Supramolecular chemistry. Part 71.[1] Evidence for hydrolytic DNA cleavage by lanthanide(III) and cobalt(III) derivatives

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Double stranded plasmidic DNA, cleaved by either europium(III) salts or by the cobalt(III)–cyclen complex, is religated for the first time without the use of enzymes, showing hydrolytic mechanisms with these chemical nucleases. The same result is observed in the hydrolysis of the dinucleotide TpT by Eu^{III}, which is achieved in 25% conversion. The only products observed by HPLC are 3′,5′-cTMP, 3′-TMP, 5′-TMP and thymidine. The nucleobase thymine itself is not detected (<1%), which demonstrates the absence of radical cleavage. The dinucleotide hydrolysis shows, with $[Eu^{III}]$ = 5 mM, rate enhancement factors of $>10^7$, which even exceed those observed with activated phenyl phosphates.

Most of the presently available chemical nucleases[2] cleave nucleic acids by redox reactions, which by radical attack lead to unnatural terminal sites. In order to make them useful for *e.g.* biotechnological applications it is desirable to obtain real chemical analogues of natural nucleases yielding undamaged nucleoside or nucleotide ends. The most promising candidates for this are lanthanoid(III) or cobalt(III) systems[3,4] which have been shown to catalyze cleavage of nucleic acids with rate enhancement factors of up to 10^7 at concentrations as low as 0.05 mM.[3c] Although several lines of evidence already support the proposal that these reagents react by hydrolysis (see below) it is desirable to accumulate more direct proof for this, and to develop ways to reconstitute intact polynucleotides, which is the aim of the present work.

Most evidence for hydrolytic cleavage by lanthanoid or cobalt derivatives until now has been based on indirect kinetic observation, such as the absence of rate changes upon addition of hydrogen peroxide, or the dramatic change in results obtained on addition of radical scavengers, *e.g.* Cu^{II} derivatives.[3c] Komiyama *et al.*[4] have obtained intact thymidine (T) by HPLC analysis from reaction of the dinucleotide TpT with trivalent lanthanides, but at conversion rates of only 0.2%. This paper describes a dinucleotide cleavage reaching significantly higher conversion with only hydrolytic products formed. Our other aim was the chemical religation of cleaved natural DNA. Barton *et al.*[5] have been able to religate a nucleic acid cleaved by transition metal complexes; the use of a natural ligase for this purpose necessarily limits the success of the religation. We approached the problem by religation attempts with a chemical ligating system described first by Behr *et al.*,[6] which has the advantage over natural ligases that it does not depend on the availability of the correct 5′-phosphate- and 3′-OH ends of the cleaved nucleic acids.

Results and discussion

Religation experiments with plasmid DNA

Plasmidic pBR 322-DNA was cleaved with europium(III) chloride as described earlier.[3b] Religation was attempted following the procedure of Behr *et al.*,[6] with imidazole cyanide[7] (ImCN) as activating agent. The activation may proceed *via* iminoimidazolide phosphoric acid esters or *via* phosphoroimidazolides[8] and requires metal ions such as Mn^{II} salts as cofactors.[6] Other than in related experiments with double stranded linear DNA,[6,9] formation of new nucleotide bonds with the plasmidic RF II does not require the presence of oligonucleotides or polyamines as templates. The proximity of the RF II nucleotide ends after cleavage from RF I leads to a new circular form RF I*, which is, however, not negatively supercoiled as is the original RF I form. The new RF I* form becomes positively supercoiled in the presence of the intercalating dye ethidium bromide (EB), which is used in the electrophoretic analysis, and moves somewhat faster in the electric field than the native RF I.[6,9]

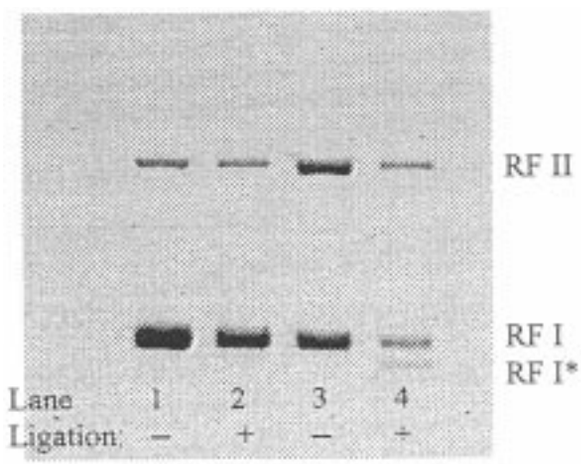


Fig. 1 Electrophoresis gel of a ligation with Eu^{3+}-cleaved pBR 322-DNA in comparison to uncleaved DNA. See Table 1 for composition of each lane.

Table 1 Optical densities (OD) of DNA bands from the ligation of Eu^{3+}-cleaved DNA (Fig. 1), corrected for background staining

Lane	Cleavage with Eu^{3+}	Addition of ligation mix	OD		
			RF I	RF II	RF I*
1	—	—	300	78	—
2	—	+	213	64	—
3	+	—	169	132	—
4	+	+	54	63	17

The observation of the new RF I* band in the chromatograms (Fig. 1) confirms religation has occurred between intact ends of the cleaved RF II form. The stainability of RF I* by the EB dye is obviously smaller than that of RF I or RF II. In contrast to RF I and RF II, where it also differs[10] by a factor of 1.22, the stainability factor of RF I* is unfortunately not known. For this reason we give in Tables 1–3 the optical densities (OD) of the bands instead of percentages. Addition of the ImCN religation mix to the original RF I (Fig. 1, Lane 1) leads within the experimental error to no change in the RF I:RF II

ratio, and to no detectable RF I* (Lane 2). The absolute optical densities, or their sum, decrease in both RF I and RF II, as they do in the other experiments (Lanes 3 and 4) for reasons unknown to us. Purification of the DNA prior to electrophoresis either by precipitation, or by ion exchange chromatography (in order to remove any metal traces that might quench fluorescence) did not yield different intensities. The decrease in OD cannot be due to further cleavage of RF II as this should show up in the build up of the linear form RF III, which is, for example, observed in radical cleavage with copper(II) and hydrogen peroxide.[3c] Only if sufficient RF II is available, after reaction with Eu^{III} ions as catalyst (Lane 3), can one detect RF I* (Lane 4). In view of the decrease of RF II after addition of the religation mix, and the absence of any other bands the low intensity of RF I* does not imply a low religation rate; better quantification must await the determination of the different stainabilities.

Similarly reduced optical densities were observed in religation attempts with plasmidic DNA which had been cleaved with the cobalt(III)–cyclen complex **1**. Although this complex is one of the most potent catalysts for the hydrolysis of activated phosphate esters,[11] until now few publications on the cleavage of nucleic acids or of oligonucleotides with this compound have been available.[3c,12] Again, the appearance of the RF I* form (Fig. 2, Table 2) provides evidence for the hydrolytic nature of the process, although the decrease of RF II is smaller than in the experiments with Eu^{III} cleaved DNA (Table 1). This is not unexpected in view of the larger catalytic efficiency of the cobalt complex, which can lead to several strand cleavages within one RF II duplex. Such multiple cleavages will be prohibitive for religation.[5] The same holds if both internucleoside bonds at one site are broken with the subsequent total loss of the linking phosphate. While our work was in progress Sargeson *et al.*[12] reported new evidence that the cobalt (III)–cyclen complex indeed leads to efficient hydrolytic DNA cleavage.

Experiments with plasmid DNA cleaved by copper(II)-complex **2** in the presence of hydrogen peroxide finally showed the absence of any RF I* form (Table 3, Fig. 3). This is to be expected due to its known radical mechanism[13] and the ensuing denatured ends in the RF II form. Thus, the appearance of the RF I* band proves the hydrolytic reaction with the Eu^{III} and Co^{III} complex.

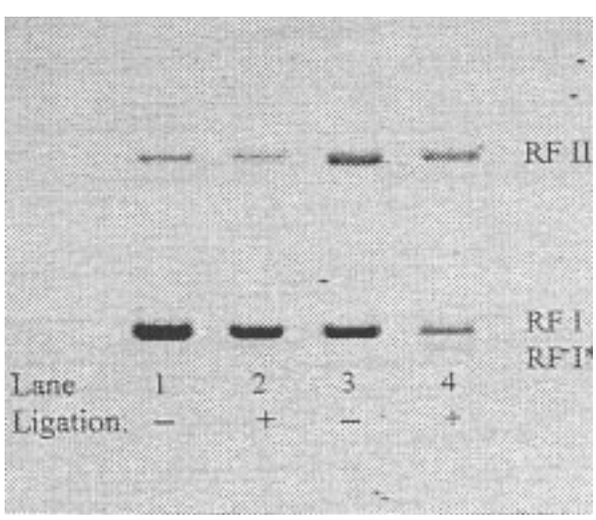
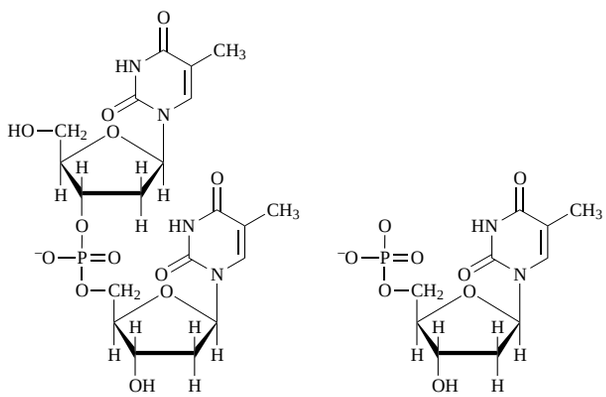
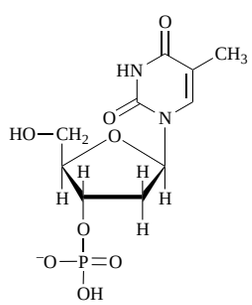


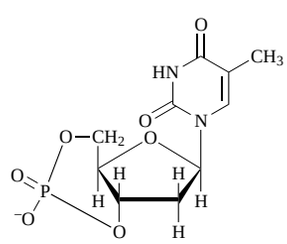
Fig. 2 Electrophoresis gel of a ligation of $[(cyclen)Co]_{aq}$-cleaved DNA. See Table 2 for composition of each lane.

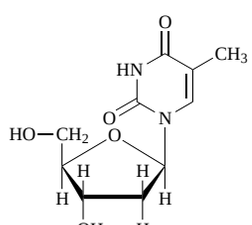
Hydrolysis of the dinucleotide TpT with Eu^{III} as catalyst

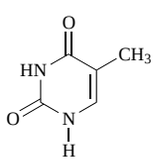
The hydrolytic nature of a dinucleotide cleavage should be evidenced also by the undamaged constituents one would expect from such a mechanism. The typical product of a radical degradation of the sugar part would be the nucleobase alone, without sugar, and/or denatured nucleobase derivatives.[2] Optimization of a HPLC separation on reversed-phase columns with a phosphate buffer–methanol gradient as eluent allowed separation of all possible components in blind runs, together with isonicotinic acid as internal standard for quantitative evaluations. The dinucleotide TpT was reacted with 5 mM $Eu^{III}Cl_3$ at 70 °C for up to 14 days, which allowed the reaction to be followed to up to 25% completion (Fig. 4). The detected products

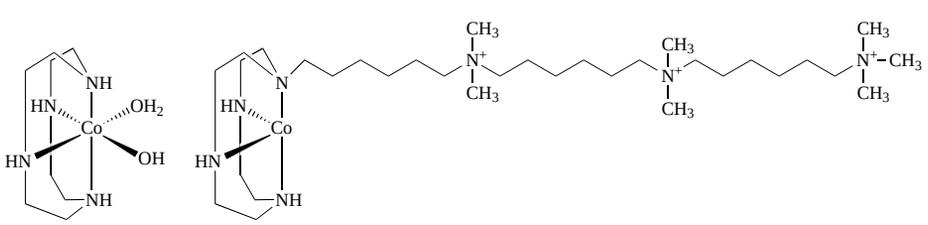












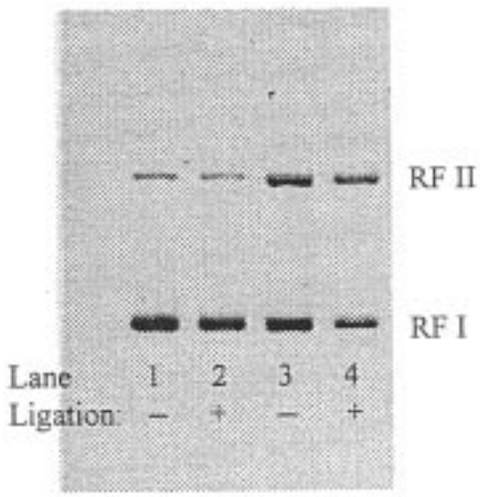
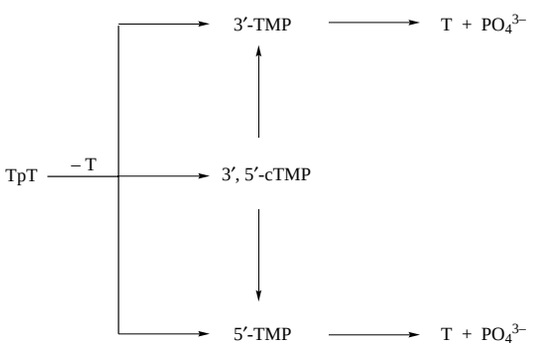


Fig. 3 Electrophoresis gel of a ligation trial of DNA cleaved in the presence of copper complex **2** and H_2O_2. See Table 3 for composition of each lane.

3′-TMP, 5′-TMP, 3′,5′-cTMP and T were all in line with a hydrolytic mechanism, and could be identified within a total of 100 ± 8%. Separate dilution experiments showed that the nucleobase thymine, which would be expected from a radical reaction, can be excluded within a limit of <1% reaction.

The products (Table 4) indicate that two processes are occurring: the cyclic ester 3′,5′-cTMP is the result of an intramolecular attack by either the 3′- or the 5′-OH. This, however, cannot be the only mechanism, since Eu^{III} also cleaves plasmid DNA, in which no 5′-OH is available.[3b,14] The possible pathways (Scheme 1) lead partially to the same products, in particular to



Scheme 1 Possible reaction products from TpT hydrolysis with Eu^{3+}

the 'normal' DNA-type hydrolysis products 3′- and 5′-TMP. The conversion of the cyclic product 3′,5′-cTMP to the monophosphates 3′- and/or 5′-TMP could not be observed within 14 days' reaction. The slow consecutive reactions of the primary products made it impossible to study the conversion range necessary for a full evaluation of the complex kinetic scheme in Fig. 5. However, the initial rate observed for the disappearance of the dinucleotide TpT yields $k = 2.8 \times 10^{-7}$ s^{-1} within ±3.5% error and allows comparison to the uncatalyzed rate, for which Chin *et al.*[15] have derived an estimated value of $k = 1 \times 10^{-16}$ s^{-1} at 25 °C, based on a linear free energy correlation of log *k* with diesters of phosphoric acid and the p*K* values of the leaving group. If we allow a limiting factor of 100 for the temperature difference between 25 and 70 °C, the uncatalyzed hydrolysis at 70 °C should proceed with $k = 10^{-14}$ s^{-1}. The rate acceleration factor then is $>10^7$ for $[Eu^{III}] = 5$ mM. This rate enhancement is even higher than that observed with the activated model ester bis(nitrophenyl) phosphate BNPP.[3a] This indicated that good leaving groups in the substrate do not enhance the efficiency of these artifical nucleases, in accordance with our earlier finding that biphenyl phosphate hydrolysis is also catalyzed more strongly than that of BNPP.

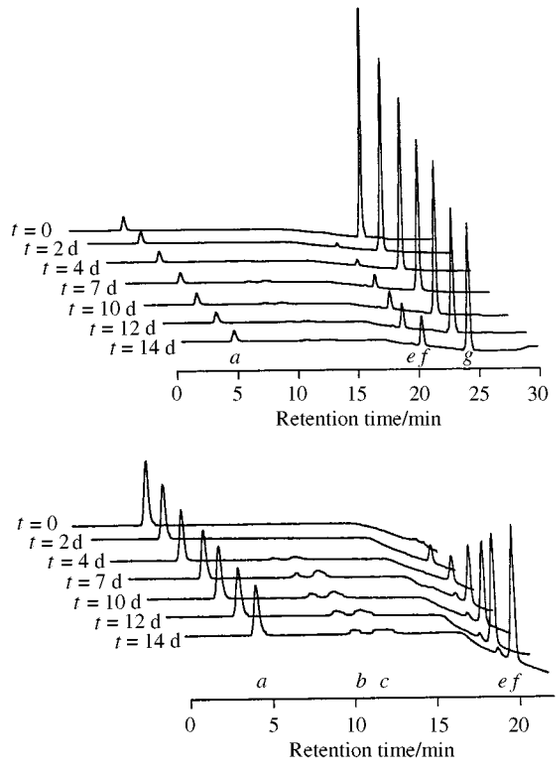


Fig. 4 HPLC chromatograms of TpT-hydrolysis with 4 mM Eu^{3+} at 70 °C and pH 7.0 in 0.01 M EPPS buffer and incubation times from 0–14 days. Whole chromatograms (top) and enlarged cutouts (bottom): (*a*) isonicotinic acid; (*b*) 5′-TMP; (*c*) 3′-TMP; (*e*) 3′,5′-cTMP, (*f*) thymidine, (*g*) TpT.

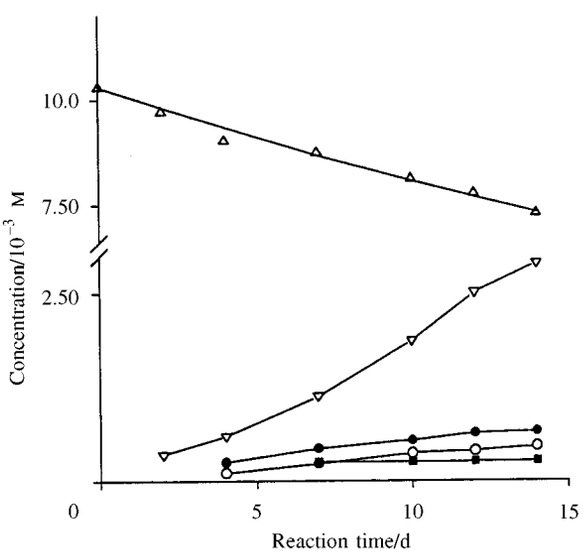


Fig. 5 Concentration of TpT (△), thymidine (▽), 3′-TMP (●), 5′-TMP (○) and 3′,5′-cTMP (■) during TpT hydrolysis with $EuCl_3$

Experimental

Determination of rate constants was performed as described earlier.[3a–c] The complex [(cyclen)$CoCl_2$]Cl was synthesized according to the literature procedure.[16]

Ligation of cleaved DNA

Commercial pBR 322-DNA (Pharmacia) (12.5 μl) was added to water (37.5 μl) (for Co^{III}–cyclen complex) or 0.01 M EPPS-buffer (for Eu^{3+} or copper complex) and the cleaving reagent (50 μl) in the same solvent. After incubation at 37 °C the reaction was stopped by addition of 0.6 M EDTA (Eu^{3+} and copper complex) or 1 M potassium cyanide (Co^{III}–cyclen complex) and

Table 2 Optical densities (OD) of DNA bands from the ligation of Co^{III}–cyclen-cleaved DNA (Fig. 2), corrected for background staining

Lane	Cleavage with Co^{III}–Cyclen	Addition of ligation mix	OD RF I	RF II	RF I*
1	—	—	100	23	—
2	—	+	75	17	—
3	+	—	71	51	—
4	+	+	32	30	17

Table 3 Optical densities (OD) of DNA bands from the ligation[a] of Cu^{II} **2**-cleaved DNA (Fig. 3) corrected for background staining

Lane	Cleavage with Cu^{II} **2**[b]	Addition of ligation mix	OD RF I	RF II	RF I*
1	—	—	187	52	<10
2	—	+	165	45	<10
3	+	—	139	120	<10
4	+	+	100	89	<10

[a] After 1 h incubation, 37 °C, EPS buffer pH 7.0. [b] With Cu^{II} in presence of 1×10^{-4} M hydrogen peroxide; for other conditions see Experimental section.

Table 4 Concentration of detected substances from TpT hydrolysis with Eu^{3+} after different incubation times

t/d	[5′-TMP]/ 10^{-6} M	[3′-TMP]/ 10^{-6} M	[3′,5′-cTMP]/ 10^{-6} M	[Thymidine]/ 10^{-6} M	[TpT]/ 10^{-5} M
0	—	—	—	—	10.30
2	—	—	—	3.49	9.71
4	1.05	2.46	—	5.89	9.03
7	2.30	4.30	2.46	11.2	8.74
10	3.66	5.53	2.48	18.6	8.12
12	3.94	6.27	2.56	25.1	7.77
14	4.55	6.54	2.63	29.0	7.29

centrifuged over an ion-exclusion column (MicroSpin S-300 HR, Pharmacia), which was equilibrated with MES-buffer pH 6 before use. The eluate was filled up to 200 µl and stored at −18 °C before use. For ligation the cleaved DNA (4 µl) was mixed with MES-buffer (4 µl), a solution of $MnCl_2 \cdot 4H_2O$ in MES-buffer (19.8 mg in 10 ml; 1 µl) and a solution of imidazole cyanide[7] in MES-buffer (16.1 mg in 3 ml; 1 µl). Ligation was carried out for 16 h at 20 °C.

HPLC measurements

These were carried out on a Waters 600E system (Millipore) in combination with a reversed-phase-ET 250/4 Nucleosil 100 $5C_{18}$-column (5 µm, Machery & Nagel). Detection of the peaks was performed online with a LC-spectrophotometer Lambda-Max 481 (Millipore) at 265 nm. Mobile phase was a phosphate buffer–methanol gradient (0–10 min: pure buffer of 1.361 g KH_2PO in 1 l water, pH 5.6; 10–35 min from 0–60% methanol). All solvents were microfiltered, degassed and saturated with helium before use.

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