Base moiety selectivity in cleavage of short oligoribonucleotides by di- and tri-nuclear Zn(II) complexes of azacrown-derived ligands†

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Cleavage of 6-mer oligoribonucleotides by the dinuclear Zn^{2+} complex of 1,3-bis[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene (**L**^{1}) and the trinuclear Zn^{2+} complex of 1,3,5-tris[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene (**L**^{3}) has been studied. The dinuclear complex cleaves at sufficiently low concentrations ($[(Zn^{2+})_2\mathbf{L}^1] \leq 0.1$ mmol L^{-1}) the $^{5'}$NpU$^{3'}$ and $^{5'}$UpN$^{3'}$ bonds (N = G, C, A) much more readily than the other phosphodiester bonds, but leaves the $^{5'}$UpU$^{3'}$ site intact. The trinuclear $(Zn^{2+})_3\mathbf{L}^3$ complex, in turn, cleaves the $^{5'}$UpU$^{3'}$ bond more readily than any other linkages, even faster than the $^{5'}$NpU$^{3'}$ and $^{5'}$UpN$^{3'}$ sites. Somewhat unexpectedly, the $^{5'}$UpNpU$^{3'}$ site is cleaved only slowly by both the di- and tri-nuclear complex. The base-moiety selectivity remains qualitatively similar, though slightly less pronounced, when the hexanucleotides are closed to hairpin loops by three additional CG-pairs of 2′-*O*-methylribonucleotides. Phosphodiester bonds within a double helical stem are not cleaved, not even the $^{5'}$UpU$^{3'}$ sites. Guanine base also becomes recognized by $(Zn^{2+})_2\mathbf{L}^1$ and $(Zn^{2+})_3\mathbf{L}^3$, but the affinity to G is clearly lower than to U. The trinuclear cleaving agent, however, cleaves the $^{5'}$GpG3′ bond only 35% less readily than the $^{5'}$UpU3′ bond.

Introduction

Dinuclear Zn^{2+} complexes have received considerable interest as chemical models of enzymes catalyzing phosphoryl transfer and as constituents of artificial RNases.[1–9] Most of the studies have been inspired by the concept of double Lewis acid activation, *i.e.* the marked acceleration of phosphodiester cleavage by dinuclear complexes capable of simultaneous binding to both of the non-bridging oxygen atoms.[10] Much less attention has been paid to the fact that dinuclear complexes may simultaneously interact with the base and phosphate moieties of RNA and, hence, induce base moiety selective cleavage. In this respect, azacrown complexes of Zn^{2+} are of particular interest, since they, besides their marked cleaving activity,[11] recognize uracil and thymine bases.[12]

We have shown previously that Zn^{2+} complexes of di- and tri-azacrown ligands exhibit as cleaving agents considerable base moiety selectivity when dinucleoside-3′,5′-phosphates are used as substrates.[8a,8c] Among the four possible dinucleoside-3′,5′-phosphates derived from A and U, the heterodimers, $^{5'}$ApU$^{3'}$ and $^{5'}$UpA$^{3'}$, are cleaved by the dinuclear Zn^{2+} complexes of 1,3- (**L**^{1}) and 1,4- (**L**^{2}) bis[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene up to two orders of magnitude more readily than $^{5'}$UpU$^{3'}$ or $^{5'}$ApA$^{3'}$. The trinuclear complex of 1,3,5-tris[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene (**L**^{3}), in turn, cleaves $^{5'}$UpU$^{3'}$ as readily as $^{5'}$ApU$^{3'}$ and $^{5'}$UpA$^{3'}$, while the cleavage of $^{5'}$ApA$^{3'}$ remains slow.[8c] Kinetic, UV-spectrophotometric and ^{1}H NMR spectroscopic studies suggest that with $^{5'}$ApU$^{3'}$ and $^{5'}$UpA$^{3'}$, one of the Zn^{2+}-azacrown moieties anchors the cleaving agent to deprotonated N3-site of the uracil base, while the other azacrown moiety serves as a cleaving agent. With $^{5'}$UpU$^{3'}$, both of the azacrown moieties are engaged in the base moiety binding. The catalytic activity of $(Zn^{2+})_2\mathbf{L}^1$ is, hence, lost, but it can be restored by addition of a third azacrown group, as with $(Zn^{2+})_3\mathbf{L}^3$. For comparison, di- and tri-nuclear Cu^{2+} complexes of calix[4]arenes bearing azacrown ligands at the upper rim have been observed to preferably cleave uracil containing dinucleoside-3,5-phosphates.[13a]

Besides uracil, guanine base offers a site of anchoring for the Zn^{2+} complexes of ligands **L**^{2} and **L**^{3}.[8a] Binding to N1-deprotonated guanine is, however, considerably weaker than binding to N3-deprotonated uracil. Hence, dinucleoside-3′,5′-phosphates that consist of one guanosine and one adenosine or cytidine are cleaved by the dinuclear Zn^{2+} complex of **L**^{2} approximately one order of magnitude less readily than their uracil counterparts, but still three times as fast as the dimers that contain only adenine and/or cytosine bases.

The present study has been undertaken to find out whether this kind of base moiety selectivity also operates at oligonucleotide level, *i.e.* in the presence of a plethora of binding sites. For this purpose, a set of 6-mer oligoribonucleotides (**1–10** in Scheme 1) have been synthesized and their susceptibility to hydrolysis by the Zn^{2+} complexes of ligands **L**^{1} and **L**^{3} has been studied. To find out whether the situation within hairpin loops is similar to that with relaxed single strands, some representatives of the hexanucleotides have been closed to hairpin loops with three additional GC pairs of 2′-*O*-methylribonucleotides (**11–13**). In addition, the cleavage of an 18-mer oligoribonucleotide hairpin (**14**) containing a $^{5'}$UpU$^{3'}$/$^{3'}$ApA$^{5'}$ site within the double helical stem has been studied to find out whether the trinuclear $(Zn^{2+})_3\mathbf{L}^3$ complex could by binding to the $^{5'}$UpU$^{3'}$ site displace the complementary strand and then cleave the intervening phosphodiester bond. In other words, could this cleaving agent be utilized to hydrolyze a phosphodiester bond within a double helical stem, which is known

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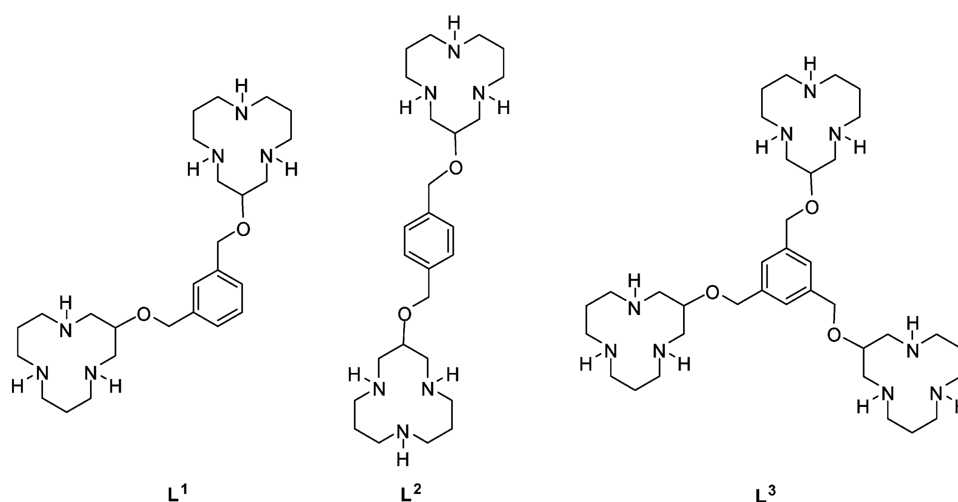


1 5'-CAACAC-3'

2 5'-CAAUAC-3'

3 5'-CAUUCA-3'

4 5'-CUAUCA-3'

5 5'-CUACUA-3'

6 5'-CAAGAC-3'

7 5'-CAGGCA-3'

8 5'-CAGUCA-3'

9 5'-CGAGCA-3'

10 5'-CGACGA-3'

11 5'-*GCG* C A A
3´-*CGC* C A C

12 5'-*GCG* C A A
3´-*CGC* A C U

13 5'-*GCG* C A U
3´-*CGC* A C U

14 5'-*GGC* GUU C *A A*
3´-*CCG* CAA G *G C*

2´-*O*-Methylribonucleosides in italics

Scheme 1 Di- and-tri-nucleating azacrown ligands and oligonucleotides used in the present study.

to withstand prolonged treatment with monomeric azacrown chelates of Zn^{2+}.[11a,11b] To the best of our knowledge, previous studies on base moiety selective cleavage of oligonucleotides by metal ion complexes are limited to the recent observation that di- and tri-nuclear Cu^{2+} complexes of azacrown-decorated calix[4]arenes prefer hydrolysis of CpA phosphodiester bonds.[13b]

Results and discussion

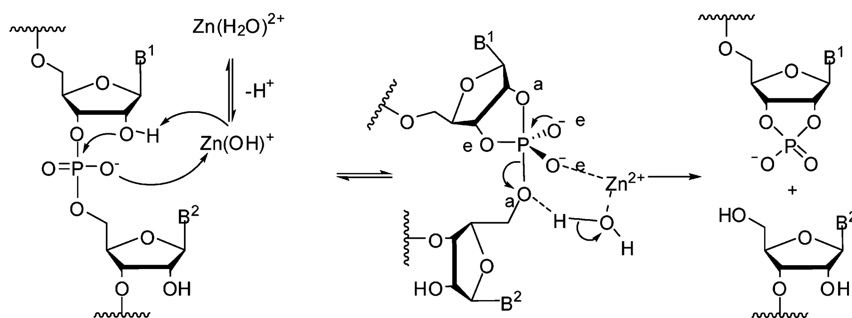
Syntheses

The synthesis of the di- and tri-nucleating azacrown ligands, 1,3-bis[(1,5,9-triazacyclododecan-3-yloxy)methyl]benzene (**L**[1]) and 1,3,5-tris[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene (**L**[3]), has been reported previously.[8c] Oligoribonucleotides **1–14** were prepared by standard phosphoramidite protocol, using 2′-*O*-triisopropylsilyloxymethyl (TOM) building blocks and CPG-succinyl-support. The TOM-protecting groups were removed by triethylamine trihydrofluoride treatment in DMSO (65 °C, 30 min), followed by treatment with aq sodium acetate (0.1 mol L^{-1}, 65 °C, 30 min). The crude oligonucleotides were purified by RP-HPLC and their identity was verified by ESI-MS.

Kinetic measurements and determination of the cleavage site

As shown previously,[14] the Zn^{2+}-promoted cleavage of oligoribonucleotides proceeds by an attack of the 2′-hydroxy group on the phosphorus atom with concomitant departure of the 5′-linked nucleoside (Scheme 2). The role of the metal ion evidently is to facilitate the proton transfer from the attacking nucleophile (2′-O) to the departing nucleophile (5′-O), the latter step being rate-limiting.[15] The 2′,3′-cyclic phosphate obtained is finally hydrolyzed to a mixture of 2′- and 3′-phosphates.

The progress of the Zn^{2+}-complex promoted cleavage of oligonucleotides **1–14** was followed at pH 7.5 and 35 °C by withdrawing aliquots from the reaction solution at suitable intervals and analyzing their composition by capillary electrophoresis. Potassium 4-nitrotoluenesulfonate was used as an internal standard. All kinetic measurements were carried out in excess of the cleaving agent, either $(Zn^{2+})_2$**L**^{1} or $(Zn^{2+})_3$**L**^{3}. The concentration of the



Scheme 2 Mechanism of Zn^{2+}-promoted cleavage of RNA phosphodiester bonds (a = apical ligand, e = equatorial ligand).

target oligonucleotide was 50 μmol L^{-1}. The disappearance of the starting material followed first-order kinetics, as exemplified by Fig. 1. The kinetic data obtained is indicated in Tables 2–3 and Figs. 2 and 3, and it is discussed in more detail below.

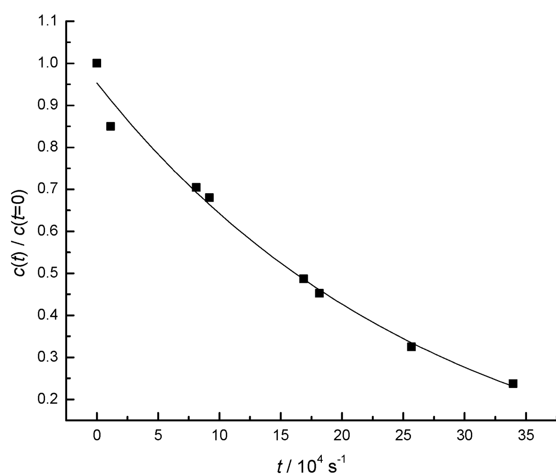


Fig. 1 Cleavage of hexanucleotide 5′-CGAGCA-3′ by the $(Zn^{2+})_3$**L**[3] complexes in HEPES buffer (0.10 mol L^{-1}, pH 7.5, 35 °C, I = 0.10 mol L^{-1}).

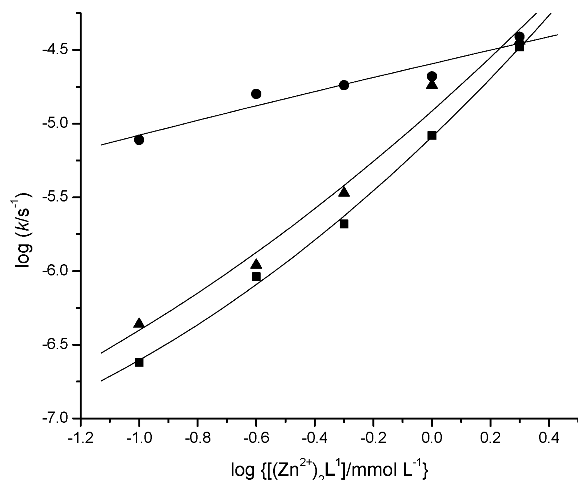


Fig. 2 Logarithmic rate constants for the breakdown of $^{5'}$CAACAC$^{3'}$ (**1**;■), $^{5'}$CAAUAC$^{3'}$ (**2**;●), and $^{5'}$CAUUCA$^{3'}$ (**3**;▲) as a function of the logarithmic concentration of $(Zn^{2+})_2$**L**[1] at pH 7.5 and 35 °C (I = 0.1 mol L^{-1}).

The preferred cleavage sites were determined for oligonucleotides that were rapidly hydrolyzed and, hence, selective cleavage of some phosphodiester bonds by the base moiety anchored cleaving agent was expected. The reactions were allowed to proceed for 2–3 half-lives and the reaction mixtures were then subjected to RP-HPLC separation. Major peaks were collected and identified by ESI-MS. The HPLC-chromatograms and MS data are given as the Supporting information. The predominant cleavage sites are listed in Table 1.

Cleavage of oligonucleotides containing only A, C and U

Figs. 2 and 3 show the logarithmic first-order rate constants for the cleavage of oligonucleotides $^{5'}$CAACAC$^{3'}$ (**1**), $^{5'}$CAAUAC$^{3'}$ (**2**), and $^{5'}$CAUUCA$^{3'}$ (**3**) as a function of the logarithmic concentration of $(Zn^{2+})_2$**L**[1] and $(Zn^{2+})_3$**L**[3], respectively. As seen, all the oligonucleotides are cleaved approximately as readily at the high concentration of the cleaving agent (2.0 mmol L^{-1}), the cleaving activity of $(Zn^{2+})_3$**L**[3] being 2- to 3-fold compared to that of $(Zn^{2+})_2$**L**[1]. Accordingly, the presence of one or two uracil bases as the preferred site of anchoring of the cleaving agent does not result in any observable rate acceleration, but random cleavage of the phosphodiester bonds predominates. The higher cleaving activity of the trinuclear complex may, at least partly, be attributed to the 1.5 times higher overall concentration of the Zn^{2+} azacrown chelate (Zn^{2+}[12]aneN_3) moieties. On going to low concentrations of the cleaving agent (0.10 mmol L^{-1}) the situation, however, dramatically changes. When the dinuclear $(Zn^{2+})_2$**L**[1] is used as a catalyst, oligonucleotide **2**, containing one U, is cleaved 30 times as fast as **1**, containing only C and A, and almost 20 times as fast as **3**, containing two adjacent U residues. According to MS-analysis of the HPLC separated products, the cleavage of **2** predominantly

Table 1 Predominant cleavage sites (↓) for the sequence-selective breakdown of 6-mer oligoribonucleotides by $(Zn^{2+})_2$**L**[1] or $(Zn^{2+})_3$**L**[3] in 0.10 mol L^{-1} HEPES buffer (0.10 mol L^{-1}, pH 7.5, 35 °C, I = 0.10 mol L^{-1})

Oligonucleotide	Complex	Sites of cleavage
2	$(Zn^{2+})_2$**L**[1]	5′-CAA↓U↓AC-3′
2	$(Zn^{2+})_3$**L**[3]	5′-CAAU↓AC-3′
3	$(Zn^{2+})_3$**L**[3]	5′-CAU↓UAC-3′
5	$(Zn^{2+})_3$**L**[3]	5′-CUAC↓U↓A-3′
6	$(Zn^{2+})_3$**L**[3]	5′-CAAG↓AC-5′
7	$(Zn^{2+})_3$**L**[3]	5′-CAG↓GCA-3′
8	$(Zn^{2+})_3$**L**[3]	5′-CA↓G↓U↓CA-3′
9	$(Zn^{2+})_3$**L**[3]	5′-C↓G↓A↓G↓CA-3′
10	$(Zn^{2+})_3$**L**[3]	5′-C↓G↓AC↓G↓A-3′

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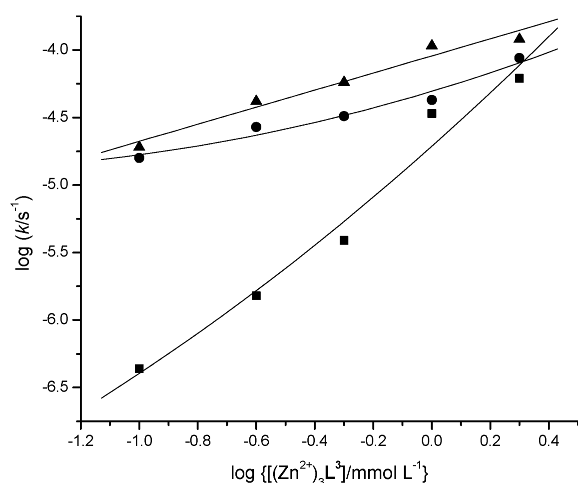

Fig. 3 Logarithmic rate constants for the breakdown of $^{5'}$CAACAC$^{3'}$ (**1**;■), $^{5'}$CAAUAC$^{3'}$ (**2**;●), and $^{5'}$CAUUCA$^{3'}$ (**3**;▲) as a function of the logarithmic concentration of $(Zn^{2+})_2$**L**^{1} at pH 7.5 and 35 °C (I = 0.1 mol L^{-1}).

takes place by rupture of the phosphodiester bonds neighboring the U residue, *i. e.* the $^{5'}$ApU$^{3'}$ and $^{5'}$UpA$^{3'}$ bonds (Table 1). Evidently anchoring of one of the Zn^{2+}[12]aneN_3 moieties of $(Zn^{2+})_2$**L**^{1} to the uracil base keeps the other Zn^{2+}[12]aneN_3 moiety in the vicinity of the neighboring phosphodiester bonds, resulting in site-selective cleavage. In the presence of two adjacent U residues, both Zn^{2+}[12]aneN_3 moieties are engaged in nucleobase binding and the cleavage of oligonucleotide **3**, hence, remains slow. Addition of a third Zn^{2+}[12]aneN_3 moiety to the cleaving agent, however, changes the situation. As seen from Fig. 3, oligonucleotide **3** is hydrolyzed in the presence of $(Zn^{2+})_3$**L**^{3} as fast as **2**. Only the $^{5'}$UpU$^{3'}$ linkage is now selectively cleaved (Table 1). With **2**, cleavage at the 3′-side of U predominates. In summary, at a low concentration of the cleaving agent, a selectivity comparable to that previously[8c] reported for dinucleoside-3′,5′-phosphates is achieved, while at high catalyst concentrations random cleavage of phosphodiester bonds predominates.

The fact that the cleavage reaction exhibits base moiety selectivity only at low concentrations of the cleaving agent is expected. We have shown previously that the second-order rate constant for the cleavage of individual phosphodiester bonds within an oligonucleotide structure by the Zn^{2+}[12]aneN_3 complex fall in the range 4.0×10^{-4} L mol^{-1} s^{-1}–2.6×10^{-3} s^{-1} under the conditions used in the present study.[11a] The cleaving activity of Zn^{2+} aqua ion is very similar, the second order rate constants ranging from 3.9×10^{-4} L mol^{-1} s^{-1} to 2.5×10^{-3} L mol^{-1} s^{-1}. In addition, studies with dinucleoside-3′,5′-phosphates indicate that neither of these catalysts exhibit any marked base selectivity. [8a,c,14b] Accordingly, an average second-order rate constant for the cleavage of a single phosphodiester bond by a Zn^{2+} azacrown complex or Zn^{2+} aqua ion is 1.5×10^{-3} L mol^{-1} s^{-1} and, hence, the rate constant for the disappearance of a 6-mer oligonucleotide is 7.5×10^{-3} L mol^{-1} s^{-1}. At the highest concentration of the cleaving agent, $[(Zn^{2+})_2$**L**$^1] = 2.0$ mmol L^{-1}, *i.e.* in 40-fold excess of the oligonucleotide target (50 μmol L^{-1}), the rate constant for the disappearance of **1–3** by random cleavage (without any base moiety anchoring) may be expected to be 3×10^{-5} s^{-1}. This estimation is in excellent agreement with the experimental values: 3.3×10^{-5} s^{-1}, 3.9×10^{-5} s^{-1} and 3.6×10^{-5} s^{-1} obtained with **1**, **2** and **3**, respectively. On going to the lowest concentration of $(Zn^{2+})_2$**L**^{1} (0.1 mmol L^{-1}), the rate of the random cleavage should be decelerated by a factor of 20 or 40, in the absence or presence of the uracil-anchoring, respectively. In fact, the random background cleavage appears to be slower, since the observed rate constant for the disappearance of **1**, *viz.* 2.4×10^{-7} s^{-1}, is considerably smaller than the predicted value 1.6×10^{-6} s^{-1}. Anyway, the random cleavage becomes so slow that the effect of uracil-anchoring becomes clearly visible. Qualitatively similar considerations apply to the cleavage promoted by $(Zn^{2+})_3$**L**^{3}. At 2 mmol L^{-1} concentration of the cleaving agent, the rate constants for the random cleavage may be estimated to be 4.5×10^{-5} s^{-1}, in reasonable agreement with the experimental value of 7.1×10^{-5} s^{-1} for **1**. At 0.1 mmol L^{-1} concentration, in turn, the predicted value for **1** is 2.3×10^{-6} s^{-1} and the observed one is again considerably smaller, *viz.* 4.4×10^{-7} s^{-1}.

It is worth noting that the lowest concentration of $(Zn^{2+})_2$**L**^{1} and $(Zn^{2+})_2$**L**^{2} (0.10 mmol L^{-1}) in the cleaving experiments is only double compared to the concentration of the oligonucleotide. Our previous studies,[8c] however, show that the formation of ternary complex between $^{5'}$UpU$^{3'}$ and $(Zn^{2+})_2$**L**^{1} is quantitative under such conditions. In other words, half of $(Zn^{2+})_2$**L**^{1} is engaged in the ternary complex with the oligonucleotide. The rest uncomplexed $(Zn^{2+})_2$**L**^{1} undoubtedly is partially dissociated, since the binary complex is considerably less stable than the ternary complex.[8c] The concentration of uncomplexed Zn^{2+} must, however, remain below 0.1 mmol L^{-1} and, as discussed above, such a low concentration does not result in marked cleavage.

Table 2 records the rate constants observed for the hydrolysis of a larger selection of A, C and U containing oligonucleotides at a low concentration (0.10 mmol L^{-1}) of $(Zn^{2+})_2$**L**^{1} or $(Zn^{2+})_3$**L**^{3}. Among the oligomers studied, **4** and **5** contain two uracil bases, like oligomer **3** discussed above, but separated by one (A) or two ($^{5'}$ApU$^{3'}$) intervening nucleosides, respectively. As indicated before, the presence of two adjacent uracil bases prevents the catalytic action of the dinuclear $(Zn^{2+})_2$**L**^{1} complex, but allows efficient cleavage of the intervening phosphodiester bond by the trinuclear $(Zn^{2+})_3$**L**^{3} complex. The dinuclear $(Zn^{2+})_2$**L**^{1} evidently bridges two uracil bases even when they are separated by one nucleoside, since

Table 2 First-order rate constants for the cleavage of oligoribonucleotides containing A, C and U by $(Zn^{2+})_2$**L**^{1} and $(Zn^{2+})_3$**L**^{3} (0.10 mmol L^{-1}) in HEPES buffer (0.10 mol L^{-1}) at pH 7.5 and 35 °C (I = 0.10 mol L^{-1})

Oligonucleotide	Complex	k/10^{-6} s^{-1}
1 $^{5'}$CAACAC$^{3'}$	$(Zn^{2+})_2$**L**^{1}	0.24 ± 0.03
	$(Zn^{2+})_3$**L**^{3}	0.44 ± 0.03
2 $^{5'}$CAAUAC$^{3'}$	$(Zn^{2+})_2$**L**^{1}	7.70 ± 0.05
	$(Zn^{2+})_3$**L**^{3}	15.6 ± 0.2
3 $^{5'}$CAUUCA$^{3'}$	$(Zn^{2+})_2$**L**^{1}	0.44 ± 0.05
	$(Zn^{2+})_3$**L**^{3}	19.8 ± 0.4
4 $^{5'}$CUAUCA$^{3'}$	$(Zn^{2+})_2$**L**^{1}	0.27 ± 0.02
	$(Zn^{2+})_3$**L**^{3}	1.84 ± 0.03
5 $^{5'}$CUACUA$^{3'}$	$(Zn^{2+})_2$**L**^{1}	1.12 ± 0.16
	$(Zn^{2+})_3$**L**^{3}	16.2 ± 0.5
11 $^{5'}$*GCG*-CAACAC-*CGC*$^{3'}$ (hairpin)	$(Zn^{2+})_2$**L**^{1}	0.48 ± 0.20
	$(Zn^{2+})_3$**L**^{3}	0.68 ± 0.05
12 $^{5'}$*GCG*-CAAUCA-*CGC*$^{3'}$ (hairpin)	$(Zn^{2+})_2$**L**^{1}	3.15 ± 0.07
	$(Zn^{2+})_3$**L**^{3}	2.57 ± 0.09
13 $^{5'}$*GCG*-CAUUCA-*CGC*$^{3'}$ (hairpin)	$(Zn^{2+})_2$**L**^{1}	0.39 ± 0.05
	$(Zn^{2+})_3$**L**^{3}	5.66 ± 0.50

oligomer **4** containing a sequence $^{5'}$UpApU$^{3'}$ remains stable in the presence of this complex, similar to **3** containing a $^{5'}$UpU$^{3'}$ site. In striking contrast to the rapid cleavage of **3** by $(Zn^{2+})_3\mathbf{L}^3$, **4** is cleaved only slowly by this complex. The intervening nucleoside (A) somehow appears to prevent the interaction of the third Zn^{2+}[12]aneN$_3$ moiety of the $^{5'}$UpApU$^{3'}$-anchored cleaving agent with the $^{5'}$UpA$^{3'}$ and $^{5'}$ApU$^{3'}$ bonds.

Oligomer **5**, having the two uracil bases separated by two nucleosides ($^{5'}$ApC$^{3'}$), behaves like oligomer **2**, containing a single U, in the sense that it is readily cleaved by $(Zn^{2+})_3\mathbf{L}^3$. However, even in this case simultaneous binding to both uracil bases seems to play a role, since cleavage by the dinuclear $(Zn^{2+})_2\mathbf{L}^1$ remains relatively slow. The reason why among the two uridine residues the one close to the 3′-terminus constitutes the predominant cleavage site of $(Zn^{2+})_3\mathbf{L}^3$ (Table 1) remains obscure.

To find out whether the base-selectivity of the cleavage reaction within hairpin loops is similar to that within relaxed single strands, hexanucleotide loops (**11–13**) were generated using three GC pairs of 2′-*O*-methylribonucleotides to close the loop. It has been reported[16] that oligoribonucleotides consisting of three closing GC-base pairs and an intervening non-self-complementary sequence of 4–9 nucleotides adopt at 37 °C and pH 7.0 (I = 0.1 mol L^{-1} or 1.0 mol L^{-1}) a hairpin loop structure. No dimerization to structures containing an internal loop has been observed at oligonucleotide concentrations less than 1 mmol L^{-1}. The melting point of these hairpins ranges from 55 to 70 °C. For example, a hairpin $^{5'}$GCGUGACAGCCGC$^{3'}$, closely resembling oligonucleotides **11–13** has a melting point of 56.8 °C. For comparison, oligomer **11** melts at 53.7 °C. Hairpin **14** is, in turn, closed by four GC-pairs. We have shown previously[11b] that hexa- and hepta-nucleotide loops closed with 4 CG base-pairs of 2′-*O*-oligoribonucleotides exhibit at pH 7.0 (I = 0.1 mol L^{-1} with NaCl) concentration independent melting points of 87 ± 1 °C and 89 ± 1 °C, respectively. Unfortunately, the melting temperatures cannot be determined in the presence of the cleaving agents, since the concomitant reasonably fast hydrolysis inevitably interferes with the measurement at elevated temperatures. Binding of the cleaving agent to a uracil base within the loop structure possibly induces some conformational changes, but it hardly results in such a strain that the hairpin structure would be opened. In all likelihood the hairpin structure still predominates at 35 °C, *i. e.* 20 °C below its inherent melting temperature.

Comparison of the cleavage rates of hairpins **11–13** with those of the corresponding linear hexanucleotides **1–3** (Table 2) reveals that the selectivity within loops is qualitatively similar to that within linear single strands, although somewhat less pronounced. For example, $^{5'}$CAUUCA$^{3'}$ (**3**) is cleaved with $(Zn^{2+})_3\mathbf{L}^3$ 45 times as rapidly as $^{5'}$CAACAC$^{3'}$ (**1**). When the same sequences are incorporated into a loop structure, the ratio of their cleavage rates is 8.3. Replacement of the non-terminal C in **1** with U to obtain **2**, in turn, accelerates the cleavage by factors 32 and 35 on using cleaving agents $(Zn^{2+})_2\mathbf{L}^1$ and $(Zn^{2+})_3\mathbf{L}^3$, respectively. Within a hairpin loop, the corresponding accelerations (**12** compared to **11**) are only 6.6 and 3.8.

Cleavage of oligonucleotides containing all four nucleotides

Previous studies[8a] with dinucleoside-3′,5′-phosphates have shown that cleaving agents $(Zn^{2+})_2\mathbf{L}^1$ and $(Zn^{2+})_3\mathbf{L}^3$ also interact with guanine base, although more weakly than with uracil base. To find out whether this weak interaction is sufficient to result in base-selective cleavage at oligonucleotide level, U in oligonucleotides **2–5** was replaced with G. The kinetic data for the cleavage of the oligonucleotides obtained (**6–10**) is given in Table 3.

Table 3 First-order rate constants for the cleavage of oligoribonucleotides containing all four nucleotides by $(Zn^{2+})_2\mathbf{L}^1$ and $(Zn^{2+})_3\mathbf{L}^3$ (0.10 mmol L^{-1}) in HEPES buffer (0.10 mol L^{-1}) at pH 7.5 and 35 °C (I = 0.10 mol L^{-1})

Oligonucleotide	Complex	$k/10^{-6}$ s^{-1}
6 $^{5'}$CAAGAC$^{3'}$	$(Zn^{2+})_2\mathbf{L}^1$	1.02 ± 0.06
	$(Zn^{2+})_3\mathbf{L}^3$	4.66 ± 0.29
7 $^{5'}$CAGGCA$^{3'}$	$(Zn^{2+})_2\mathbf{L}^1$	1.92 ± 0.12
	$(Zn^{2+})_3\mathbf{L}^3$	12.8 ± 0.91
8 $^{5'}$CAGUCA$^{3'}$	$(Zn^{2+})_2\mathbf{L}^1$	2.17 ± 0.05
	$(Zn^{2+})_3\mathbf{L}^3$	6.43 ± 0.11
9 $^{5'}$CGAGCA$^{3'}$	$(Zn^{2+})_2\mathbf{L}^1$	1.39 ± 0.06
	$(Zn^{2+})_3\mathbf{L}^3$	4.30 ± 0.06
10 $^{5'}$CGACGA$^{3'}$	$(Zn^{2+})_2\mathbf{L}^1$	1.93 ± 0.04
	$(Zn^{2+})_3\mathbf{L}^3$	4.37 ± 0.08
14 $^{5'}$*GGC*-GUUC*AACG*GAAC-*GCC*$^{3'}$ (hairpin)	$(Zn^{2+})_2\mathbf{L}^1$	0.48 ± 0.20
	$(Zn^{2+})_3\mathbf{L}^3$	0.68 ± 0.05

Several lines of evidence indicate that guanine base is recognized by $(Zn^{2+})_2\mathbf{L}^1$ and $(Zn^{2+})_3\mathbf{L}^3$, but binding to G is clearly weaker than to U. Firstly, oligonucleotide **2** incorporating a single U is cleaved 7.5 and 3.3 times as fast as its G-substituted counterpart **6** by $(Zn^{2+})_2\mathbf{L}^1$ and $(Zn^{2+})_3\mathbf{L}^3$, respectively. The predominant cleavage site of **6** is at the 3′-side of the G residue (Table 1). Secondly, $^{5'}$GpG$^{3'}$-containing **7** is cleaved by dinuclear $(Zn^{2+})_2\mathbf{L}^1$ 4.4 times as fast as the $^{5'}$UpU$^{3'}$-containing **3**. In other words, $(Zn^{2+})_2\mathbf{L}^1$ is not arrested to a $^{5'}$GpG$^{3'}$-site as quantitatively as to a $^{5'}$UpU$^{3'}$-site. In striking contrast to the slow hydrolysis of the $^{5'}$UpU$^{3'}$-containing **3** compared to its $^{5'}$ApU$^{3'}$-containing counterpart **2**, the $^{5'}$GpG$^{3'}$-containing **7** is cleaved even faster than the $^{5'}$ApG$^{3'}$-containing **6**. With trinuclear $(Zn^{2+})_3\mathbf{L}^3$, **7** is cleaved 35% more slowly than **3**, the cleavage expectedly taking place between the two guanosines. Thirdly, replacement of the $^{5'}$ApU$^{3'}$ site in **2** with a $^{5'}$GpU$^{3'}$ site (**8**) results in a 3.5-fold deceleration on the cleavage by $(Zn^{2+})_2\mathbf{L}^1$. The guanine base evidently competes with the scissile phosphodiester linkages for the remaining Zn^{2+}[12]aneN$_3$ moiety of the uracil-anchored cleaving agent. $(Zn^{2+})_3\mathbf{L}^3$-promoted cleavage is decelerated by a factor of 2.4, most likely due to lower affinity to the $^{5'}$GpU$^{3'}$ site. Hydrolysis of the $^{5'}$GpU$^{3'}$ bond clearly predominates, but the neighboring phosphodiester bonds are also cleaved to a detectable extent. Finally, oligonucleotides **9** and **10** containing two G residues separated by one and two nucleosides, respectively, are cleaved by both the di- and tri-nuclear complex approximately as rapidly as oligonucleotide **6** incorporating only a single G. Phosphodiester bonds on both sides of the G residues are cleaved. Accordingly, no indication of simultaneous binding of the guanine bases to either of the cleaving agents is obtained.

It has been known since the early studies of Usher and McHale[17] that the phosphodiester bonds are hydrolyzed within a double helix much less readily than within a single strand, because stacking between the neighboring base-pairs prevents the 5′-linked nucleoside to adopt within the phosphorane intermediate an

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apical position required for the PO5′ bond rupture. More recent studies have shown that this is also the case on using Zn^{2+}[12]aneN_3 as a cleaving agent.[11a,11b] Since Zn^{2+}[12]aneN_3 binds to N3 and O^4 of uracil, *i.e.* to atoms engaged in Watson-Crick hydrogen bonding within a double helix, one might speculate that $(Zn^{2+})_3$**L**^{3} could possibly perform a chain invasion at a $^{5'}$UpU$^{3'}$ site and cleave the intervening phosphodiester bond. To find out whether this really is the case, an 18-mer hairpin structure (**14**) consisting of a $^{5'}$ApApCpG$^{3'}$ tetraloop of 2′-*O*-methylribonucleosides and a potential $^{5'}$UpU$^{3'}$/$^{3'}$ApA$^{5'}$ cleavage site in the stem was prepared. The stem additionally contained one CG-pair on both sides of the $^{5'}$UpU$^{3'}$/$^{3'}$ApA$^{5'}$ site and three terminal GC-pairs of 2′-*O*-methylribonucleotides. Both agents, $(Zn^{2+})_2$**L**^{1} and $(Zn^{2+})_3$**L**^{3}, cleaved this hairpin only slowly and at a comparable rate. Evidently, neither the di- nor tri-nuclear complex was able to cleave phosphodiester bonds within the double helical stem.

Conclusion

Cleavage of short oligoribonucleotides by a di- and tri-nuclear Zn^{2+} complex of [12]aneN_3-derived ligands containing additionally an ether oxygen as a potential H-bond acceptor site has been studied. The results show that the marked base-moiety selectivity previously[8a,c] reported for dinucleoside-3′,5′-phosphates is sufficient to allow selective cleavage of short oligonucleotides in spite of the presence of a plethora of potential binding sites. Accordingly, the dinuclear complex cleaves at sufficiently low concentrations ($[(Zn^{2+})_2\mathbf{L}^1] \leq 0.1$ mmol L^{-1}) the $^{5'}$NpU$^{3'}$ and $^{5'}$UpN$^{3'}$ bonds (N = G, C, A) more readily than the other phosphodiester bonds, but leaves the $^{5'}$UpU$^{3'}$ sites intact. The trinuclear $(Zn^{2+})_3$**L**^{3} complex, in turn, cleaves the $^{5'}$UpU^3 bonds more readily than any other linkages, even faster than the $^{5'}$NpU$^{3'}$ and $^{5'}$UpN$^{3'}$ sites. Interestingly, an intervening nucleoside between two U residues ($^{5'}$UpNpU$^{3'}$), however, prevents the catalytic action. Within a double helix, a $^{5'}$UpU$^{3'}$ bond is not cleaved. In addition to uracil, guanine base is also recognized by $(Zn^{2+})_2$**L**^{1} and $(Zn^{2+})_3$**L**^{3}, but the affinity to G is clearly lower than to U. The trinuclear cleaving agent still cleaves the $^{5'}$GpG3′ bonds only 35% less readily than the $^{5'}$UpU3′ bonds. The base-moiety selectivity remains qualitatively similar, though somewhat less pronounced, when the sequences are closed to hexanucleotide hairpin loops by three additional CG-pairs of 2′-*O*-methylribonucleotides.

While the observations discussed above encourage one to continue development of small molecule cleaving agents specific for sequences of only a few bases, it is quite clear that the complexes studied in the present paper do not yet allow manipulation of really long RNA sequences. Introduction of a single uracil base into a 6-mer oligonucleotide results in a 30-fold rate-acceleration. Since the rate of the random background cleavage is proportional to the number of phosphodiester bonds in the oligomer, the site-selectivity of the cleavage is gradually lost on approaching 30 nucleotides long sequences. Similarly, though binding to two contiguous uracil bases is preferred over binding to a single uracil, the difference in stability is not sufficient for selective cleavage of UpU sites within long RNA sequences. Nevertheless, the cleaving agents studied may well find useful applications as chemical probes for the structural elucidation of medium size RNA molecules, such as tRNA.

Experimental section

Synthesis of oligonucleotides and cleaving agents

Ligands **L**^{1} and **L**^{3} were prepared as described previously.[8c] Oligoribonucleotides **1–14** were synthesized by conventional phosphoramidite strategy from 2′-*O*-triisopropylsilyloxymethyl (2′-*O*-TOM) protected phosphoramidite building blocks (Glenn Research) on a 1 μmol L^{-1} scale, following the standard RNA-coupling protocol of ABI-392 DNA/RNA synthesizer. The 5′-O-DMTr group was removed on-support, except in case of oligonucleotide **14**. Detritylation was then carried out after chromatographic purification by treatment with 80% aq acetic acid (300 μL) at room temperature for 1 h. The solvent was removed by evaporation and the residue was dissolved in water (500 μL). The aqueous layer was washed with EtOAc (2 × 500 μL). The 2′-*O*-TOM protecting groups were removed with triethylamine trihydrofluoride in DMSO (65 °C, 30 min) followed by treatment with aq sodium acetate (0.1 mol L^{-1}, 65 °C, 30 min). The crude oligonucleotides were purified and desalted by RP-HPLC, except oligonucleotide **14** which was desalted by NAPTM-5 column. Chromatographic conditions: a Hypersil ODS column (250 × 4.6 mm, particle size 5 μm), flow rate 1 mL min^{-1}, buffer A = aq NH_4OAc (50 mmol L^{-1}), buffer B = 1:1 mixture of buffer A and MeCN, a linear gradient from 0 to 25% buffer B in buffer A in 25 min, followed by a linear gradient from 25 to 40% buffer B in buffer A in 5 min. All aqueous solutions were prepared in sterilized water and sterilized equipment was used for their handling.

The identity of **1–14** was verified by ESI-MS (Bruker micrOTOF-Q ESI-MS system). **1**: *m/z* obsd. (calcd.) 919.2 (919.6) $[M-2H]^{2-}$, 612.4 (612.7) $[M-3H]^{3-}$, 459.1 (459.3) $[M-4H]^{4-}$. **2**: *m/z* obsd. (calcd.) 919.7 (920.0) $[M-2H]^{2-}$, 612.8 (613.1) $[M-3H]^{3-}$, 459.3 (459.5) $[M-4H]^{4-}$. **3**: *m/z* obsd. (calcd.) 908.2 (908.6) $[M-2H]^{2-}$, 605.1 (605.4) $[M-3H]^{3-}$, 453.6 (453.8) $[M-4H]^{4-}$. **4**: *m/z* obsd. (calcd.) 908.1 (908.6) $[M-2H]^{2-}$, 605.1 (605.4) $[M-3H]^{3-}$. **5**: *m/z* obsd. (calcd.) 1817.3 (1818.2) $[M-H]^{-}$, 908.1 (908.6) $[M-2H]^{2-}$. **6**: *m/z* obsd. (calcd.) 939.2 (939.6) $[M-2H]^{2-}$, 625.8 (626.1) $[M-3H]^{3-}$, 469.1 (469.3) $[M-4H]^{4-}$. **7**: *m/z* obsd. (calcd.) 947.2 (947.6) $[M-2H]^{2-}$, 631.1 (631.4) $[M-3H]^{3-}$, 473.1 (473.3) $[M-4H]^{4-}$. **8**: *m/z* obsd. (calcd.) 927.7 (928.1) $[M-2H]^{2-}$, 618.1 (618.4), $[M-3H]^{3-}$, 463.3 (463.5). **9**: *m/z* obsd. (calcd.) 947.2 (947.6) $[M-2H]^{2-}$, 631.1 (631.4) $[M-3H]^{3-}$, 473.1 (473.3) $[M-4H]^{4-}$. **10**: *m/z* obsd. (calcd.) 947.2 (947.6) $[M-2H]^{2-}$, 631.1 (631.4) $[M-3H]^{3-}$, 473.1 (473.3) $[M-4H]^{4-}$. **11**: *m/z* obsd. (calcd.) 1290.9 (1290.7) $[M-3H]^{3-}$, 967.9 (967.7), $[M-4H]^{4-}$, 774.1 (774.0) $[M-5H]^{5-}$, 644.9 (644.8) $[M-6H]^{6-}$, 552.7 (552.7) $[M-7H]^{7-}$. **12**: *m/z* obsd. (calcd.) 1291.2 (1291.1) $[M-3H]^{3-}$, 968.2 (968.2) $[M-4H]^{4-}$, 774.3 (774.4) $[M-5H]^{5-}$, 645.1 (645.2) $[M-6H]^{6-}$, 552.8 (552.9) $[M-7H]^{7-}$. **13**: *m/z* obsd. (calcd.) 1283.5 (1283.7) $[M-3H]^{3-}$, 962.4 (962.5) $[M-4H]^{4-}$, 769.7 (769.8) $[M-5H]^{5-}$, 641.3 (641.3) $[M-6H]^{6-}$, 549.5 (549.6) $[M-7H]^{7-}$. **14**: *m/z* obsd. (calcd.) 1959.3 (1959.3) $[M-3H]^{3-}$, 1469.2 (1469.2) $[M-4H]^{4-}$, 1175.2 (1175.2) $[M-5H]^{5-}$, 979.1 (979.1) $[M-6H]^{6-}$, 839.1 (839.1) $[M-7H]^{7-}$, 734.1 (734.1) $[M-8H]^{8-}$, 652.4 (652.4) $[M-9H]^{9-}$.

Kinetic experiments

The reactions were carried out in Eppendorf tubes which were immersed in a thermostated water bath at 35 ± 0.1 °C. The

solutions of the Zn^{2+} complexes were prepared by mixing stoichiometric amounts of ligand $\mathbf{L}^1$ or $\mathbf{L}^3$ and $Zn(NO_3)_2$ in sterilized water overnight at room temperature. HEPES buffer (0.10 mol L^{-1}, pH 7.5), the target oligoribonucleotide (50 μmol L^{-1}) and potassium 4-nitrobenzenesulfonate (internal standard, 0.10 mmol L^{-1}) were then added to the solution. The ionic strength was adjusted to 0.10 mol L^{-1} with $NaNO_3$. The total volume of the reaction mixture was 200 μL. Aliquots of 20 μL were withdrawn at suitable time intervals and cooled immediately on an ice-water bath. The reaction was quenched by adding aq hydrogen chloride (1 μL of 1.0 mol L^{-1} solution). The aliquots were analyzed on a Beckman Coulter P/ACE-MDQ Capillary Electrophoresis System using a fused silica capillary (75 μm inner diameter, 50 cm effective length) and citrate buffer (0.2 mol L^{-1}, pH 3.1) as a background electrolyte. The voltage applied was –20 kV and the temperature of the capillary was kept at 25 °C. Between each analytical run, the capillary was flushed for 3 min with water, aq HCl (10 mmol L^{-1}) and the background electrolyte. The samples were injected using hydrodynamic injection with 2 psi for 8 s. The oligonucleotides and the internal standard were detected by a UV detector at a wavelength of 254 nm.

First-order rate constants were calculated for the disappearance of the target oligoribonucleotide by applying the integrated first-order rate law to the diminution of the signal of the starting material. For normalizing, the integrated peak areas of the starting material and internal standards were first divided by the migration times of the corresponding peaks. Then the starting material was divided by the internal standard and the first-order rate constant k was calculated from the first-order rate law.

Determination of the cleavage sites

The $(Zn^{2+})_3\mathbf{L}^3$-promoted hydrolysis of hexanucleotides **2**, **3**, and **5–10**, and the $(Zn^{2+})_2\mathbf{L}^1$-promoted hydrolysis of **2**, was allowed to proceed from 2 to 3 half-lives in Eppendorf tubes immersed in a thermostated water bath at 35 ± 0.1 °C. The reaction conditions were as described above, except that the total volume of the reaction mixture was larger, either 400 μL or 600 μL. The reactions were quenched by adding aq. hydrogen chloride solution (20 μL or 30 μL of 1.0 mol L^{-1} solution). The reaction mixtures were cooled immediately on an ice-water bath and stored in a freezer before analysis. The composition of the reaction mixtures were analyzed by RP-HPLC. The cleavage products were separated and collected, and finally verified by MS analysis. Chromatographic conditions: Hypersil ODS column (250 × 4.6 mm, particle size 5 μm), flow rate 1 mL min^{-1}, buffer aq. NH_4OAc (A = 0.05 mol L^{-1}), buffer B = 1:1 mixture of buffer A and MeCN, a linear gradient from 0 to 15% buffer B in buffer A in 25 min, followed by a linear gradient from 15 to 30% buffer B in buffer A in 10 min. The results obtained are given as Supporting Information.

Melting temperature measurements. The melting curves (absorbance *versus* temperature) were measured at 260 nm on a Perkin-Elmer Lambda 2 UV-vis spectrometer equipped with a Peltier Temperature controller. The temperature was changed at a rate of 0.5 °C/min (from 20 to 90 °C). The measurements were performed in 10 mmol L^{-1} HEPES buffer (pH 7.5) containing 0.1 mol L^{-1} NaCl. The concentration of the oligonucleotide was 6 μmol L^{-1}.

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