Double-Strand Hydrolysis of Plasmid DNA by Dicerium Complexes at 37 °C

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Abstract: Significant effort has been made to develop synthetic metal complexes that hydrolyze DNA. Here we report a new dicerium complex, Ce_2(HXTA) (HXTA = 5-methyl-2-hydroxy-1,3-xylene-α,α-diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid), which can hydrolyze DNA at pH 8 and 37 °C. This complex hydrolyzes DNA restriction fragments to give products with high regioselectivity, affording >90% 5′-OPO_3 and 3′-OH ends, like the products of DNA hydrolyzing enzymes. Ce_2(HXTA) also hydrolyzes Litmus 29 plasmid DNA to afford both nicked and linear DNA. Analysis of the relative amounts of supercoiled, nicked, and linear DNA present show that there is one double-strand cleavage per ten single-strand cleavages, indicating that the linear DNA formed cannot be the result of two random single-strand cleavage events. The kinetics of nicked and linear DNA formation are comparable, both being associated with apparent first-order rate constants of approximately 1×10^{-4} s^{-1} for complex concentrations of 10^{-5}−10^{-4} M. These observations suggest that similar factors affect the hydrolysis of the first and second DNA strands and that cleaving the phosphodiester bond is likely the rate determining step in both cases. This is the first detailed study of a metal complex shown to mimic DNA hydrolases in their capability to effect double-strand DNA hydrolysis regioselectively at the 3′-O−P bond.

Introduction

To overcome the remarkable stability of the phosphodiester bond in DNA, which under physiological conditions has a $t_{1/2}$ estimated to be 130 000 years,[1] Nature has evolved a number of enzymes such as restriction endonucleases and topoisomerases that efficiently catalyze DNA hydrolysis.[2] Considerable effort has been expended to develop small molecules that hydrolyze DNA,[2,3] in order to develop hydrolytic agents that have potential applications in molecular biology and therapy.[4] Most useful would be synthetic reagents that hydrolyze DNA efficiently, preferably in a double-stranded fashion, to afford 5′-OPO_3 and 3′-OH ends, as observed for many DNA hydrolases. Most of the synthetic nucleases reported thus far do not produce predominantly 5′-OPO_3 and 3′-OH ends and typically only catalyze single-strand cleavage. Double-strand cleavage of DNA by small molecules has been reported, but these agents such as bleomycin and the enediyne based drugs cleave DNA by oxidative mechanisms.[5−9]

Our goal has been to develop synthetic complexes capable of hydrolytic double-strand DNA cleavage. Lewis acidic metal ions, particularly lanthanide ions, are known to carry out DNA hydrolysis. Lanthanide ions and their complexes have been demonstrated to hydrolyze dideoxyribonucleotides and single-strand oligomer DNA,[10−14] and there are detailed reports on Ce^{4+} hydrolysis of oligomer DNA[15] and 2′-deoxythymidine 3′:5′-cyclic monophosphate (cTMP).[16] We have recently reported the first synthetic lanthanide complexes, Ce_2(HPTA) and La_2(HPTA) (for the structure of HPTA, see Figure 1), capable of double-strand DNA hydrolysis.[17] These complexes work effectively at higher temperatures, i.e., 55 °C, but are inefficient at 37 °C. DNA can be hydrolyzed at 37 °C by a number of other complexes, including lanthanide complexes of polyalcohol and macrocyclic ligands,[18] Co^{3+} complexes of polyamine ligands,[19,20] and Cu^{2+} complexes of kanamycin and neamine.[21] These systems have not been reported to carry out the double-strand hydrolysis of DNA. Barton's group has designed a zinc-peptide attached to a rhodium intercalator that cleaves plasmid DNA to yield both nicked and linear DNA, but an analysis of

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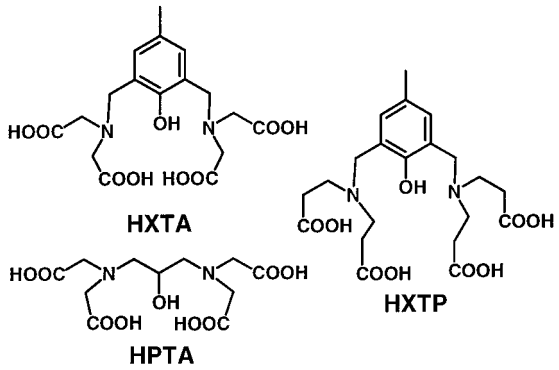

Figure 1. Structures of the HXTA, HXTP, and HPTA ligands.

the possible double-strand hydrolysis reaction has not been reported.[22] Here we follow up on our earlier report of dicerium and dilanthanide complexes that catalyze double-strand DNA hydrolysis at 55 °C[17] with a detailed study of a new dicerium complex Ce_2(HXTA) (for the structure of HXTA, see Figure 1), which is capable of carrying out double-strand DNA hydrolysis efficiently at 37 °C. Furthermore, Ce_2(HXTA) exhibits high regioselectivity in cleaving the 3′-O−P bond.

Materials and Methods

Substances. The Litmus 29 plasmid DNA, prepared from DH5α cells, was purified by using Qiagen Plasmid Maxi Kits. The enzymes *Hin*d III, *Pvu* II, calf intestinal phosphatase, and RQ DNase I were purchased from Promega, *Bam*H I and T4 polynucleotide kinase were purchased from New England Biolabs, and the Klenow fragment of DNA polymerase I was purchased from Gibco BRL. MPE was purchased from Sigma. All radioactive nucleotides were ordered from Amersham Life Sciences. Metal salts were purchased from Aldrich. All materials used were molecular biology grade when available, otherwise the purest available material was used.

The ligand HXTA was synthesized by a modification of published procedures.[23] To a 100 mL aqueous solution of 16.7 g (0.125 mol) of iminodiacetic acid and 6.8 mL (6.75 g, 0.063mol) of *p*-cresol was added 10.5 g (0.25 mol) of NaOH in 40 mL of water cooled in an ice−water bath. Upon dissolution, 15 mL of 37% formaldehyde was added dropwise at 0 °C. The solution was stirred for 30 min, heated at 70 °C for 4 h, and then concentrated to dryness. Recrystallization of the solid from methanol yielded colorless crystals of Na_4HXTA. Yield ∼90%. ^{1}H NMR in D_2O (δ, ppm): 6.83 (s, 2H), 3.60 (s, 4H), 3.04 (s, 8H), and 2.05 (s, 3H). Elemental analysis (calculated) for $C_{17}H_{18}N_2O_9Na_4 \cdot 1.7H_2O$: C 39.52 (39.49), H 4.44 (4.17), N 5.28 (5.41). The HXTP ligand was also synthesized as the tetrasodium salt. Six milliliters of a 37% formaldehyde solution (80 mmol) was added in a dropwise fashion to a 50 mL aqueous solution of disodium iminodipropionate (10 g, 48 mmol) and *p*-cresol (2.5 mL, 24 mmol) at 0 °C. This mixture was kept at 0 °C for 30 min and then stirred at room temperature for 48 h. The solvent was removed by rotary evaporation, and the resulting residue was recrystallized from methanol (15.6 g, 60% yield). ^{1}H NMR in D_2O (δ, ppm): 6.85 (s, 2H), 3.63 (s, 4H), 2.73 (t, 8H, $J_{CH,CH}$ = 7.8 Hz), 2.30 (t, 8H, $J_{CH,CH}$ = 7.8 Hz), 2.06 (s, 3H). Elemental analysis (calculated) for $C_{21}H_{26}N_2O_9Na_4 \cdot 3.6H_2O$: C 41.56 (41.54), H 5.37 (5.51), N 4.54 (4.61).

Plasmid DNA Cleavage. The plasmid DNA cleavage reactions were carried out in a total reaction volume of 70 μL containing cleavage agent, 7 μg of Litmus 29 plasmid DNA (2820 base pairs), and 7 μL of 100 mM pH 8.0 Tris buffer. $La(NO_3)_3$, $Ce(NO_3)_3$, $Nd(NO_3)_3$, $Eu(NO_3)_3$, $Yb(NO_3)_3$, $Fe(NO_3)_3$, $Zn(NO_3)_2$, $(NH_4)_2Fe(SO_4)_2$, and $(NH_4)_2Ce(NO_3)_6$ were the sources of the metal ions used in the cleavage reactions. Solutions (0.1 M) of HXTA and HXTP were made with sterile Millipore H_2O, adjusted to pH 7, and sterile filtered prior to use. The reactions were carried out at 37 and 55 °C in a covered heating block to prevent solvent evaporation in the course of the experiment. Samples were frozen at −20 °C in a dye solution (0.04% bromophenol blue, 0.04% xylene cyanol FF, and 5% glycerol) prior to running the gel. All gels were run on 1.2% agarose slab gels for 120 min at 70 V. Gels were stained with ethidium bromide and pictures taken with Polaroid 3000_{ISO} type 667 film. Bands on the gels were quantified by using a Microtek Scanmaker E6 and the software program NIH Image 1.60. This quantification was confirmed by directly digitizing the gel image with use of a Biorad GelDoc and the software Molecular Analyst. The amount of supercoiled DNA was multiplied by a factor of 1.22 to account for reduced ethidium bromide intercalation into supercoiled DNA.

The values for n_1 and n_2 were determined from the aforementioned gel quantification and formulas based on the Freifelder−Trumbo relationship.[24] The amount of supercoiled DNA is expressed in $f_I = \exp(-(n_1 + n_2))$, where f_I is the fraction of supercoiled DNA and n_1 and n_2 are the number of single-strand and double-strand DNA cuts, respectively. The amount of linear DNA is expressed in $f_{III} = n_2 \exp(-n_2)$, where f_{III} is the fraction of DNA in the linear form. The n_1 and n_2 values reported in this paper represent 42 data points taken from 9 gels for Ce_2(HXTA). Alternatively, this method of analysis was confirmed by using Cowan's methodology.[25] In this method $n_2 = f_{III}/(1 - f_{III})$ and $n_1 = -\ln(f_I(1 + n_2))$ are the formulas used and are specific for cases where double-strand cuts are the dominant mechanism for generating linear DNA.

Kinetics. Kinetic determinations on the cleavage of supercoiled plasmid DNA by Ce_2(HXTA) were carried out by quantifying the nicked and linear DNA fractions as described above. Plots of ln(nicked fraction) or ln(linear fraction) values versus time afforded a straight line, the slope of which provides the apparent first-order rate constant. Kinetic measurements with bis(*p*-nitrophenyl)phosphate (BNPP) were performed on a Beckman DU-650 spectrophotometer at 37 °C in 100 mM pH 8.0 Tris buffer. Reactions with 0.1 mM Ce_2(HXTA) and 1 mM dApdA were performed at 37 °C for 24 h in 10 mM pH 8.0 Tris buffer. At the end of the reaction, Ce_2(HXTA) was precipitated with 1 M KH_2PO_4. The supernatant was then applied on a C18 reverse phase column for the analysis of hydrolysis products, using as eluant a 50:50 mixture of MeOH:KH_2PO_4 at pH 4.5 with a flow rate of 0.5 mL/min. The Ce_2(HXTA) reaction products were confirmed via known standards.

End Analysis of Restriction Fragments. 5′-end labeled restriction fragments were prepared by using the following procedure. Litmus 29 plasmid DNA was treated with 50 U of *Hin*d III followed by ethanol precipitation. The DNA was then treated with 3 U of calf intestinal phosphatase followed by heat treatment at 75 °C for 10 min in the presence of 10 mM pH 8 EDTA. After standard phenol:chloroform: isoamyl alcohol treatment and ethanol precipitation the DNA was 5′-end-labeled with 2 U T4 polynucleotide kinase in the presence of γ-^{32}P-ATP. The DNA was then treated with phenol:chloroform:isoamyl alcohol and run through a Sephadex G-50 column equilibrated to pH 8 with Tris buffer. After NH_4OAc/ethanol precipitation, the DNA was treated with 4 U of *Pvu* II and purified with a 6% nondenaturing PAGE gel. The DNA was visualized by exposure to X-ray film and purified from the gel by using the crush and soak method.[26]

3′-end labeled restriction fragments were prepared using the following procedure. Litmus 29 DNA (2 μg) was treated with 15 U of *Bam*H I at 37 °C followed by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. The DNA was then treated with 4 U of the Klenow fragment and α-^{32}P-dATP on ice with use of standard fill-in conditions, provided by Gibco BRL, and extracted with phenol: chloroform:isoamyl alcohol followed by a Sephadex G-50 spin column and ethanol precipitation. The sample was then treated with 20 U of *Pvu* II at 37 °C and purified on a 6% nondenaturing PAGE gel. The

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DNA was visualized by exposure to X-ray film and purified from the gel by using the crush and soak method.[26]

The 5′- and 3′-end-labeled restriction fragments (800000−1000000 cpm) were treated with Ce_2(HXTA) in the presence of 0.25 μg/10 μL unlabeled linear fragments of Litmus 29 in 10 mM pH 8.0 Tris in a total volume of 100 μL. All reactions were done in triplicate. After 5 h of incubation at 55 °C, or 24 h at 37 °C, the samples were ethanol precipitated and suspended in formamide dye buffer. Maxam Gilbert reactions followed standard molecular biology protocols.[26] DNase reactions were carried out in 10 mM pH 8.0 Tris buffer for 15 min with serial dilutions of DNase I. Fe(II)MPE reactions were carried out with 10 μM FeMPE, 0.1 μg/μL cold carrier DNA, 1 mM DTT, and 10 mM pH 8.0 Tris buffer at 37 °C for 1 h. The samples were run on a 20% denaturing PAGE 7 M urea gel for 210 min at 50 W, then exposed to Fuji RX X-ray film. Only parts of the high-resolution gels are shown in Figure 3A, B for clarity. The entire gels are shown in Figures S1 and S2 in the Supporting Information.

Oligonucleotide Cleavage. The 18-mer oligonucleotide HO-dA(pdA)$_{16}$pdA (MW + Na = 5577 g/mol) was purchased from Synthetic Genetics. The cleavage experiment was carried out at 40 °C for 3 h in a 100 μL total reaction volume with 20 μL of 1 μg/μL oligonucleotide solution, 20 μL of 10 mM pH 8.0 Tris buffer, 40 μL of 25 mM Ce_2HXTA, and 20 μL of Milli-Q water. The cleaved samples were then concentrated and prepared for mass spectrometry by using the Millipore ZipTip$_{AX}$ protocol. Mass spectrometry data were obtained on a Bruker Biflex III MALDI-TOF mass spectrometer in positive linear mode using as matrix 1 part of 50 mg/mL ammonium citrate in Milli-Q water to 9 parts of 50 mg/mL 3-hydroxypicolinic acid in 1:1 (v/v) acetonitrile/Milli-Q water.

Results and Discussion

Reactivity with Plasmid DNA. We previously reported that Ce_2(HPTA) can effect double-strand hydrolysis of plasmid DNA at 55 °C.[17] These results led us to examine the DNA cleaving ability of Ce^{4+} complexes of two related dinucleating ligands, HXTA and HXTP (Figure 1), in an attempt to find hydrolytic agents that can cleave DNA under more physiological conditions. Litmus 29 plasmid DNA was thus treated with Ce_2(HXTA) and Ce_2(HXTP) at pH 8.0 and 37 °C. Results typical for this experiment are shown in Figure 2. In Figure 2a, 25 μM Ce_2(HXTA) was exposed to 0.1 μg/μL DNA with aliquots removed every 20 min. After 120 min (lane 8) we observe 20% nicked DNA. In Figure 2b, 100 μM Ce_2(HXTA) was exposed to 0.1 μg/μL DNA with aliquots removed every 120 min. After 720 min we observed 17% supercoiled, 63% nicked, and 20% linear DNA (Figure 2b, lane 8). No DNA cleavage was observed under identical reaction conditions in the absence of both Ce^{4+} and HXTA (Figure 2a,b, lane 2) or in the absence of either component (data not shown). At 55 °C, Ce_2(HXTA) cleavage of Litmus 29 plasmid DNA afforded 16% supercoiled, 62% nicked, and 21% linear DNA after 3 h. This result is similar to data reported for Ce_2(HPTA),[17] which give 21% supercoiled, 46% nicked, and 21% linear DNA (Table 1) under identical conditions. At 37 °C, however, Ce_2(HPTA) gives only 55% supercoiled, 42% nicked, and 3% linear DNA after 12 h of reaction. The corresponding 37 °C experiments with Ce_2(HXTP) (Table 1) give results similar to Ce_2(HPTA) with 42% nicked DNA and 3% linear DNA after 12 h. We also explored whether combinations of other metal ions with HXTA could cleave DNA. The addition of 2 equiv of La^{3+}, Ce^{3+}, Nd^{3+}, or Eu^{3+} to 1 equiv of HXTA did not form a reagent that showed DNA cleavage activity within a 3-h period at 37 °C. With Yb^{3+}, 24% nicked DNA was observed over a 3-h time period (data not shown), compared to 48% for Ce_2(HXTA) over the same time period. These results clearly indicate that Ce_2(HXTA) is a superior double-strand DNA cleavage agent, so our subsequent efforts focused on the reactivity of the Ce_2(HXTA) complex.

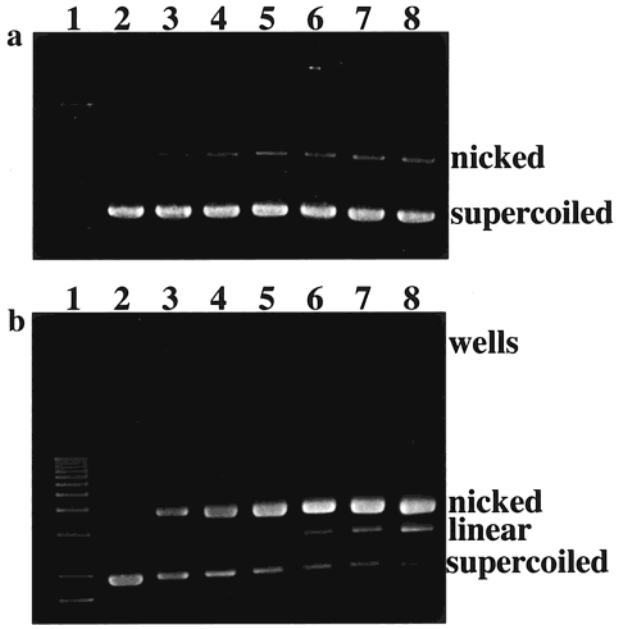

Figure 2. (a) Agarose gel electrophoresis of Litmus 29 plasmid DNA treated with 50 μM $(NH_4)_2Ce(NO_3)_6$ and 25 μM HXTA. Lane 1 is a kilobase molecular weight ladder. Lane 2 is a control experiment consisting of 1 μg of Litmus 29 DNA incubated for 120 min at 37 °C in 10 mM pH 8.0 Tris buffer. Lanes 3−8 represent 1 μg of DNA reacted for 20 (lane 3), 40 (lane 4), 60 (lane 5), 80 (lane 6), 100 (lane 7), and 120 min (lane 8) with cleavage agent at 37 °C in 10 mM pH 8.0 Tris buffer. (b) Agarose gel electrophoresis of Litmus 29 plasmid DNA treated with 200 μM $(NH_4)_2Ce(NO_3)_6$ and 100 μM HXTA. Lane 1 is a kilobase molecular weight ladder. Lane 2 is a control experiment consisting of 1 μg of Litmus 29 DNA incubated for 720 min at 37 °C in 10 mM pH 8.0 Tris buffer. Lanes 3−8 represent 1 μg of DNA reacted for 120 (lane 3), 240 (lane 4), 360 (lane 5), 480 (lane 6), 600 (lane 7), and 720 min (lane 8) with cleavage agent at 37 °C in 10 mM pH 8.0 Tris buffer.

Table 1. Relative Amounts of the Three Forms of Plasmid DNA after Treatment by Ce_2L

complex	T, °C	rxn time, h	rel amounts, % supercoiled	nicked	linear	n_1/n_2
Ce_2(HXTA)						
100 μM	37	6	25	65	10	12
100 μM	37	12	17	63	20	6
50 μM	37	6	36	57	7	13
50 μM	37	12	25	61	14	8
100 μM	55	3	16	62	21	5
Ce_2(HXTP)						
100 μM	37	6	55	42	3	19
Ce_2(HPTA)						
100 μM	55	6	25	59	16	6
100 μM	55	6	47	39	14	4
100 μM	55	6	29	56	16	4
100 μM	55	6	21	46	33	2

Nature of the Cleavage Ends. DNA strand breakage by small molecules can occur by either oxidative or hydrolytic strand scission. These two general mechanisms can be distinguished by examining the products generated by cleaving 5′- and 3′-^{32}P end-labeled DNA restriction fragments. Figure 3 shows magnified sections of these gels that provide information regarding the nature of the cleavage ends, while the entire high-resolution gels are provided in the Supporting Information. Control experiments show that no significant cleavage of the restriction fragments occurs in the absence of Ce_2(HXTA) (see Figures S1 and S2 in the Supporting Information).

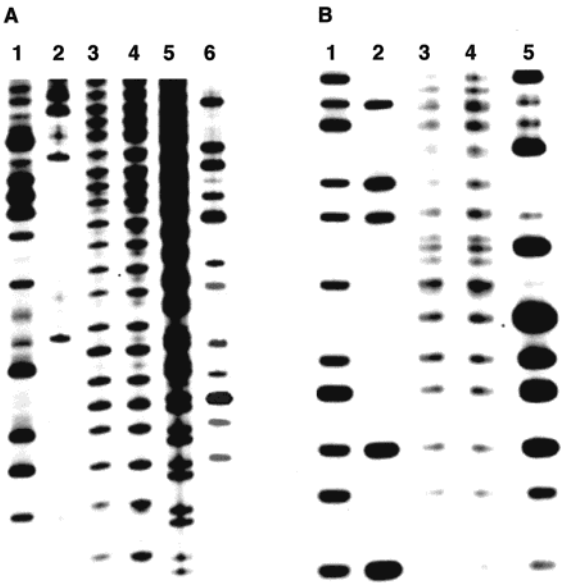

Figure 3. A: Section of a high-resolution denaturing PAGE gel of cleavage products of a 5′-end-labeled 192-bp restriction fragment by treatment with Ce_2(HXTA). Lane 1, C+T Maxam and Gilbert sequencing; lane 2, G Maxam and Gilbert sequencing; lane 3, DNA cleaved by Ce_2(HXTA) at 37 °C for 24 h; lane 4, DNA cleaved by Ce_2(HXTA) at 55 °C for 5 h; lane 5, DNA cleaved by FeMPE; lane 6, DNA cleaved by DNase I. No DNA cleavage was observed in the absence of Ce_2-HXTA; see lanes 7 and 8 of Figure S1 in the Supporting Information. B: Section of a high-resolution denaturing PAGE gel of cleavage products of a 3′-end-labeled 186-bp restriction fragment by treatment with Ce_2(HXTA). Lane 1, A+G Maxam and Gilbert sequencing; lane 2, G Maxam and Gilbert sequencing; lane 3, DNA cleaved by Ce_2-(HXTA) at 37 °C for 24 h; lane 4, DNA cleaved by Ce_2(HXTA) at 55 °C for 5 h; lane 5, DNA cleaved by DNase I. No DNA cleavage was observed in the absence of Ce_2HXTA; see lanes 6 and 7 of Figure S2 in the Supporting Information.

The nature of the 3′-end products of DNA cleavage was determined by cleaving a 5′-end ^{32}P-labeled 192-base-pair restriction fragment with Ce_2(HXTA) (Figure 3A). These DNA cleavage products (lanes 3 and 4 at 37 and 55 °C, respectively) were compared with the products produced by Maxam and Gilbert sequencing, which generates 3′-OPO_3 ends (C+T, lane 1; G, lane 2) and DNase I cleavage (lane 6), which produces 3′-OH ends. Examination of lanes 3 and 4 shows that the majority product bands of Ce_2(HXTA) cleavage consistently comigrate with the products of DNase I-catalyzed DNA cleavage, indicating that the majority products have 3′-OH ends. Furthermore, the majority product bands do not comigrate with the 3′-OPO_3 products of Maxam and Gilbert DNA cleavage in most cases. There are two ambiguous instances; the fourth and fifth bands from the bottom in lanes 3 and 4 appear to migrate slightly ahead of the corresponding DNase I bands in lane 6 but slightly behind the corresponding bands in the C+T lane in lane 1. Lane 5 shows cleavage products derived from treatment of the restriction fragment with FeMPE, O_2, and DTT, which produces 3′-OPO_3 and 3′-$OPO_3CH_2COO^-$ (phosphoglycolate) ends. Although most of lane 5 is overexposed, it is clear from a comparison of the bottoms of lanes 3 and 4 with the bottom of lane 5 that Ce_2(HXTA) cleavage does not produce 3′-phosphoglycolate ends. Scrutiny of lane 4 (Ce_2(HXTA) cleavage at 55 °C) shows that some of the bands have an accompanying minor component that comigrates with the corresponding band in the Maxam and Gilbert lanes, indicating the presence of some 3′-OPO_3 ends. These minority components are estimated to represent less than 10% of the corresponding 3′-OH band. Furthermore, these minor components are much less discernible in lane 3 (Ce_2(HXTA) cleavage at 37 °C), if at all. These results indicate that Ce_2(HXTA) cleavage is quite selective for cleaving the 3′-O−P bond and that this regioselectivity is more pronounced at 37 °C than at 55 °C.

Table 2. Relative Amounts of the Various DNA Hydrolysis End Products

		rel amounts, %				
complex	substrate	3′-OPO_3	3′-OH	5′-OPO_3	5′-OH	ref
Ce_2(HXTA)	duplex DNA	5	95	90	10	TW[a]
Ce_2(HPTA)	duplex DNA	10	90	60	40	17
Ce^{4+}(1 mM)	s.s. DNA	33	66	33	66	15
Ce^{4+}(10 mM)	s.s. DNA	0	100	0	100	15

[a] TW = this work.

The preference of Ce_2(HXTA) for cleaving the 3′-O−P phosphate diester bond is corroborated by an examination of the 5′-ends derived from cleavage of a 3′-end ^{32}P-labeled 186-base-pair restriction fragment. The gel section shown in Figure 3B compares the 5′-ends of DNA cleaved by Ce_2(HXTA) at 37 and 55 °C (lanes 3 and 4, respectively) with the 5′-ends of DNA obtained by the Maxam and Gilbert chemical sequencing method (A+G, lane 1; G, lane 2) and DNase I treatment (lane 5). Almost all of the bands derived from Ce_2HXTA cleavage comigrate with the bands derived from DNase I treatment and Maxam and Gilbert sequencing, both of which produce 5′-OPO_3 ends. There are, however, two bands that appear in the middle of lanes 3 and 4 (Figure 3B, bands 8 and 10 from the top) that do not correspond to 5′-OPO_3 ends and would appear to correspond to the 5′-OH fragments of bands 9 and 11. These bands, though less intense than the corresponding 5′-OPO_3 band, represent significant deviations from the generalization that Ce_2(HXTA) preferentially cleaves the 3′-O−P bond. Further work will be needed to clarify why the cleavage of these particular phosphodiester bonds is not as regioselective as cleavage at other sites.

The absence of ends ascribable to oxidative cleavage in the high-resolution PAGE analysis of the cleavage products of the restriction fragments was corroborated by studies of the 18-mer oligonucleotide, HO-dA$(pdA)_{16}$pdA. The single-stranded oligonucleotide was treated with 10 mM Ce_2(HXTA) at 37 °C for 1−3 h. MALDI-TOF analysis of the oligonucleotide solution after cleavage showed a prominent peak at *m*/*z* 5577, corresponding to the molecular ion plus sodium $(M+Na)^+$. Less intense peaks near the molecular ion were observed at *m*/*z* 5460, 5265, and 4954. The *m*/*z* 5460 peak corresponds to the loss of a deoxyadenosyl group from the molecular ion plus the matrix 3-hydroxypicolinic acid, while *m*/*z* 5265 and 4954 peaks correspond to losses of dAp and dApdAp fragments from the molecular ion plus sodium, respectively. There are no peaks corresponding to oligonucleotide fragments with phosphoglycolate ends.

Our restriction fragment experiments show that Ce_2(HXTA) has a strong preference for cleaving the 3′-O−P bond, generating fragments with predominantly 3′-OH ends and 5′-OPO_3 ends (with some exceptions discussed above). These are precisely the types of ends generated by DNA cleaving enzymes, such as endonucleases and DNase I. This hydrolytic preference is similar to that observed for Ce_2(HPTA) (Table 2), which was estimated to afford 90% 3′-OH ends as well. Ce_2(HPTA), however, gives fragments with a 60:40 ratio of 5′-OPO_3 and 5′-OH ends, suggesting that some of the 5′-OPO_3 ends are further hydrolyzed. For comparison, Komiyama and co-workers found that the cleavage of single-strand oligonucleotide DNA

by Ce^{4+} results in a 2:1 ratio of hydroxy to phosphate ends at both the 5′ and 3′ ends,[15] suggesting that the free ion exhibits no preference as to which O−P bond is cleaved and significant further hydrolysis of the product phosphates occurs. Contrasting this is a recent report showing that cyclic 3′,5′-TMP is cleaved by Ce^{4+}at the 5′−O−P bond seven times more often than at the 3′−O−P bond,[16] opposite to that for Ce_2(HXTA). The preference of Ce_2(HXTA) and Ce_2(HPTA) to cleave DNA at the 3′-O−P bond to generate 5′-OPO_3 and 3′-OH products must derive from the effect the polycarboxylate ligands have on the Ce^{4+} ion. The HPTA and HXTA ligands, in conjunction with Ce^{4+}, represent the first systems capable of directing lanthanides to generate "natural" DNA cleavage ends. More detailed studies, beyond the scope of this paper, are clearly needed to fully elucidate this preference.

Double-Strand DNA Cleavage. The linear DNA shown in Figure 2b can be generated from supercoiled plasmid DNA by two general mechanisms. Linear DNA formation utilizing a single-strand DNA break mechanism is typified by the DNA footprinting reagent FeMPE[27] and the enzyme human DNase I.[4] In this mechanism one DNA strand is cleaved, then the cleavage agent leaves the area of the first strand break to carry out strand breaks elsewhere on the DNA. It can take ∼120 random single-strand breaks to generate linear DNA with a plasmid the size of Litmus 29.[28] The other general mechanism by which linear DNA is formed is double-strand cutting. In this case a second strand break occurs on the opposite DNA strand within 16 nucleotides of an initial strand break to form a double-strand cut. A double-strand cutting reagent is indicated if this occurs more often than random chance, as determined by a Poisson distribution initially utilized by Friefelder and Trumbo.[24] This method of analysis was used to determine that Fe^{2+}bleomycin, the best characterized DNA double-strand cutting small molecule, generates linear DNA through double-strand cuts.[29] The Friefelder−Trumbo relationship was further developed by Cowan and co-workers,[25] and used by Lazarus and co-workers to determine that hyperactive mutants of DNase I generate linear DNA by double-strand cutting.[4] We analyzed our results by both the Friefelder−Trumbo and Cowan methodologies.

Experiments, such as those shown by Figure 2b, were analyzed to determine if linear DNA formation was due to double-strand cuts, single-strand breaks, or a mixture of double-strand cuts and single-strand breaks. By quantifying the amounts of supercoiled, nicked, and linear DNA in each sample, and using the Friefelder−Trumbo relationship, we were able to determine the number of single-strand breaks, conveniently expressed as n_1, and double-strand cuts, conveniently expressed as n_2.[24,29] The Ce_2(HXTA) complex gives an average of 10 ± 3 single-strand breaks for every 1 double-strand cut, i.e., an n_1/n_2 ratio of 10 ± 3. An n_1/n_2 value in this range indicates that linear DNA is generated by double-strand cuts rather than single-strand breaks. To confirm this conclusion we then analyzed our results using the model developed by Cowan and co-workers that specifically addresses situations where there are mixed single-strand breaks and double-strand cuts.[25] The values derived for n_1 were then plotted against the fractions of supercoiled, nicked, and linear DNA. Figure 4a shows the plot of n_1 versus the DNA fractions for Ce_2(HXTA), while Figure 4b shows the corresponding plot for Ce_2(HPTA) for comparison. For both plots, linear DNA formation is observed at $n_1 < 2$, which is consistent with a mechanism where double-strand cuts are exclusively responsible for generating linear DNA. Furthermore, this second analysis also gives $n_1/n_2 = 10$ for Litmus 29 plasmid DNA cleavage by Ce_2(HXTA).

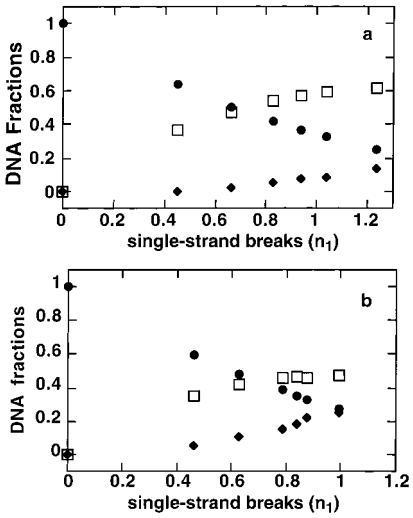

Figure 4. (a) A plot of the fractions of supercoiled (●), nicked (□), and linear (◆) DNA versus the number of single-strand cuts for 100 μM Ce_2(HXTA). (b) A plot of the fractions of supercoiled (●), nicked (□), and linear (◆) DNA versus the number of single-strand cuts for 100 μM Ce_2(HPTA) (data from ref 17)

The double-strand DNA cleaving ability of the Ce_2(HXTA) complex at 37 °C is quite novel. To date the only other small molecules reported to carry out hydrolytic double-strand cleavage of DNA are Ce_2(HPTA) and La_2(HPTA);[17] these two complexes carry out DNA cleavage at 55 °C and afford n_1/n_2 ratios of 4 and 9, respectively. For comparison, Fe^{2+}bleomycin, the best studied oxidative cleavage agent that generates double-strand DNA cleavage, gives n_1/n_2 values ranging from 3 to 20, depending on the system studied.[29−36] The n_1/n_2 ratio of 10 for Ce_2(HXTA) is within the reported range of values for Fe^{2+}bleomycin and similar to values we reported for Ce_2(HPTA) and La_2(HPTA). Ce_2(HPTA) and La_2(HPTA) only work effectively as double-strand cleavage agents at 55 °C, whereas Ce_2(HXTA) works effectively at 37 °C. This is the same temperature regime where both Fe^{2+}bleomycin and most restriction endonucleases are reported to be effective. Ce_2(HXTA) thus represents the first small molecule reported to be capable of double-strand DNA hydrolysis under physiologically relevant conditions.

DNA Cleavage Kinetics. The efficacy of Ce_2(HXTA) at DNA cleavage led us to investigate the kinetics of phosphodiester bond hydrolysis by this complex. Since bis(*p*-nitrophenyl) phosphate (BNPP) is typically used as a model substrate for such hydrolytic reactions, we first investigated the kinetics of BNPP hydrolysis by Ce_2(HXTA). Analysis of hydrolysis

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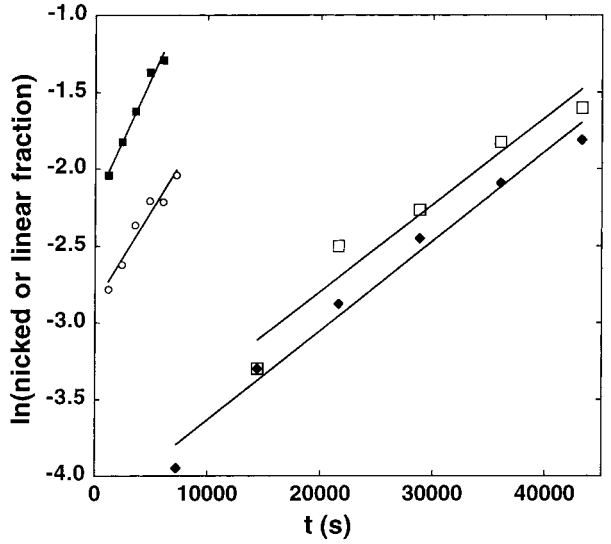

Figure 5. Kinetic plots for the formation of nicked DNA generated by the cleavage of Litmus 29 DNA by 10 (■) and 25 μM Ce_2(HXTA) (○) and the formation of linear DNA generated by the cleavage of Litmus 29 DNA by 50 (□) and 100 μM Ce_2(HXTA) (◆).

experiments with 1 mM BNPP and catalyst over a 40-min period at 37 °C gave a second-order rate constant k of 0.10(1) M^{-1} s^{-1} or 1.0×10^{-5} s^{-1} for 0.1 mM catalyst.

The kinetics of supercoiled plasmid DNA cleavage by Ce_2(HXTA) at 37 °C into nicked and linear DNA could be followed by gel electrophoresis, as illustrated by the gels shown in Figure 2. Figure 2a shows a typical agarose gel generated by exposing Litmus 29 to 25 μM Ce_2(HXTA) over a 2-h time course. The 2-h period was chosen to emphasize single-strand cleavage. A straight line was obtained in semilog plots of [nicked DNA] vs time, indicating that nicked DNA formation follows first-order kinetics, and a k_{obs} of $1.5(4) \times 10^{-4}$ s^{-1} (Figure 5) was determined. A similar k_{obs} was obtained with 10 μM complex. Linear DNA formation by Ce_2(HXTA) could be observed with longer reaction times and higher concentrations (50−100 μM) of complex. The kinetic data, derived from gels similar to those depicted by Figure 2b, show that linear DNA formation also follows first-order kinetics (Figure 5), with k_{obs} values of $(5-8) \times 10^{-5}$ s^{-1}, slightly smaller than those observed for nicked DNA formation by Ce_2(HXTA). The fact that comparable k_{obs} values were observed for the 10−100 μM complex suggests the participation of a preequilibrium step in which a DNA:complex adduct is formed followed by rate determining scission of the phosphodiester bond. Our study represents the only report to date of kinetic studies for linear DNA formation by a synthetic complex utilizing a hydrolytic DNA cleavage mechanism.

Kinetic studies of the formation of nicked DNA have been reported for other metal complexes. These results, summarized in Table 3, show rate constants similar to those we have found. It is not straightforward to compare these data with each other, since the studies were not necessarily carried out under analogous conditions and the substrate/catalyst ratios differ over a large range of values. The complexes with the highest hydrolysis rate constants are Pr_2(macro) and Co(cyclen) complexes studied by Schneider, but the k_{cat} values reported derive from the use of millimolar concentrations of complex. The hydrolysis efficiency of Ce_2(HXTA) is comparable, though somewhat smaller. However, much lower concentrations of complex are required to achieve this hydrolytic efficiency, which may reflect the much greater affinity of our complex for DNA. When

Table 3. Comparison of the Kinetic Data Reported for Plasmid DNA Cleavage at 37 °C

cleavage agent	[agent], μM	[$ROPO_2OR$], μM	k, s^{-1}	ref
nicked DNA formation				
Co^{3+}(tamen)[a]	1000	23	5×10^{-5}	20
$Pr^{3+}{}_2$(macro)[b]	10−1000	39	2.8×10^{-4}	37
Co^{3+}(*N*-alkyl-cyclen)[c]	10−5000	39	$(2.2-2.7) \times 10^{-4}$	19
Cu^{2+}(neamine)	5	50	3×10^{-4}	21
$Zn^{2+}{}_2$(peptide)[d]	5	80	$2.5(2) \times 10^{-5}$ [b]	22
$Ce^{4+}{}_2$(HXTA)	10	300	1.4×10^{-4}	TW[e]
$Ce^{4+}{}_2$(HXTA)	25	300	$1.5(4) \times 10^{-4}$	TW[e]
linear DNA formation				
$Ce^{4+}{}_2$(HXTA)	50	300	$(5-8) \times 10^{-5}$	TW[e]
$Ce^{4+}{}_2$(HXTA)	100	300	$(5-8) \times 10^{-5}$	TW[e]

[a] tamen = 6-(4-amino-2-azabutyl)-6-methyl-1,4-diazacycloheptane [b] macro = 30-membered macrocyclic ring with six nitrogen and four oxygen ligating atoms; k is the k_{cat} value derived from a Michaelis−Menten analysis of a plot of k_{obs} versus [complex]. [c] cyclen = 1,4,7,10-tetraazacyclododecane, R = H or cationic alkyl side chain; k is the k_{cat} value derived from a Michaelis−Menten analysis of a plot of k_{obs} versus complex concentration. [d] peptide = hexadecapeptide tethered to a DNA-intercalating Rh complex; k is the value determined by monitoring the disappearance of supercoiled plasmid. [e] TW = this work.

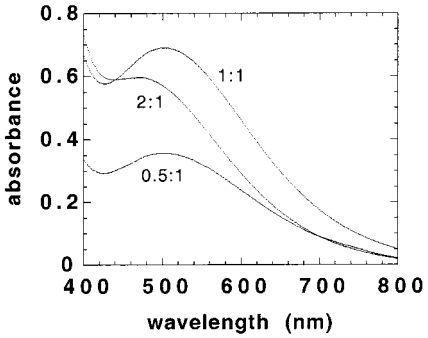

Figure 6. UV−vis spectra of aqueous solutions with Ce:HXTA ratios of 0.5:1, 1:1, and 2:1 at pH 8.

compared to DNA cleaving enzymes, however, Ce_2(HXTA) is about 4 orders of magnitude less efficient than Type-II restriction endonucleases, which typically exhibit k_{cat} values in the range of 1.7×10^{-2} to 1.7×10^{-1} s^{-1} for the formation of linear DNA.[38]

Nature of the Hydrolytic Agent. Because attempts to crystallize the Ce_2HXTA complex were unsuccessful, we performed titration experiments to gain insight into the nature of the hydrolytic agent. UV−vis titration experiments were carried out to ascertain the ligand-to-metal stoichiometry of the Ce_2(HXTA) complex. It was expected that a phenolate-to-metal charge-transfer transition would be observed in the visible region upon metal coordination. Thus titration of HXTA with up to 1 equiv of Ce^{4+} at pH 8.0 resulted in the formation of a purple complex (λ_{max} 502 nm; ϵ 6910 $L \cdot mol^{-1} \cdot cm^{-1}$; Figure 6). Subsequent addition of Ce^{4+} to a Ce^{4+}:HXTA ratio of 2:1 resulted in a blue shift to 472 nm (ϵ 5970 $L \cdot mol^{-} \cdot cm^{-1}$). These observations suggest that Ce^{4+} can form 1:1 and 2:1 complexes with HXTA. The spectral behavior is analogous to the binding of Fe^{3+} to HXTA at pH 4;[23] the 1:1 Fe:HXTA complex exhibits a λ_{max} of 550 nm, while the 2:1 Fe:HXTA complex has a λ_{max} of 472 nm. The latter complex has been crystallographically characterized as [Fe_2(HXTA)(μ-OH)(H_2O)$_2$], where the di-iron(III) center is bridged by a hydroxide and the phenoxo group

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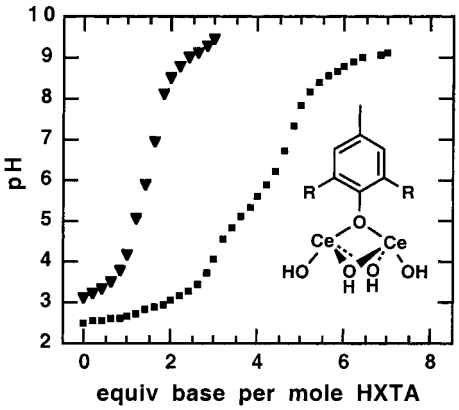

Figure 7. Potentiometric titration data for Ce(HXTA) (▼) and Ce_2(HXTA) (■).

of the dinucleating ligand. Based on the Fe^{3+} precedent, the blue shift observed upon binding two Ce^{4+} ions suggests the formation of a phenoxo-bridged dicerium complex analogous to Fe_2(HXTA). This complex appears stable under the reaction conditions, as indicated by the persistence of its absorption spectrum over a 24-h period at 37 °C.

Potentiometric titrations were also carried out on the 1:1 and 2:1 Ce:HXTA complexes (Figure 7) to determine whether any of the solvent molecules expected to be coordinated to the metal center were ionized at pH 8. With the addition of 1 equiv of Ce^{4+} to a solution of the tetrasodium salt of HXTA, we found that 2 equiv of base were required to return the solution to a pH of 8.0, indicating that Ce^{4+} binding to HXTA resulted in the release of two protons. The two protons are most likely from the un-ionized phenol and a coordinated water, i.e.

$$Ce^{4+} + H{-}HXTA^{4-} + H_2O \rightarrow [Ce(HXTA)(OH)]^{2-} + 2H^+$$

With the addition of 2 equiv of Ce^{4+} to HXTA, 5 equiv of base were required to reach pH 8 (Figure 7). One of these five protons derives from the ligand, and the other four presumably arise from coordinated solvent molecules, i.e.,

$$2Ce^{4+} + 4H_2O + H{-}HXTA^{4-} \rightarrow [Ce_2(HXTA)(OH)_4]^{-} + 5H^+$$

The titration shows two inflection points, one near pH 3 involving three protons and another near pH 6 involving the remaining two protons. A species with a $Ce_2(\mu\text{-}OH)_2(OH)_2^{4+}$ core has been proposed by Komiyama and co-workers as the catalytically active Ce^{4+} DNA cleavage agent in Ce-oxide gels.[1] On the basis of this precedent, we propose that the Ce_2HXTA complex may have a triply bridged $Ce_2(\mu\text{-ArO})(\mu\text{-OH})_2(OH)_2$ core (Figure 7 inset). The first inflection would involve the ionization of the three moieties that give rise to the bridging ligands, i.e., the phenol and two water ligands, while the second inflection would involve the ionization of two terminal water ligands.

The mechanism for DNA hydrolysis by Ce_2(HXTA) is analogous to that proposed earlier for Ce_2(HPTA).[17] Attack of DNA by the complex may result in the displacement of a bridging hydroxide and formation of a (μ-phosphato)dicerium complex. Coordinated in this manner, the phosphodiester bond experiences double Lewis acid activation.[39] Cleavage is then initiated by an in-line nucleophilic attack of one of the terminal hydroxides, resulting in inversion of configuration at phosphorus as observed in a study by Cullis.[16]

The three dicerium complexes observed thus far to carry out double-strand hydrolysis of plasmid DNA exhibt the following order of increasing effectivity: HPTA < HXTP < HXTA. The position of the HPTA complex in this series can be rationalized by the presence of the more basic alkoxo bridge (vs a phenoxo bridge in the other complexes) that would decrease the Lewis acidity of the cerium centers in the complex. The HXTA and HXTP ligands differ in their respective formation of five- and six-membered chelate rings, which should also affect the Lewis acidity of the metal centers. The six-membered rings can probably better accommodate the relatively large Ce^{4+} ions and thus lower their Lewis acidity. It would have been useful to have structural data on the two complexes for comparison, but we have been unable to obtain crystals of either complex.

Summary. Three features make the DNA cleavage chemistry of Ce_2(HXTA) particularly notable. First and foremost is the observation of double-strand hydrolysis. To date, only the related Ce_2(HPTA) and La_2(HPTA) complexes[17] and probably the Zn-oligopeptide of Barton[22] are capable of double-strand DNA hydrolysis. Ce_2HPTA is capable of performing a double-strand scission for every five single-strand cleavage events but is effective only at 55 °C. Ce_2(HXTA) is also effective at 55 °C with a n_1/n_2 value of 5, but works as well at a more physiological temperature of 37 °C with an n_1/n_2 value of 10 ± 3. Second, Ce_2(HXTA) appears to be rather ineffective at further hydrolyzing the phosphomonoesters formed at 37 °C. In contrast, about one-third of the phosphomonoester bonds are hydrolyzed in the cases of Ce_2(HPTA) (at 55 °C) and the cerium-hydroxide gel. Third, DNA hydrolysis by Ce_2(HXTA) and Ce_2(HPTA) is quite regioselective, producing 90−95% of hydrolysis products due to cleavage of the 3′-O−P bond. In contrast, the cerium hydroxide gel shows no regioselectivity at all.[15] These comparisons suggest that the structure imposed by the dinucleating ligands may be important for eliciting the observed regioselectivity of Ce_2(HXTA) and Ce_2(HPTA).

To date, Ce_2(HXTA) represents the synthetic complex that comes the closest to mimicking the hydrolysis chemistry catalyzed by restriction endonucleases. While its hydrolytic cleavage reaction is NOT sequence-specific, Ce_2(HXTA) targets the 3′-O−P bond of the phosphodiester regioselectively and can effect a significant amount of double-strand cleavage. Moreover this chemistry occurs under reasonable physiological conditions, i.e., 37 °C and pH 8. By suitable attachment of DNA recognition elements to the dinucleating ligand framework, it should then be possible to develop a synthetic DNA hydrolytic agent with the desired sequence specificity.

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Supporting Information Available: Figures S1 and S2 showing complete high-resolution gels of Ce_2(HXTA) cleaved restriction fragments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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