Acceleration of the Zn^{2+}-promoted phosphodiester hydrolysis of oligonucleotides by the 3′-terminal monophosphate group: intrastrand participation over several nucleoside units

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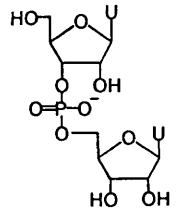
The Zn^{2+}-promoted hydrolysis of the 5′-terminal ribonucleoside phosphodiester bond in chimeric ribo/deoxyribo oligonucleotide 3′-monophosphates, $Up(Tp)_4$ and $Up(Tp)_9$, and their dephosphorylated analogue, $Up(Tp)_3T$, has been studied at various metal ion and substrate concentrations, and in the presence and absence of deoxyribooligonucleotide 3′-monophosphates, $(Tp)_n$, containing no cleavable ribonucleoside phosphodiester bond. The results strongly suggest that the rate-accelerating effect of the 3′-terminal monophosphate group on the phosphodiester hydrolysis is of intramolecular origin: the Zn^{2+} ion bridges the favoured site of coordination, *i.e.* the terminal monophosphate group, and the cleaving phosphodiester bond. The 3′-monophosphate group also causes the reaction order in $[Zn^{2+}]$ to deviate from unity, the values obtained with $Up(Tp)_3T$, $Up(Tp)_4$ and $Up(Tp)_9$ being 1.1, 1.4 and 1.7, respectively. Possibly, the intramolecular participation of the 3′-monophosphate bound Zn^{2+} ion is facilitated by another Zn^{2+} ion that stabilizes the folded conformation of the oligonucleotide chain in the reactive Zn^{2+}/substrate macrochelate.

Introduction

Metal-ion-promoted phosphodiester hydrolysis of RNA and its model compounds has become an object of wide interest, since metal-ion-based agents cleaving RNA in a sequence selective manner are expected to find applications in the antisense oligonucleotide strategy.[1] Both the mechanisms of these reactions[2,3] and their possible applications[4,5] have recently been reviewed.

Mechanistic studies on the metal-ion-promoted hydrolysis of simple RNA mimetics, such as uridylyl-3′,5′-uridine (3′,5′-UpU; **1**), suggest that the actual catalyst most likely is the hydroxo ligand of the phosphate-bound metal ion.[2] This serves as an intracomplex general base deprotonating the 2′-hydroxy function, and hence assisting the nucleophilic attack on the phosphorus, the nucleophilicity of which is simultaneously enhanced by the coordinated metal ion [Scheme 1(*a*)]. The proposed intracomplex general base catalysis cannot be strictly distinguished from a mechanism where the metal ion is directly



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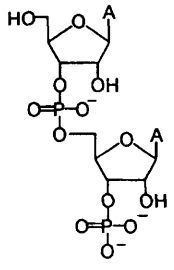
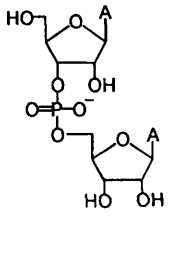


2a



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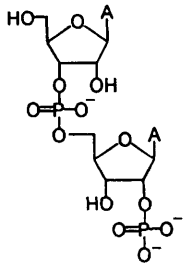

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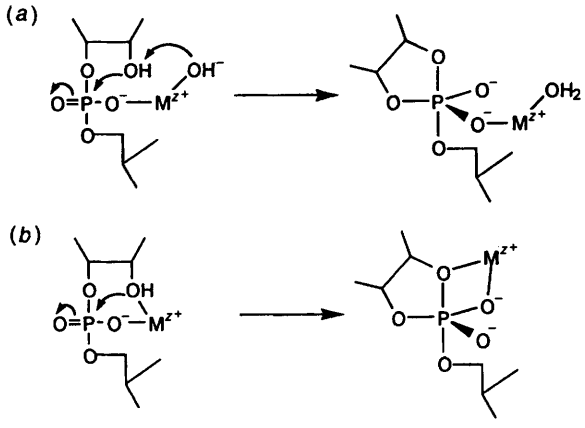
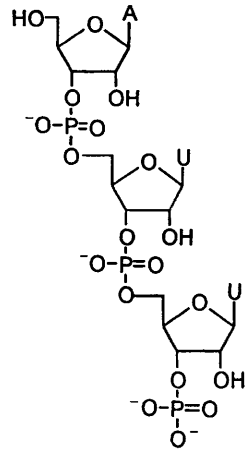


Scheme 1

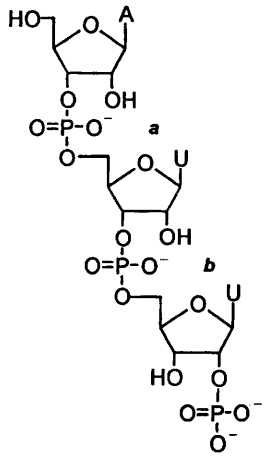
coordinated to the attacking nucleophile [Scheme 1(*b*)]. This question is not, however, a very crucial one, since in both cases the coordinated metal ion plays essentially the same dual role: decreases the electron density at phosphorus and increases the nucleophilicity of 2′-O.

With oligomeric substrates containing several phosphodiester bonds, the mechanism seems to be more complicated. Neighbouring phosphate groups accelerate the reaction. For example, an individual phosphodiester bond within a polyuridylic acid strand [poly(U)] is hydrolysed 130 times more readily than that of 3′,5′-UpU **1** at $[Zn^{2+}] = 10$ mM (M = mol dm^{-3}).[6] A rate acceleration of this magnitude can hardly be entirely attributed to enhanced electrostatic interaction between the metal ion and the polyanionic substrate, as compared to that between the metal ion and monoanionic 3′,5′-UpU. For comparison, the hydronium-ion-catalysed hydrolysis of poly(U) is only ten times faster than that of 3′,5′-UpU.[7] In other words, the bifunctional catalysis described above cannot adequately explain the reactivity difference between poly(U) and 3′,5′-UpU, but additional factors, such as multidentate binding of Zn^{2+} to more than one phosphate group, have to be considered.

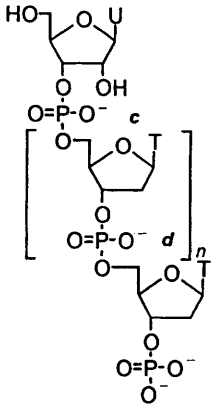
Butzow and Eichhorn[8] had observed already in the 1970s that the terminal phosphate group of 3′,5′-ApAp(3′) **2a**



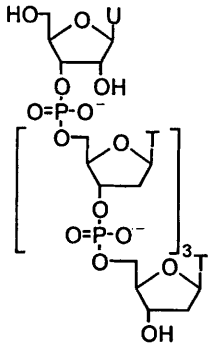
3a



3b



4: $n = 3$
5: $n = 8$



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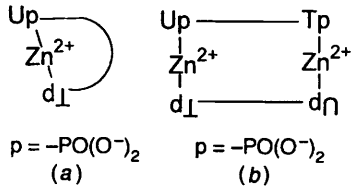
enhances the hydrolysis of the phosphodiester bond: the phosphodiester bond of **2a** is hydrolysed by Zn^{2+} ions two orders of magnitude faster than that of 3',5'-ApA **2b**. This finding has later been verified by several groups.[9-12] It was also shown that the 2'-phosphate group of 3',5'-ApAp(2') **2c** results in only a modest rate enhancement.[8] Our previous studies[13] on trimeric substrates **3a,b** also corroborated the rate-accelerating effect of the 3'-monophosphate group, and revealed that the hydrolysis of both of the phosphodiester bonds of **3a** are almost equally accelerated. Furthermore, it was observed that although the 2'-monophosphate group of **3b** does not accelerate the cleavage of the adjacent phosphodiester bond (the 3'-terminal bond *b*), it enhances the hydrolysis of the next linkage (the 5'-terminal bond *a*) as efficiently as the 3'-monophosphate group. Subsequent studies were carried out with chimeric ribo/deoxyribo oligonucleotides **5**, **6** containing one hydrolysable ribonucleoside 3'-phosphodiester bond (*c*) and an increasing number (n = 1–7) of hydrolytically stable deoxyribonucleoside bonds (*d*). The results obtained showed that introduction of additional phosphodiester bonds (*d*) accelerates the cleavage of the ribonucleoside bond (*c*) by one order of magnitude, and the presence of a 3'-monophosphate function still results in an extra rate-enhancement. The latter acceleration seems to be 20-fold when the total number of phosphodiester bonds is one and four-fold when it is nine. In other words, rather surprisingly the rate-accelerating effect of the terminal monophosphate function seemed to extend over several nucleoside units.

Table 1 Pseudo-first-order rate constants for the Zn^{2+}-promoted hydrolysis of the ribonucleoside phosphodiester bond in chimeric ribo/deoxyribo oligonucleotides in the absence and presence of additional oligodeoxyribonucleotides. $[Zn^{2+}]$ = 10 mM, pH 5.5 with HEPES buffer,[a] I = 0.1 M with $NaClO_4$, T = 333.2 K

Substrate (conc/μM)	Oligodeoxyribonucleotide (conc/μM)	$k/10^{-5}$ s^{-1}
$Up(Tp)_4$ (20)	None	4.4 ± 0.1
$Up(Tp)_4$ (22)	$(Tp)_5$ (82)	4.83 ± 0.08
$Up(Tp)_4$ (22)	$(Tp)_5$ (154)	4.9 ± 0.1
$Up(Tp)_3T$ (55)	None	0.191 ± 0.007
$Up(Tp)_3T$ (80)	$(Tp)_4$ (370)	0.21 ± 0.02

[a] HEPES/HEPESNa 0.1/0.005 M; the pH at 333.2 K was calculated by using the known temperature dependence of the buffer acid.[15]

Evidently, the terminal monophosphate group offers a good dianionic binding site for Zn^{2+}, and hence accelerates the hydrolysis.† The question that still remains is whether this effect is transmitted in an intramolecular or intermolecular manner. The intramolecular mode involves the coordination of the metal ion to the terminal monophosphate group and one phosphodiester bond of the same molecule [Scheme 2(*a*)]. In



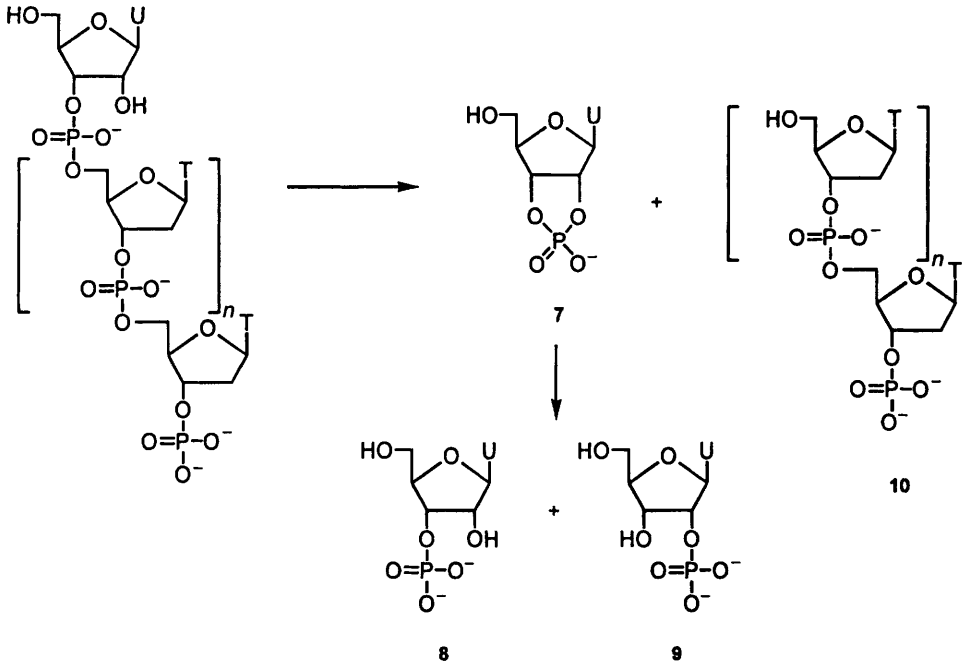
Scheme 2

the intermolecular mode the metal ion is bound to the terminal monophosphate group of one molecule, and simultaneously to a phosphodiester bond of another molecule [Scheme 2(*b*)]. Both modes may involve additional metal ions that play a structural role by reducing the electrostatic repulsion between the negatively charged phosphodiester bonds. The present paper is aimed at distinguishing between the intra- and intermolecular modes of participation of the 3'-terminal monophosphate group by using the Zn^{2+}-promoted cleavage of chimeric ribo/deoxyribo oligonucleotides as a model reaction. Moreover, the composition of the reactive Zn^{2+}/substrate complex is elucidated by kinetic measurements.

Results and discussion

Hydrolysis of chimeric ribo/deoxyribo oligonucleotide $Up(Tp)_4$ **4** and its 3'-dephosphorylated analogue $Up(Tp)_3T$ (**6**) were followed by HPLC in large excess of Zn^{2+} ions ([oligomer] = 20 μM, $[Zn^{2+}]$ = 10 mM, pH = 5.5, T = 333.2 K, I = 0.1 M). With both compounds, the 5'-terminal uridine 3'-phosphodiester bond (bond *c*) was cleaved releasing uridine 2',3'-cyclic monophosphate (2',3'-cUMP; **7**), which was subsequently hydrolysed to a mixture of uridine 2'- and 3'-monophosphates (2'- and 3'-UMP; **9** and **8**). No sign of hydrolysis of the thymidine 3'-phosphodiester bonds (bond *d*) could be observed (Scheme 3). As reported previously,[13] the 3'-phosphorylated oligomer **5** was cleaved more than 20 times as quickly as its 3'-hydroxy analogue **6** (Table 1). The disappearance of both **5** and **6** obeyed first-order kinetics. This, as such, suggests that the reactive species contains only one substrate molecule. One

† The pK_a of the 3'-terminal monophosphate group may be estimated to be 5.7 and the logarithmic stability constant for the 1:1 complex of Zn^{2+} with the dianionic ligand 2.7.[16] Accordingly, Zn^{2+} efficiently competes with proton for the phosphate dianion under experimental conditions.



Scheme 3

may argue, however, that the cleaved tail, $(Tp)_4$, may take the place of $Up(Tp)_4$ in a bimolecular Zn^{2+}/substrate complex [Scheme 2(*b*)], and hence the observed first-order kinetics would not be a sufficient piece of evidence for the intramolecular mode of participation of the 3′-phosphate group. To eliminate this possibility the following experiments were carried out.

First, the rate constant for the hydrolysis of $Up(Tp)_3T$ **6** was determined in excess of $(Tp)_4$ (**10**; $n = 3$). As seen from Table 1, the presence of $(Tp)_4$ in up to five-fold excess did not accelerate the Zn^{2+}-promoted hydrolysis of $Up(Tp)_3T$. In other words, a terminal monophosphate group, when not present in the same oligomer as the cleavable uridine phosphodiester bond, could not accelerate the hydrolysis. This argues against the intermolecular participation of the monophosphate group, but as formation of a bimolecular complex depicted in Scheme 2(*b*) may be favoured by the presence of a monophosphate group in both molecules involved, further experiments were carried out.

Secondly, the rate constant for the hydrolysis of $Up(Tp)_4$ and $Up(Tp)_9$ were measured at various initial substrate concentrations, 20–200 μM. As seen from Fig. 1, the hydrolysis rate is almost independent of the substrate concentration, or is perhaps slightly decreased with the increasing substrate concentration. In the large excess of Zn^{2+} ions employed, the mole fraction of the bimolecular complex [Scheme 2(*b*)] may be expected to increase with increasing total concentration of the oligomeric ligand, independently of how many metal ions are involved in the complex formation. Accordingly, the hydrolysis rate should increase with increasing oligomer concentration, if the reactive species were of the type depicted in Scheme 2(*b*). Since this is not the case, the 3′-terminal monophosphate group in all likelihood accelerates the cleavage of the 5′-terminal uridine phosphodiester bond intramolecularly [Scheme 2(*a*)]. In fact, the hydrolysis appears to be slightly retarded at high oligomer concentrations. Accordingly, one might speculate that under such conditions interchain bridging of oligomeric substrates by Zn^{2+} ions starts to play a role, and this binding mode is rate retarding compared to the intrachain bridging. Consistent with these conclusions, addition of $(Tp)_5$ to the reaction mixture of $Up(Tp)_4$ did not accelerate the hydrolysis, as is clearly seen from the rate constants listed in Table 1.

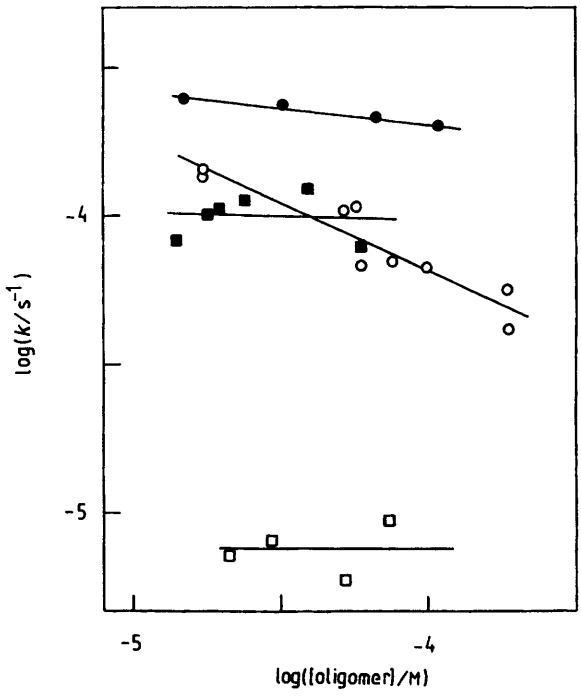


Fig. 1 The effect of substrate concentration on the Zn^{2+}-promoted hydrolysis of the ribonucleoside phosphodiester bond in chimeric ribo/deoxyribo oligonucleotides. The pseudo-first-order rate constants were obtained at pH 5.5 adjusted with a HEPES buffer, $I(NaClO_4)$ 0.1 M, $T = 333.2$ K. Notation: $Up(Tp)_4$ at $[Zn^{2+}] = 20$ mM (●), $Up(Tp)_4$ at $[Zn^{2+}] = 10$ mM (○), $Up(Tp)_9$ at $[Zn^{2+}] = 10$ mM (■) and $Up(Tp)_3T$ at $[Zn^{2+}] = 10$ mM (□).

As mentioned above, the intramolecular participation of the 3′-monophosphate group in the hydrolysis of the 5′-terminal uridine phosphodiester bond requires that the oligonucleotide is significantly folded; otherwise an interaction between groups that are several nucleotide units apart seems improbable. This kind of folded stucture may be expected to be stabilized by metal ions that bridge between the negatively charged phosphodiester bonds. To learn more about the importance of this stabilization, the reaction order in $[Zn^{2+}]$ was determined

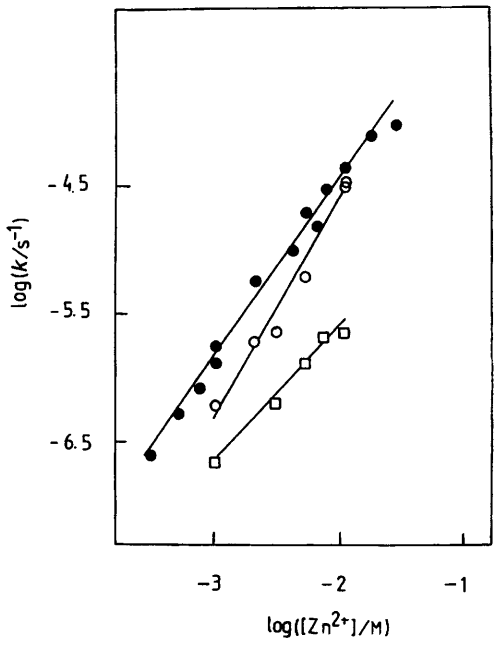


Fig. 2 The effect of the concentration of Zn^{2+} on the hydrolysis of the ribonucleoside phosphodiester bond in chimeric ribo/deoxyribo oligonucleotides. The pseudo-first-order rate constants were obtained at pH 5.5 adjusted with a HEPES buffer, I(NaClO$_4$) 0.1 M, T = 333.2 K. Notation: Up(Tp)$_4$ (●), Up(Tp)$_9$ (○) and Up(Tp)$_3$T (□).

for the hydrolysis of Up(Tp)$_3$T, Up(Tp)$_4$ and Up(Tp)$_9$ at 0.3 mM < [Zn^{2+}] < 30 mM (I = 0.1 M), keeping the initial concentration of the oligomeric substrate constant. The results obtained are shown in Fig. 2. As shown, the hydrolysis of Up(Tp)$_3$T, bearing no monophosphate group at the 3′-terminus, exhibits first-order dependence on [Zn^{2+}], analogously to the hydrolysis of the isolated phosphodiester bond of 3′,5′-UpU.[14] In contrast, the hydrolysis of both 3′-phosphorylated oligomers is more than first order in the metal ion concentration: 1.42 ± 0.05 with Up(Tp)$_4$ and 1.7 ± 0.2 with Up(Tp)$_9$. In other words, the rate-accelerating effect of the 3′-monophosphate group becomes more marked at high metal ion concentrations, and the susceptibility of the acceleration to the metal ion concentration rises on increasing the distance between the monophosphate group and the cleavable phosphodiester bond. Up(Tp)$_3$T, having no 3′-terminal monophosphate group, is also hydrolysed more quickly than 3′,5′-UpU, and the acceleration probably results from didentate binding of Zn^{2+} to the substrate. However, now the rate-accelerating effect of additional phosphodiester bonds is independent of the metal ion concentration; the reaction order in [Zn^{2+}] is unity as with 3′,5′-UpU. In this case the didentate coordination, which enhances binding of Zn^{2+} to the scissile bond, takes place at two adjacent phosphodiester bonds (one of which is the cleavable one). There is no reason to believe that didentate binding of Zn^{2+} to the cleavable bond and any of the phosphodiester bonds further within the chain would be as favourable. Accordingly, no special chain-folding is needed to obtain the reactive Zn^{2+}-oligomer complex, and hence its formation is not enhanced by additional metal ions. With Up(Tp)$_4$ and Up(Tp)$_9$, the preferred site of co-ordination, *viz.* the monophosphate group, is situated several nucleoside units away from the cleavable bond. Accordingly, simultaneous binding of a Zn^{2+} ion to this site and the cleavable ribonucleoside phosphodiester bond may be facilitated by another metal ion (or metal ions), which reduces the electrostatic repulsion between the noncleavable deoxyribonucleoside phosphodiester bonds, and hence stabilizes the folded conformation. This is reflected in the reaction kinetics as positive deviation from first-order dependence on [Zn^{2+}]. This kind of conformational stabilization by extra metal ions becomes more important with increasing chain length, and hence Up(Tp)$_9$ exhibits a higher reaction order in [Zn^{2+}] than Up(Tp)$_4$. It is worth noting that the Zn^{2+}-promoted hydrolysis of poly(U), proceeding in all likelihood *via* intermediary formation of oligoribonucleotide 2′- and 3′-monophosphates, also shows a positive deviation from first-order dependence on [Zn^{2+}], the reaction order being 1.2.[6] When this value was determined, the reaction was followed until 20% of all phosphodiester bonds were cleaved. Accordingly, the chain length varies during a kinetic run, being on average five at the end of the measurement. Furthermore, one should bear in mind that the first bond ruptures must proceed without participation of the terminal monophosphate functions. Accordingly, the relatively small positive deviation from first-order dependence on [Zn^{2+}] does not contradict the higher values of reaction order obtained with Up(Tp)$_n$.

In summary, the results of the present study strongly suggest that the previously reported[13] rate-accelerating effect of a terminal 3′-monophosphate group on the metal-ion-promoted phosphodiester hydrolysis of RNA oligomers is intramolecular in origin. The terminal monophosphate group offers a good binding site for the metal ion, and the same metal ion simultaneously interacts with the cleaving phosphodiester bond, in spite of the fact that these two binding sites may be situated several nucleoside units apart. The folding of the oligonucleotide chain that enables such a binding mode is enhanced by additional metal ions.

Experimental

Materials

The oligonucleotides employed in the kinetic measurements, *viz.* Up(Tp)$_3$T **6**, Up(Tp)$_4$ **4**, Up(Tp)$_9$ (**5**), (Tp)$_4$ (**10**; n = 3) and (Tp)$_5$ (**10**; n = 4), were synthesized by the phosphoramidite approach on solid support as described previously.[13] Uridine monophosphates (**7**–**9**) used as reference compounds were commercial products of Sigma. All the other reagents were of analytical grade.

Kinetic measurements

The kinetic experiments were performed at 333.2 K. The temperature was adjusted with a water bath and kept constant within 0.1 K. The reactions were carried out in tightly stoppered tubes. The pH of the reaction solutions was adjusted with HEPES buffer (HEPES/HEPESNa 0.1/0.005 M) and the ionic strength with $NaClO_4$. The pK_a of the buffer acid was extrapolated to 333.2 K by the known temperature dependence.[15] The reported pH values are the calculated ones, but in several cases the pH was checked at 333.2 K by a direct measurement before and after a kinetic run. The substrate was added as a concentrated aqueous solution.

Aliquots of 0.5 ml were withdrawn at suitable intervals, and their compositions were analysed by HLPC, monitoring the formation of uridine 2′,3′-cyclic monophosphate **7**. Moreover, it was checked that the (Tp)$_n$ or (Tp)$_n$T oligomers were not hydrolysed under the reaction conditions. The reactions were followed for about two halflives. The separations were carried out on a Hypersil RP-18 (250 × 4 mm, 5 μm) column, using a mixture of acetonitrile and acetic acid buffer (0.1 M, pH 4.3, containing 0.1 M NH_4Cl) as an eluent. The initial oligomer concentrations were determined as follows. The first aliquot of every kinetic run was treated with a mixture of phosphodiesterase I and alkaline phosphatase to release the 5′-terminal uridine nucleotide as uridine. The aliquot treated with the enzyme mixture was left to stand for several hours to ensure that the digestion was complete, and then analysed by HPLC as described above. The area of the signal observed was converted to concentration

with the aid of a calibration line based on uridine standards of known concentrations. The concentrations of the solutions of $(Tp)_n$ oligomers were determined similarly by using thymidine standards and taking the number of nucleosidic units in the oligomer into account.

Calculation of the rate constants

The mole fraction of the initial product, uridine 2',3'-cyclic monophosphate **7**, was determined as a function of time. The pseudo-first-order rate constant for its hydrolysis was determined under the same conditions. The pseudo-first-order rate constant for the release of **7** from the oligomeric starting material (**5** or **6**) was then calculated by applying the rate equation of two consecutive first-order reactions [eqn. (1)].

$$x_t(\mathbf{7}) = [k/(k_d - k)][e^{-kt} - e^{-k_d t}] \qquad (1)$$

In eqn. (1) k stands for the first-order rate constant for the formation of **7** (= rate constant for the disappearance of the starting oligonucleotide) and k_d for the rate constant of the disappearance of **7**.

$x_t(\mathbf{7})$ is the mole fraction of uridine 2',3'-cyclic monophosphate at time t.

Acknowledgements

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