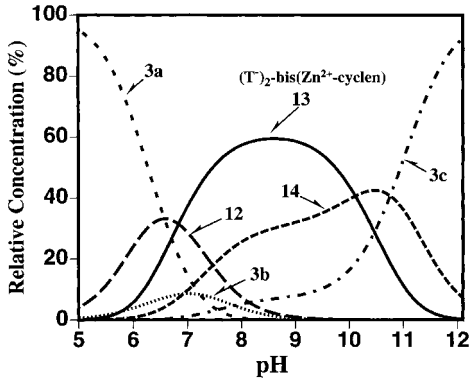


Figure 5. Species distribution that results from an aqueous solution of 1 mM **3a** in the presence of 2 mM T at 25 °C with $I = 0.10$ ($NaNO_3$).

Interaction of Zn^{II}-benzylcyclen (15) with thymidine (T) and thymidylylthymidine (TpT): In order to obtain reference values, potentiometric pH titrations of Zn^{II}-(1-benzyl-1,4,7,10-tetraazacyclododecane) (**15**, Zn^{II}-benzylcyclen)[24] in the presence of T (in 1:1 molar ratio) or TpT (in 2:1 molar ratio) were conducted under the same conditions (see Figure 6). A similar extent of the decrease in the buffer pH (Figure 6c) implies that two molecules of Zn^{II}-benzylcyclen (**15**) independently interact with each T part of TpT, like the interaction of two T with bis(Zn^{II}-cyclen) **3a** (cf. Figure 3c). From a comparison of the decrease in pH for imide deprotonation in Figures 3d and 6c, it is intuitively understood that TpT forms a stronger complex with **3a** than with two equivalents of **15**. Complexation equilibria for **15** in the presence of T and TpT (see

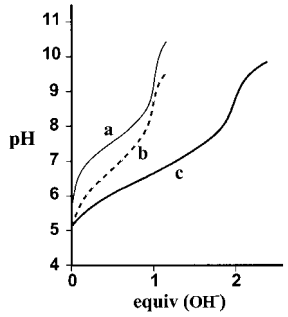
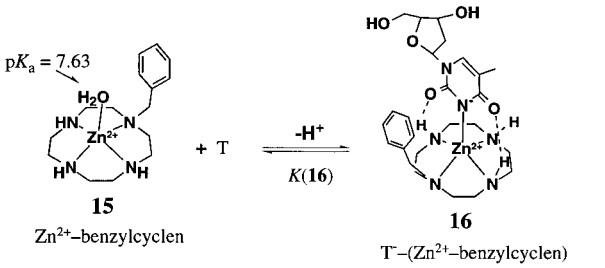
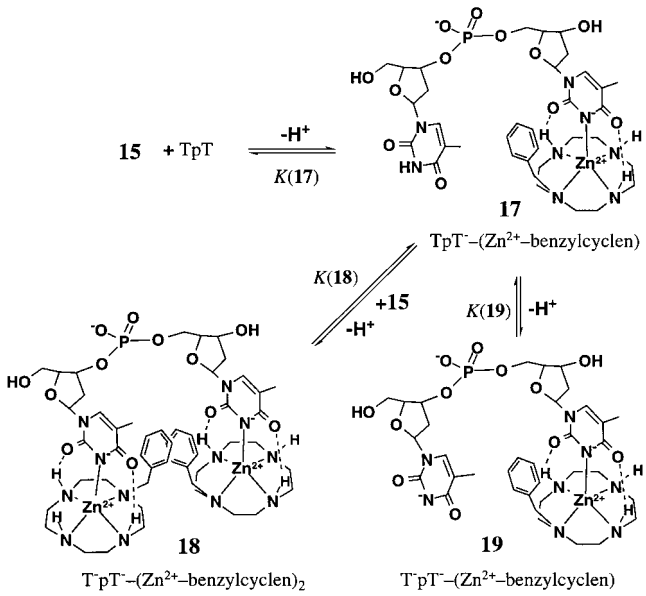


Figure 6. Potentiometric pH titration curves for Zn^{II}-benzylcyclen (**15**) at 25 °C with $I = 0.10$ ($NaNO_3$): a) 1 mM **15**; b) 1 mM **15** + 1.0 mM T; c) 2 mM **15** + 1.0 mM TpT. Equiv(OH^-) is the number of equivalents of base added.

Schemes 6 and 7, respectively) were determined from Figure 6b and c. The complexation constants for [T^--(Zn^{II}-benzylcyclen)] (**16**), [TpT^--(Zn^{II}-benzylcyclen)] (**17**), [T^-pT^--(Zn^{II}-benzylcyclen)$_2$] (**18**), and [T^-pT^--(Zn^{II}-benzylcyclen)] (**19**) are defined by Equations (8) – (11). The obtained log K(**16**), log K(**17**), log K(**18**), and log K(**19**) values are -3.9 ± 0.1, -3.6 ± 0.1, -3.9 ± 0.1, and -10.4 ± 0.1, respectively. The 1:1 complexation constant log K(**16**) is almost the same as those for complexes **2** [log K(**2**) = −4.2][13] and **12** [log K(**12**) = −3.7]. In this case the relative concentration of the T^-pT^- complex with two Zn^{II}-benzylcyclen molecules (**18**) in a mixture of TpT (1 mM) and **15** (2 mM) is only 52% at pH 7.4.



Scheme 6.



Scheme 7.

$$\mathbf{15} + T \rightleftarrows [T^-\text{-}(Zn^{II}\text{-benzylcyclen})]\ (\mathbf{16}) + H^+ \qquad K(\mathbf{16}) = [\mathbf{16}]a_{H^+}/[\mathbf{15}][T] \quad (8)$$

$$\mathbf{15} + TpT \rightleftarrows [TpT^-\text{-}(Zn^{II}\text{-benzylcyclen})]\ (\mathbf{17}) + H^+ \qquad K(\mathbf{17}) = [\mathbf{17}]a_{H^+}/[\mathbf{15}][TpT] \quad (9)$$

$$\mathbf{15} + \mathbf{17} \rightleftarrows [T^-pT^-\text{-}(Zn^{II}\text{-benzylcyclen})_2]\ (\mathbf{18}) + H^+ \qquad K(\mathbf{18}) = [\mathbf{18}]a_{H^+}/[\mathbf{15}][\mathbf{17}] \quad (10)$$

$$\mathbf{17} \rightleftarrows [T^-pT^-\text{-}(Zn^{II}\text{-benzylcyclen})]\ (\mathbf{19}) + H^+ \qquad K(\mathbf{19}) = [\mathbf{19}]a_{H^+}/[\mathbf{17}] \quad (11)$$

Quantitative measurement of interaction of tris(Zn^{II}-cyclen) (4) with tymidylylthymidylylthymidine (TpTpT): Since the stoichiometric 1:1 complexation has been established by the UV spectrophotometric titration (Figure 2d) and FAB-MS data, the interaction of tris(Zn^{II}-cyclen) (**4**; 1.0 mM) with thymidylylthymidylylthymidine (TpTpT; 1.0 mM) was investigated in more detail by the potentiometric pH titrations with $I = 0.10$ ($NaNO_3$) at 25 °C. Typical titration curves for TpTpT and **4** are shown in Figure 7a and b, respectively, both of which

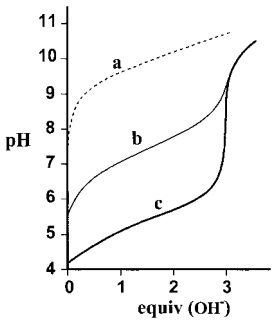


Figure 7. Potentiometric pH titration curves for TpTpT and tris(Zn^{II}-cyclen) (**4a**) at 25 °C with $I = 0.10$ ($NaNO_3$): a) 1 mM TpTpT; b) 1 mM **4a**; c) b + 1 mM TpTpT. Equiv(OH^-) is the number of equivalents of base added.

show dissociation of three protons at $0 < \text{equiv}(OH^-) < 3$. The deprotonation constants ($= -\log([A^-]a_{H^+}/[HA])$ are estimated to be 6.98 ± 0.02 for **4a** ⇄ **4b** + H^+, 7.41 ± 0.02 for **4b** ⇄ **4c** + H^+, 8.02 ± 0.02 for **4c** ⇄ **4d** + H^+, 9.37 ± 0.05 for TpTpT ⇄ $TpTpT^-$ + H^+, 10.10 ± 0.05 for $TpTpT^-$ ⇄ TpT^-pT^- + H^+, and 10.31 ± 0.05 for TpT^-pT^- ⇄ $T^-pT^-pT^-$ + H^+.

Next, tris(Zn^{II}-cyclen) **4a** (1.0 mM) was titrated under the same conditions (Figure 7c), but in the presence of equimolar amounts of TpTpT. We saw a great fall of the buffer pH region (cf. Figure 3c and d) with a break at equiv(OH^-) = 3; this indicates extremely facile deprotonation of "three thymidyl imides" with concomitant complexation. Since **4** is stable (i.e., negligible Zn^{II} dissociation) at mM concentration at pH 5 in aqueous solution (see Experimental Section), the analysis was applied to the titration data at pH > 5. These data were fitted to three complexation equilibria [see Scheme 8 and Eqs (12) – (14)] that did not include such equilibria as formation of Zn^{II}-bound OH^- species found for the T/bis(Zn^{II}-cyclen) system. Furthermore, no more deprotonation (e.g., dissociation of **6** to **4d** + $T^-pT^-pT^-$ + 3H^+) was ob-

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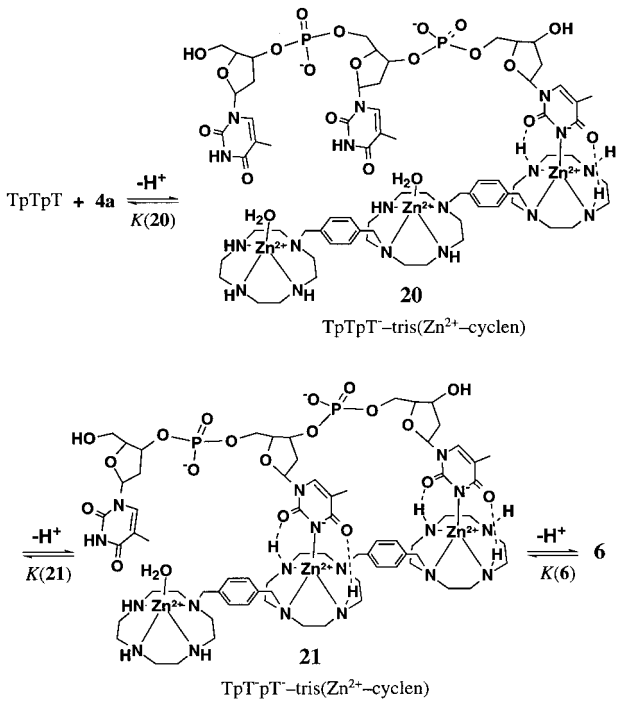


Scheme 8.

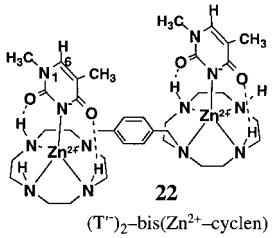
served until pH 12, which indicates an extremely high affinity of $T^-pT^-pT^-$ for **4a**. The obtained complexation constants $\log K$(**20**), $\log K$(**21**), and $\log K$(**6**) are -0.6 ± 0.1, -5.5 ± 0.1, and -6.4 ± 0.1, respectively. From the complexation and deprotonation constants for **4a** and TpTpT, the dissociation constant K_d (= [uncomplexed TpTpT][uncomplexed **4a**]/[**6**]) at pH 7.4 and 25 °C is estimated to be extremely small with a value of 8.0×10^{-10} M.

$$\mathbf{4a} + \mathrm{TpTpT} \rightleftarrows [\mathrm{TpTpT^-}\text{-tris}(\mathrm{Zn^{II}}\text{-cyclen})]\ (\mathbf{20}) + \mathrm{H^+} \qquad (12)$$
$$K(\mathbf{20}) = [\mathbf{20}]a_{\mathrm{H^+}}/[\mathbf{4a}][\mathrm{TpTpT}]$$

$$\mathbf{20} \rightleftarrows [\mathrm{TpT^-pT^-}\text{-tris}(\mathrm{Zn^{II}}\text{-cyclen})]\ (\mathbf{21}) + \mathrm{H^+} \quad K(\mathbf{21}) = [\mathbf{21}]a_{\mathrm{H^+}}/[\mathbf{20}] \qquad (13)$$

$$\mathbf{21} \rightleftarrows [\mathrm{T^-pT^-pT^-}\text{-tris}(\mathrm{Zn^{II}}\text{-cyclen})]\ (\mathbf{6}) + \mathrm{H^+} \quad K(\mathbf{6}) = [\mathbf{6}]a_{\mathrm{H^+}}/[\mathbf{21}] \qquad (14)$$

Replacement reaction of 1-methylthymine (T′) with thymidylylthymidine (TpT) on bis(Zn^{II}-cyclen) (3a): All the attempts to isolate the bis(Zn^{II}-cyclen) complexes with T or TpT failed, but one homologous complex with 1-methylthymine (T′, $pK_a = 10.04\pm0.05$ at 25 °C) was isolated as its diperchlorate salt, $[(T'^-)_2$-**3a**$][ClO_4]_2$ (**22**). The potentiometric pH titration of **3a** (1.0 mM) with T′ (2.0 mM) was conducted at 25 °C and the result was analogous to that with T. The 1:1 and



1:2 complexation constants {$\log([T'^-$-**3a**$]a_{H^+}/[T'][$**3a**$])$ and $\log([$**22**$]a_{H^+}/[T'][T'^-$-**3a**$])$} have almost the same value, -3.9 ± 0.1 and -3.8 ± 0.1, respectively; these in turn are almost the same as those found for complexes formed with T ($\log K$(**12**) = −3.7 and $\log K$(**13**) = −3.8). In this case, the Zn^{II}-cyclen units of **3a** act as an independent recognition site for the thymine imido anion.

Comparison of proton NMR spectra of 1-methylthymine T′ (Figure 1d), the complex **22** (Figure 1e), and complex **3** (Figure 1c) in D_2O with $I = 0.10$ ($NaNO_3$) at pD 8.4 and 35 °C showed significant upfield shifts of the HC(6) protons of T′ and the aromatic protons (ArH) of **3** upon complexation to give **22**: shift in δ from 7.46 to 7.31 for HC(6) and from 7.43 to 7.23 for ArH. The aromatic NMR signals of T′ (10 mM) gradually moved upfield as complex **3** (final concentration of 5 mM) was added, which is similar to the case for complex **5** (Figure 1a and b).

Next, we examined, by ^{1}H NMR spectroscopy, the displacement of T′ by TpT when **22** was mixed with equimolar amounts of TpT in D_2O solution (see Figure 1e and f) with $I = 0.10$ ($NaNO_3$) at 35 °C and pD = 8.4. We found the prompt and complete replacement of the two 1-methylthymine anions (T'^-) by TpT (i.e., **22** + TpT ⇄ **5** + 2 T′), which supports **5** being overwhelmingly more stable than **22**, a conclusion derived from the potentiometric pH titrations. The NMR peak assignment of the mixture was conducted with the reference spectra for [T^-pT^--bis(Zn^{II}-cyclen)] (**5**, 5 mM) and free 1-methylthymine (T′; Figure 1b and d).

Isothermal titration calorimetric study of [T^--(Zn^{II}-benzylcyclen)] (16), [T^-pT^--bis(Zn^{II}-cyclen)] (5), other deoxydinucleotides-bis(Zn^{II}-cyclen) complexes, and [$T^-pT^-pT^-$-tris(Zn^{II}-cyclen)] (6): To demonstrate the selective TpT recognition by bis(Zn^{II}-cyclen) (**3**), 2′-deoxyguanylylthymidine (GpT), 2′-deoxycytidylylthymidine (CpT), and 2′-deoxyadenylylthymidine (ApT) were also investigated by isothermal calorimetric titrations[25] with $I = 0.10$ ($NaNO_3$) at 25 °C in 10 mM HEPES buffer solution (pH 7.4). For a check, the cumulative heat (mJ) of the complexation reaction was plotted for titration of **3** (0.25 mM) with TpT (10 mM; see Figure 8a). The result indicated a linear increase

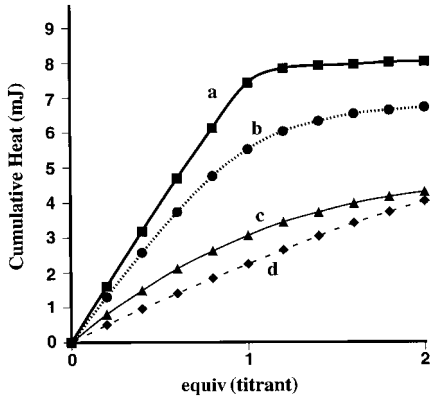


Figure 8. Isothermal calorimetric titration curves for bis(Zn^{II}-cyclen) **3** (0.25 mM, 1 mL) at 25 °C and pH 7.4 (10 mM HEPES buffer) with $I = 0.10$ ($NaNO_3$): a) with 10 mM TpT; b) with 10 mM GpT; c) with 10 mM CpT; d) with 10 mM ApT. Equiv(titrant) is the number of equivalents of titrant added.

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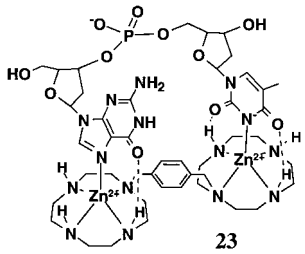
(i.e., exothermic, see Experimental Section) until ca. 0.8 equivalents of TpT had been added and a gentle curve near equimolar mixture that was followed by a plateau with additional TpT; this suggests that the 1:1 complexation of [T⁻pT⁻-bis(Zn^{II}-cyclen)] (**5**) is almost finished at the concentration level of 0.25 mM TpT. The computation of the titration data gave a dissociation constant of $(1.0 \pm 0.1) \times 10^{-6}$ M (= [uncomplexed TpT][uncomplexed **3**]/[**5**] at pH 7.4 (in HEPES buffer), which is a good match with the K_d value of 6.3×10^{-7} M, determined above by potentiometric pH and UV titrations under the same conditions.

Less-sharp titration curves (Figure 8b–d) were obtained with GpT, CpT, and ApT under the same conditions, which implies weaker interactions of these with bis(Zn^{II}-cyclen) (**3**). The dissociation constant K_d for GpT (= [uncomplexed GpT][uncomplexed **3**]/[1:1 complex]) was estimated to be $(1.3 \pm 0.1) \times 10^{-5}$ M. The structure **23** is proposed for the 1:1 complex; this is based on the reported G and T⁻ binding



23

GpT⁻–bis(Zn^{2+}–cyclen)

modes with 1-[(9-acridinyl)methyl]-1,4,7,10-tetraazacyclododecane Zn^{II} complex at physiological pH[14] and the extremely small interaction of Zn^{II}-cyclen with phosphodiester linkage of polyT[17, 19] and phosphodiester anion (e.g., bis(butyl) phosphate).[21] The stoichiometric 1:1 complexation (to give **23**) was independently established by UV titration of GpT (0.50 mM, $\varepsilon = 1.83 \times 10^4$ M^{-1}cm^{-1} at 267 nm) with complex **3** (0–1.00 mM). The UV titration curve was similar to that for [T⁻pT⁻-bis(Zn^{II}-cyclen)] (**5**, see Figure 2c) and gave an identical value of $(1.3 \pm 0.2) \times 10^{-5}$ M. The decrease in UV absorbance (at 267 nm, $\Delta\varepsilon = 1.4 \times 10^3$) on going from GpT to **23** ($\varepsilon = 1.69 \times 10^4$) was smaller than that on going from TpT to **5** ($\Delta\varepsilon = 4.1 \times 10^3$). The much larger dissociation constants for the 1:1 complexations ($> 10^{-4}$ M) were considered for other deoxydinucleotides CpT and ApT from the lesser complexation heats. However, the computation failed to give accurate K_d values that fit to the experimental curves, possibly due to multiple (1:1, 1:2, etc.) complexation modes of **3** with these deoxydinucleotides.

The earlier potentiometric pH titration study established the much stronger interaction ($K_d = 8.0 \times 10^{-10}$ M) of tris(Zn^{II}-cyclen) (**4**) with TpTpT. We checked this by the isothermal titration technique with a much lower concentration of **4** (50 μM) with TpTpT titrant (2.5 mM), whereby the heat generated was sufficient to permit studying the exothermic complexation reaction (see Experimental Section). The results showed a linear increase of cumulative heat up to the addition of an equimolar amount of TpTpT and a sharp break at [**4**] = [TpTpT], followed by a plateau at additional TpTpT. This fact implies that the 1:1 complexation is stoichiometric under these conditions and its apparent dissociation constant should be less than 10^{-7} M, which supports the result from the pH titration study.

Discussion

From this study, bis(Zn^{II}-cyclen) (**3a**) and tris(Zn^{II}-cyclen) (**4a**) were found to be excellent host molecules for TpT and TpTpT, respectively. Although the final products [T⁻pT⁻-bis(Zn^{II}-cyclen)] (**5**) and [T⁻pT⁻pT⁻-tris(Zn^{II}-cyclen)] (**6**) could not be isolated, the stoichiometric formation of **5** and **6** in aqueous solution, in analogous fashions to the well established [T⁻-(Zn^{II}-cyclen)] (**2**),[12–19] is now evident by combined studies of the UV spectrophotometric, potentiometric pH, ^{1}H NMR, and isothermal calorimetric titrations, and FAB-MS measurements.

The 1:1 affinities may most simply be compared in terms of the dissociation constants K_d values at a common pH of 7.4; the order of affinity is [T⁻pT⁻pT⁻-tris(Zn^{II}-cyclen)] (**6**) (K_d = 0.80 nM) > [T⁻pT⁻-bis(Zn^{II}-cyclen)] (**5**) (K_d = 0.63 μM) ≫ [T⁻-(Zn^{II}-benzylcyclen)] (**16**) (K_d = 0.50 mM) ≅ [T⁻-(Zn^{II}-cyclen)] (**2**) (K_d = 0.79 mM).

Bis(Zn^{II}-cyclen) (**3**) binds with two thymidines with almost the same complexation constant ($\log K$(**12**) = −3.7 and $\log K$(**13**) = −3.8) as those for Zn^{II}-cyclen (**1**) ($\log K$(**2**) = −4.2) and Zn^{II}-benzylcyclen (**15**) ($\log K$(**16**) = −3.9). In a similar way, TpT forms complexes with one or two molecules Zn^{II}-benzylcyclen with similar complexation constants ($\log K$(**17**) = −3.6 and $\log K$(**18**) = −3.9). These facts indicate that each Zn^{II}-cyclen unit independently acts as a binding site for a thymine base with almost the same affinity (i.e., each K_d value is ca. 10^{-3} M at pH 7.4). The greater stability (ca. 10^3 times) for **5** than for [(T⁻)$_2$-bis(Zn^{II}-cyclen)] (**13**) or for [T⁻pT⁻-(Zn^{II}-benzylcyclen)$_2$] (**18**) may remind us of a "chelate effect". A further 10^3 times greater "chelate effect" might then be anticipated, as was the case for complex **6** with respect to complex **5**.

The minimum energy calculation (MM2) gives the likely structures for **5** and **6** in Figure 9. It is of interest that in the structures **5** and **6**, the distance between the two T planes of TpT is about 10 Å, which is extremely elongated from the usual distance (ca. 3.5 Å) between two adjacent nucleobases in double-helical DNA. This fact, along with the much stronger affinity of **3a** to TpT than that of **1a** to T, well explains our previous finding that bis(Zn^{II}-cyclen) **3** far more effectively disrupts polyA-polyT double strand than **1**.[19]

From the isothermal calorimetric titrations, interaction between **3** and other deoxydinucleotides were determined. The dissociation constants for the GpT complex **23** (K_d = 1.3×10^{-5} M), CpT complex ($K_d > 10^{-4}$ M), and ApT complex ($K_d > 10^{-4}$ M) are significantly larger than that ($K_d = 1.0 \times 10^{-6}$ M) for TpT complex **5** at pH 7.4 in HEPES buffer. Thus, complex **3** virtually recognizes only TpT, but not GpT, CpT, and ApT at the concentration level of μM order. The K_d value of 1.3×10^{-5} M for GpT complex is smaller than that (7.9×10^{-4} M) for complex **2**, which suggests an additional interaction between G and the Zn^{II}-cyclen unit, as depicted in **23**.

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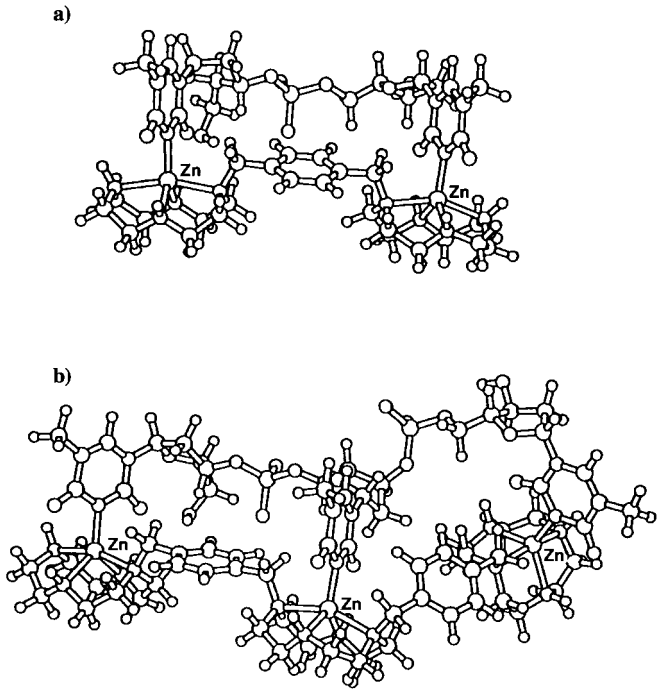

Figure 9. Minimum-energy structures: a) [T⁻pT⁻-bis(Zn^{II}-cyclen)] (**5**); b) [T⁻pT⁻pT⁻-tris(Zn^{II}-cyclen)] (**6**).

In conclusion, we have demonstrated that bis(Zn^{II}-cyclen) (**3**) and tris(Zn^{II}-cyclen) (**4**) are new types of sequence-selective ligands, which are extremely efficient in binding to TpT and TpTpT, respectively, in aqueous solution at physiological pH. The recognition occurs at the concentrations of μM and nM order, respectively, in pH 7.4 aqueous solution. These Zn^{II}-polymacrocyclic complexes and their modifications would find wide biochemical and medicinal applications by a mechanism entirely different from those of conventional DNA-motif recognizing drugs.[27]

Experimental Section

General information: All reagents and solvents used were of analytical reagent grade (purity > 99%) and used without further purification. All aqueous solution were prepared with deionized and distilled water. Anhydrous acetonitrile (CH_3CN) was distilled from calcium hydride. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JEOL JNM LA-500 spectrometer at 35 °C. 3-(Trimethylsilyl)propionic-2,2,3,3-[D_4] acid sodium salt in D_2O and tetramethylsilane in $CDCl_3$ and [D_6]DMSO were used as internal references for NMR measurements. The pD values in D_2O were corrected for a deuterium isotope effect using pD = [pH meter reading] + 0.40. Elemental analysis was performed on a Perkin Elmer CHN Analyzer 2400. Fast atom bombardment mass spectra (FAB-MS) were recorded in the positive ion mode with xenon primary atom beam in conjunction with glycerol matrix on a JEOL JMS-SX102 mass spectrometer. Thin-layer (TLC) and column chromatographies were performed with Merk Art. 5554 (silica gel) TLC plates and Fuji Silysia Chemical FL-100D (silica gel), respectively. Thymidylylthymidine (TpT, $C_{20}H_{30}N_4O_{14}PNa \cdot 0.16\,Et_2O$), 2′-deoxyguanylylthymidine (GpT, $C_{20}H_{29}N_7O_{13}PNa \cdot 0.13\,Et_2O$), 2-deoxyadenylylthymidine (ApT, $C_{20}H_{29}N_7O_{12}PNa \cdot 0.18\,Et_2O$), 2-deoxycytidylylthymidine (CpT, $C_{19}H_{29}N_4O_{13}PNa \cdot 0.08\,Et_2O$), and thymidylylthymidylylthymidine (TpTpT) were prepared as their sodium salts by reported methods,[26] and were characterized by elemental analysis (CHN, within ± 0.3%) and ^1H NMR spectrsocopy. A Good's buffer HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulufonic acid) was commercially available from Dojindo and used without further purification.

Synthesis of 1-bromomethyl-4-(4,7,10-tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-ylmethyl)benzene (8): A solution of $(Boc)_3$-cyclen[21] **7** (3.2 g, 6.8 mmol) in $CHCl_3$ (150 mL) was added dropwise to a mixture of 1,4-bis(bromomethyl)benzene (3.6 g, 14 mmol) and Na_2CO_3 (1.0 g, 9.4 mmol) in $CHCl_3$ (120 mL) at 50 °C. The reaction mixture was stirred at 60 °C for 3 days. After inorganic salts had been removed by filtration, the solvent was evaporated. The residue was purified by silica gel column chromatography (eluent: AcOEt/*n*-hexane 1:5). After evaporation of the solvent, the residue was crystallized from *n*-hexane to obtain **8** as colorless prisms (3.0 g, 67% yield). IR (KBr pellet): $\tilde{\nu} = 3489, 3053, 1690, 1462, 1404, 1366, 1277, 1250, 1155, 1124, 1096, 1049, 864, 774, 594\ cm^{-1}$; ^1H NMR ($CDCl_3$): $\delta = 1.42$ (br, 18H; CH_3), 1.48 (br, 9H; CH_3), 2.65 (br, 4H; NCH_2), 3.26–3.71 (m, 28H; NCH_2, $ArCH_2N$), 4.46 (s, 2H; $ArCH_2Br$), 7.22–7.33 (m, 4H; ArH); ^{13}C NMR ($CDCl_3$): $\delta = 28.51, 28.71, 47.59, 47.99, 49.913, 54.89, 55.80, 57.10, 79.42, 79.55, 128.92, 130.58, 136.85, 137.45, 155.48, 155.78, 156.15$.

Synthesis of 1,7-bis(4-(1,4,7,10-tetraazacyclododecan-1-ylmethyl)benzyl)-1,4,7,10-tetraazacyclododecane octahydrobromic acid salt (10·8HBr): A mixture of **8** (2.6 g, 4.0 mmol), Na_2CO_3 (0.43 g, 4.1 mmol), and 1,7-bis(diethoxyphosphoryl)-1,4,7,10-tetraazacyclododecane[22] **9** (0.73 g, 1.6 mmol) in anhydrous CH_3CN (120 mL) was stirred at 80 °C for 2 days. After inorganic salts had been removed by filtration, the solvent was evaporated. The residue was purified by silica gel column chromatography (eluent: AcOEt). After evaporation of the solvent, the residue was dissolved in EtOH (10 mL). Aqueous HCl (36%, 10 mL) was added slowly to the EtOH solution. The reaction mixture was stirred at 50 °C for 12 h. After the solvent had been evaporated, the residue was dissolved in H_2O (25 mL) and the solution pH was adjusted to 12 with NaOH aqueous solution (5 M). The alkaline solution was extracted with $CHCl_3$ (50 mL × 10). The organic layer was dried over anhydrous Na_2SO_4, and then the solvent was evaporated. The residue was crystallized from 48% aqueous HBr/EtOH to obtain $\mathbf{10} \cdot 8HBr \cdot 4H_2O$ as colorless prisms (1.5 g, 65% yield). IR (KBr pellet): $\tilde{\nu} = 2830, 1619, 1446, 1396, 1362, 1082, 1005, 968, 764\ cm^{-1}$; ^1H NMR (D_2O): $\delta = 2.94–3.28$ (m, 48H; NCH_2), 3.94 (s, 4H; $ArCH_2$), 4.00 (s, 4H; $ArCH_2$), 7.46–7.51 (m, 8H; ArH); ^{13}C NMR (D_2O): $\delta = 44.92, 45.00, 45.48, 47.14, 50.16, 50.95, 59.02, 59.38, 133.52, 137.11, 137.87$; $C_{40}H_{88}N_{12}O_4Br_8$ (1440.4): calcd C 33.4, H 6.2, N 11.7; found C 33.4, H 6.4, N 11.8.

Synthesis of 1,7-bis(4-(1,4,7,10-tetraazacyclododecan-1-ylmethyl)benzyl)-1,4,7,10-tetraazacyclododecane tris(zinc(II)) complex ([4a][NO_3]$_6$): The pH of a solution of $\mathbf{10} \cdot (HBr)_8 \cdot 4H_2O$ (1.0 g, 0.69 mmol) in H_2O (25 mL) was adjusted to 12 with NaOH aqueous solution (5 M). The alkaline solution was extracted with $CHCl_3$ (50 mL × 10). After the organic layer had been dried over anhydrous Na_2SO_4, the solvent was evaporated. $Zn(NO_3)_2 \cdot 6H_2O$ (0.70 g, 2.4 mmol) was added slowly to a solution of the residue in EtOH (100 mL) at 60 °C. After evaporation of the solvent, the residue was crystallized from H_2O/MeOH to obtain [**4a**][NO_3]$_6$ as colorless prisms (0.68 g, 73% yield). IR (KBr pellet): $\tilde{\nu} = 3489, 3225, 2880, 1638, 1385, 1092, 984, 826\ cm^{-1}$; ^1H NMR (D_2O): $\delta = 2.71–3.02$ (m, 40H; NCH_2), 3.26 (br, 8H; NCH_2), 4.02–4.05 (br, 11H; NH, $ArCH_2$), 4.27 (br, 2H; NH) 7.45 (br, 8H; ArH); ^{13}C NMR (D_2O): $\delta = 44.96, 45.07, 45.75, 46.51, 47.32, 47.44, 51.84, 51.99, 58.17, 58.32, 134.18, 134.75, 134.79$; $C_{40}H_{78}N_{18}O_{21}Zn_3$ (1343.3): calcd C 35.8, H 5.9, N 18.8; found C 35.8, H 6.0, N, 18.8. The stability of **4** in mM concentration was determined by ^1H NMR spectra of **4a** (1 mM) in 10% (v/v) D_2O/H_2O at pH 5. Only one species was detected and the solution pH remained unchanged within 0.05 of a pH unit after 2 h under an argon atmosphere.

Synthesis of 1,4-bis(1,4,7,10-tetraazacyclododecane-1-ylmethyl)benzene bis(zinc(II)) bis(1-methylthymine$^-$) complex ([22][ClO_4]$_2 \cdot 4H_2O$): A solution of 1-methylthymine (13.4 mg, 0.10 mmol) in H_2O (5 mL) and an NaOH aqueous solution (0.1 M, 0.1 mL) were added to a solution of [**3**][ClO_4]$_4 \cdot 2H_2O$[20] (50 mg, 0.05 mmol) in H_2O (10 mL). The reaction mixture was stirred at room temperature for 10 min. After evaporation of the solvent, the residue was crystallized from H_2O to obtain [**22**][ClO_4]$_2 \cdot 4H_2O$ (41 mg, 76% yield) as colorless needles. IR (KBr pellet): $\tilde{\nu} = 3295, 2932, 1658, 1638, 1574, 1551, 1474, 1447, 1634, 1335, 1144, 1092, 972, 941, 858, 783, 625, 467\ cm^{-1}$; ^1H NMR (D_2O): $\delta = 1.82$ (s, 6H; CH_3), 2.75–3.01 (m, 28H; NCH_2), 3.20–3.30 (m, 12H; NH, NCH_2, NCH_3), 3.69 (br, 4H; $ArCH_2$), 3.83 (br, 4H; NH), 7.23 (s, 4H; ArH), 7.32 (s, 2H;

HC(6)); ^{13}C NMR ([D$_6$]DMSO): $\delta = 12.65, 35.39, 42.09, 43.62, 44.34, 48.77, 54.36, 108.28, 130.81, 132.19, 141.39, 157.36, 171.79$; $C_{36}H_{68}N_{12}O_{16}Cl_2Zn_2$ (1126.7): calcd C 38.4, H 6.1, N 14.9; found C 38.6, H 6.1, N 15.1.

Potentiometric pH titrations: The electrode system (Orion Research Expandable Ion Analyzer EA920 with a Ross Combination pH Electrode 8102 BN) was calibrated daily as follows: An aqueous solution (50 mL) containing HCl (4.00 mM) and $NaNO_3$ (96.0 mM; i.e., $I = 0.10$) was prepared under an argon (> 99.999% purity) atmosphere at $25.0 \pm 0.1\,^{\circ}C$ and then first pH value (pH_1) was read. After NaOH (4.00 mL, 0.100 M) was added to acidic solution, the second pH value (pH_2) was read. The theoretical pH values corresponding to pH_1 and pH_2 were calculated: $pH_1' = 2.481$ and $pH_2' = 11.447$, with K_w ($= a_{H^+}a_{OH^-}$) $= 10^{-14.00}$, K_w' ($= [H^+][OH^-]$) $= 10^{-13.79}$, and $f_{H^+} = 0.825$. The corrected pH values ($-\log a_{H^+}$) were obtained using following equations: $a = (pH_2' - pH_1')/(pH_2 - pH_1)$; $b = pH_2' - a\,pH_2$; pH = a(pH-meter reading) + b.

The potentiometric pH titrations were carried out with $I = 0.10$ ($NaNO_3$) at $25.0 \pm 0.1\,^{\circ}C$, where at least two independent titrations were always performed. Deprotonation constants and complexation constants K (defined in the text) were determined by means of the program BEST.[23] The pH sigma-fit values defined in the program are smaller than 0.02. The obtained constants, which contained an $[H^+]$ term, were converted to corresponding mixed constants with the equation $[H^+] = a_{H^+}/f_{H^+}$. The species distribution values (%) against pH ($= -\log a_{H^+}$) were obtained by use of the program SPE.[23]

Isothermal titration calorimetric study: Heats of reaction were determined on a Calorimetry Sciences Isothermal Titration Calorimeter 4200 at $25.0\,^{\circ}C$ and pH 7.4 (10 mM HEPES buffer) with $I = 0.10$ ($NaNO_3$). The calorimeter was calibrated by use of the heat of protonation of tris(hydroxymethyl)-aminomethane (250 mM, 1.0 mL) in H_2O at $25.0\,^{\circ}C$, for which a heat value of 474.7 mJ is obtained for a 10 μL injection of 1.00 mM aqueous HCl. A sample solution of **3** (0.25 mM), **4** (50 μM) or **15** (1.0 mM) in the HEPES buffer (1.0 mL) was loaded into a calorimeter cell. After the cell temperature had become constant at $25.0\,^{\circ}C$, the guest molecule of TpT (10 mM), GpT (10 mM), CpT (10 mM), ApT (10 mM), T (25 mM), or TpTpT (2.5 mM) in the HEPES buffer was added, for which at least three independent titrations were performed. All the calorimetric data were analyzed for dissociation constant K_d with the programs Data Works and Bind Works provided by the Calorimetry Sciences, and the average values were calculated. The programs were used for analysis of spectrophotometric UV titration too, for which the UV absorption decrease at each titration point was used instead of the heat of reaction.

FAB-MS measurements: FAB-MS (positive mode) was measured for 1:1 mixture (30 mM) TpT and **3**, and for 1:1 mixture (30 mM) TpTpT and **4** both at pH 8 in aqueous solution. In the former case a major peak at m/z 1119 with Zn isotopic peaks (1118, 1120 etc) were observed, which proved the formation of **5** (m/z 1120.84). In the latter case, a major peak at m/z 1763 with Zn isotopic peaks (1764, 1767 etc) was seen, which supported the formation of **6** (m/z 1762.81).

Molecular mechanics calculations: Structure minimization for **5** and **6** was accomplished with molecular mechanics (MM2) and molecular dynamics (MD) package (CAChe Version 3.9) provided by the Oxford Molecular Group. Initial component parts of bis(Zn^{II}-cyclen) **3** and its homologue tris(Zn^{II}-cyclen) **4** in **5** and **6** were constructed by use of a crystal structure of bis(Zn^{II}-cyclen) unit in bis(Zn^{II}-cyclen) complex with barbital dianion,[20] and coordination bonds between Zn^{II} ions and imido N^- atoms of T^-pT^- and $T^-pT^-pT^-$ were added. The structures were first minimized by MM2 method (block-diagonal Newton-Raphson method, until the change in total energy (ΔE_{total}) became less than 0.01 kJ mol^{-1}) with MM2 parameters at 300 K; the resulting structure was submitted to MD simulation at 800 K with MM2 parameters, for which $sp^3d_{z^2}$ trigonal bipyramidal configuration was selected for the Zn^{II} ions. The lowest energy structures for **5** and **6** (Figure 9) were obtained by further optimization by the same MM2 method at 300 K until the change in total energy (ΔE_{total}) became less than 0.001 kJ mol^{-1}. The energy terms (kJ mol^{-1}) for the MM2 force field are bond strech (E_{bs}), bond angle (E_{ba}), dihedral angle (E_{da}), improper torsion (E_{it}), van der Waals (E_{vdw}), electrostatic (E_e), and hydrogen bond (E_{hb}): $E_{total} = -402.7$ kJ mol^{-1} for **5** ($E_{bs} = 47.4$, $E_{ba} = 311.7$, $E_{da} = 48.6$, $E_{it} = 0.1$, $E_{vdw} = 25.1$, $E_e = -807.5$, and $E_{hb} = -28.2$); $E_{total} = -563.0$ kJ mol^{-1} for **6** ($E_{bs} = 72.4$, $E_{ba} = 496.8$, $E_{da} = 60.6$, $E_{it} = 0.2$ $E_{vdw} = 47.5$, $E_e = -1205.3$, and $E_{hb} = -35.3$).

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