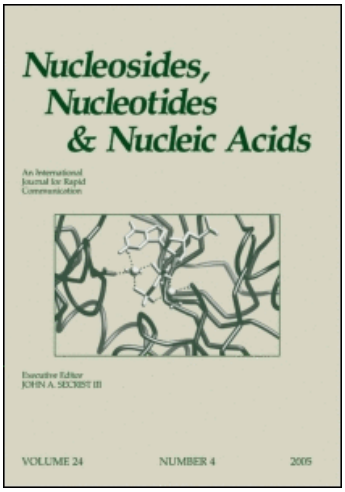

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CATALYTICALLY SIGNIFICANT MACROCHELATE FORMATION IN Zn^{2+} PROMOTED HYDROLYSIS OF OLIGORIBONUCLEOTIDES: MODEL STUDIES WITH CHIMERIC PHOSPHODIESTER/METHYLPHOSPHONATE OLIGOMERS

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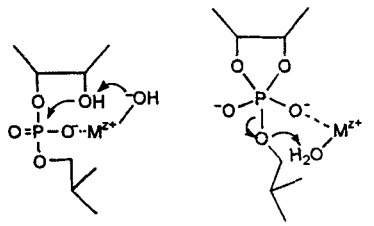
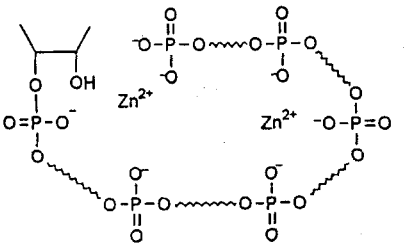
ABSTRACT. The kinetics of Zn^{2+} promoted hydrolysis of chimeric oligonucleotides Up[Tp(Me)]$_3$T and Up[Tp(Me)]$_3$Tp was studied. The results were compared to those obtained with corresponding phosphodiester oligonucleotides, and discussed in terms of an oligonucleotide-metal ion macrochelate formation.

We have recently been studying the mechanism of metal ion promoted cleavage of phosphodiester bonds of RNA and its model compounds. The central features of the catalysis have been established with 3',5'-dinucleoside monophosphates[1] (3',5'-NpN) and 2',3'-cyclic monophosphates.[2] Consistent with the results of several other groups,[3-6] only one metal ion has been shown to be involved. As has been discussed before, we prefer a mechanism where a metal aquo ion assists the cleavage by acting as intracomplex general acid/base catalyst (*Scheme 1*),[7,8] although mechanisms involving direct coordination of the metal ion to the entering and/or leaving nucleophile are also consistent with the kinetic evidence.[9]



Scheme 1



Scheme 2

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Studies with dinucleoside diphosphates[3,4,10,11] (3',5'-NpNp3') and short oligonucleotides[10,12] have, however, shown that 3',5'-NpN does not adequately mimic the metal ion promoted cleavage of RNA. As an indication of this, individual phosphodiester bonds of oligonucleotides[10] and polynucleotides[13,14] are cleaved up to 100 times as fast as that of 3',5'-NpN. To elucidate the origin of this rate-enhancement, we have previously studied the hydrolysis of a series of chimeric ribo/2'-deoxyribo oligonucleotides with and without a 3'-terminal monophosphate function.[15] We observed that the adjacent phosphodiester bonds accelerated the Zn^{2+}-promoted cleavage of a ribo phosphodiester bond by one order of magnitude; the 3'-terminal monophosphate function resulted in an additional 10-fold rate-enhancement. With the 1,5,9-triazacyclododecane chelate of Zn^{2+} as a catalyst, the rate-enhancing effects were considerably smaller. Oligonucleotides not bearing the 3'-phosphate function were observed to be only slightly more reactive than 3',5'-NpN, and the 3'-phosphate resulted in a 3- to 5-fold rate acceleration. A likely explanation for these results is an enhanced binding of the catalyst due to multidentate co-ordination. Zn^{2+} aquo ion most likely bridges two phosphate groups, while the ability of the Zn^{2+} chelate to form a similar macrochelate is restricted by the triazacyclododecane ligand. It is also worth noting that the terminal monophosphate group does not only enhance the reactivity of the neighbouring phosphodiester bond, but the cleavage of the 5'-terminal ribo phosphodiester bond of even a 9-mer is nearly as fast as that of a dinucleoside diphosphate.[15] This suggests that Zn^{2+} may bridge two phosphate groups that are several nucleosidic units apart, and hence a back-folding of the oligonucleotide chain must take place. The possibility of an intermolecular rate enhancement by another oligonucleotide molecule has been excluded by the studies on the effect of the oligonucleotide concentration on the cleavage rate.[16]

Further information about the reactive structures has been obtained by studying the effect of metal ion concentration on the hydrolysis of oligonucleotides.[16] An oligonucleotide having a terminal monophosphate function shows a clearly larger than first-order dependence of rate on the metal ion concentration, suggesting that the most reactive structure contains more than one metal ion bound to one oligonucleotide. The macrochelate structure discussed above, where one metal ion bridges the terminal monophosphate group and the scissile phosphodiester bond has been suggested to be

stabilised by another metal ion that is bound to the intervening phosphodiester bonds. A schematic representation of the macrochelate suggested[15,16] is shown in *Scheme 2*. Consistent with this proposal, oligonucleotides without the terminal monophosphate group show only a first-order dependence of rate on metal ion concentration.

To obtain further support to the mechanistic proposals described above, we now report on the hydrolysis of chimeric oligonucleotides **1** and **2** having only one 3'-ribo phosphodiester bond, the rest of the internucleosidic bonds being replaced with methylphosphonate linkages. The synthesis of these molecules has been reported elsewhere.[17] Methylphosphonate function is a poor co-ordination site for metal ions, since it does not bear a negative charge. Methylphosphonate oligomers can hence be regarded as models of phosphodiester oligonucleotides where co-ordination to phosphate groups other than the scissile phosphodiester bond and the 3'-terminal phosphate group has been blocked. Another substrate employed in this study is 3',5'-ApUp(3'), a dinucleoside diphosphate having the terminal monophosphate, but no additional phosphodiester bonds. The results obtained with these substrates help to evaluate the importance of the additional phosphodiester bonds as metal ion co-ordination sites and the role of the second metal ion in the catalysis.

RESULTS AND DISCUSSION

The pair of methylphosphonate oligonucleotides used as substrates in this study is shown by structures **1** and **2.** The hydrolysis of the only scissile phosphodiester bond of these molecules was followed by capillary zone electrophoresis (CZE). Since all the reaction products could be separated by CZE, it could be seen that the only reaction occurring under the experimental conditions was the cleavage of the phosphodiester bond. The methyl phosphonate linkages were completely stable. Dephosphorylation of the 3'-terminal phosphomonoester function was not observed either. Since methyl phosphonate linkages are chiral, methyl phosphonate oligonucleotides exist as mixtures of several diastereometrically different compounds that can not be separated by CZE. Different stereoisomers should, however, be identical in for our purposes, since metal ions are not expected to bind methyl phosphonate linkages in any case, at least not to any significant

extent, due to a the lack of the negative charge. Oligonucleotides containing phosphotriester linkages in place of the methylphosphonate linkages of **1** and **2** were our first choice for model compounds, but the triester bonds turned out to be too unstable under the experimental conditions. Phosphodiester oligonucleotides **3** and **4**, 3',5'-UpU (**5**) and 3',5'-ApUp(3') (**6**) were studied for comparative purposes.

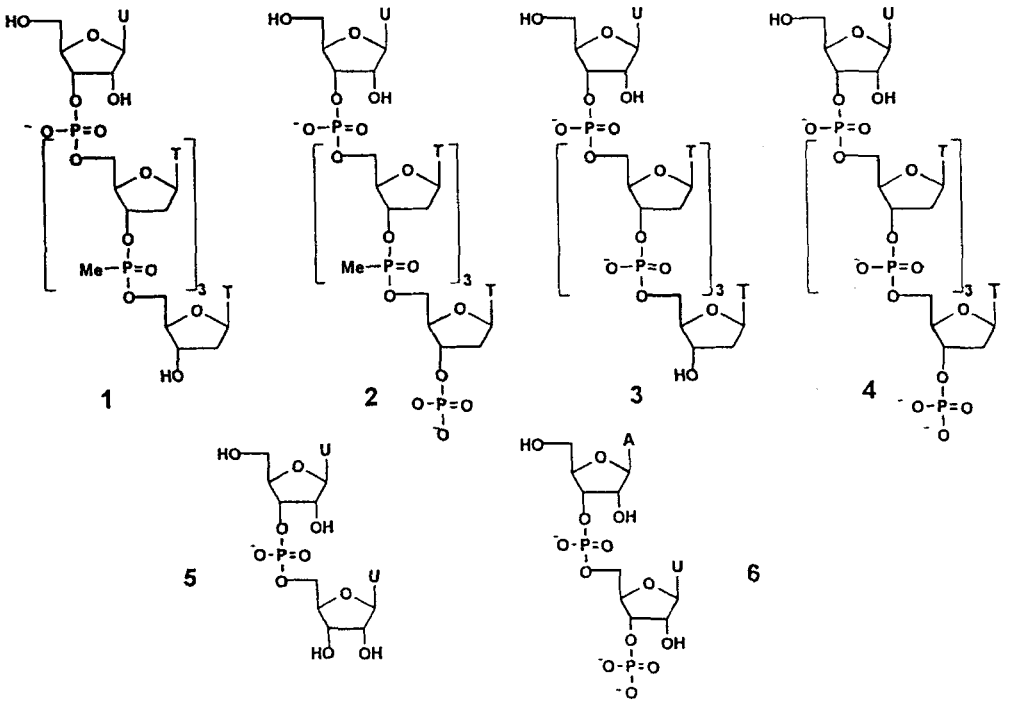

TABLE 1 records the first-order rate constants obtained for the Zn^{2+}-promoted cleavage of **1-6** at sub-saturating metal ion concentration, *i.e.* under conditions where the hydrolysis of all of the compounds still clearly shows a first-order or higher dependence on the metal ion concentration. As seen, the least reactive of the compounds studied are 3',5'-UpU (**5**) and the chimeric methylphosphonate oligomer **1** not bearing a 3'- terminal monophosphate function. **1** is cleaved only two times as fast as 3',5'-UpU. Evidently the three additional methylphosphonate linkages in **1** do not result in a similar rate acceleration as the three additional phosphodiester bonds in **3**: the cleavage rate of **1** is less than 20 % of that of **3**. In contrast to the phosphodiester bonds, the methylphosphonate linkages do not appear to contribute markedly to binding of the catalytically active metal ion.

TABLE 1. Comparison of rate constants of the hydrolysis of compounds **1-6** at a sub-saturating metal ion concentration. 10 mM $Zn(NO_3)_2$ in 0.1 M MES buffer (40 % MES/60 % MESNa), at pH 5.95 and 60 °C.

Substrate	k / 10^{-6} s^{-1}	Substrate	k / 10^{-6} s^{-1}
5	0.23±0.04	**2**	2.6±0.1
1	43.0±0.6	**6**	43.0±0.6
3	3.1±0.1	**4**	28.0±0.07

As seen from TABLE 1, the insertion of a 3'-phosphate function in **1** results in a 4.6-fold rate acceleration (compare **2** to **1**). This acceleration is about half of that observed with the corresponding phosphodiester oligomers, **4** compared to **3**. A nearly 5-fold acceleration is, however, sufficiently large to conclude that the 3'-terminal phosphate group in **2** is still able to enhance the binding of the catalytically active metal ion, in spite of the fact the most favoured co-ordination site, the 3'-phosphate, and the scissile bond are four nucleoside units apart, and the intervening methylphosphonate groups do not efficiently participate in the complex formation.

The dependence of the cleavage rate of oligomer **2** on the metal ion concentration was determined in two different concentration range. At the first stage, a relatively high temperature, 60 °C, and low concentrations of Zn^{2+} were employed. Under these conditions, the cleavage of **4** has previously been shown to exhibit a reaction-order of 1.5 with respect to the concentration of Zn^{2+}.[16] This has been taken as an indication of the involvement of two metal ions in the reaction, one of them playing a catalytic and the other one a structural role. The cleavage of **3**, in contrast, is known to be strictly first-order in the Zn^{2+} concentration,[16] consistent with only one metal ion being involved in the catalysis. The results of the present work (FIG.1) show that in contrast to the situation with **4**, the corresponding 3'-phosphorylated methylphosphonate oligomer **2** also exhibits only a first-order dependence of rate on the metal ion concentration. In other words, only one metal ion participates in the catalysis. While the 3'-phosphate still is a kinetically significant binding site, as is shown by the fact that **2** is five times as reactive as **1**, an additional metal ion is not used to enhance the interaction of the 3'-phosphate bound Zn^{2+} with the scissile phosphodiester bond.

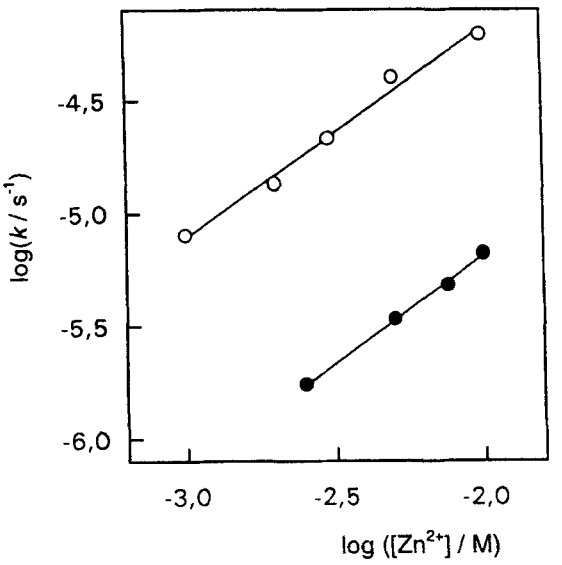

FIG.1. The effect of the $Zn(NO_3)_2$ concentration on the hydrolysis of **2** (filled circles) and **6** (open circles) in 0.1 M MES buffers, I=0.1 M (adjusted with $NaNO_3$), at pH 5.95 and 60 °C.

3',5'-ApUp(3'), **6,** shows a first-order dependence of rate on the Zn^{2+} concentration (FIG.1) consistent with the results obtained earlier by Job's method.[3,4] The behaviour is hence similar to that of **2**. Neither **6** nor **2** contain anionic phosphorus centers in addition to the 3'-phosphate and the scissile phosphodiester bond, and hence the formation of the assumed reactive species, viz. a macrochelate where one Zn^{2+} ion bridges the 3'-phosphate and the scissile bond, cannot be facilitated by another metal ion, in contrast to the situation with the phosphodiester oligomer **4**. A comparison of the cleavage rate of **6** with that of **4** also elucidates the role of the second metal ion utilised in the hydrolysis of **4**. Since **6** and **4**, using one and two metal ions, respectively, are approximately as reactive, it seems logical to assume that the role of the second metal ion involved in the cleavage of **4** is structural rather than catalytic. The longer the reacting oligomer is, the less favoured is the macrochelate formation, both entropically and due to increasing electrostatic repulsion between the phosphodiester bonds, and hence additional metal ions are needed to facilitate the back-folding. Consistent with this suggestion, our earlier results[16] with a longer counterpart of **4** , *viz.* a 3'-phosphorylated 10-mer, showed that in this case the second metal ion is even more crucial. The observed reaction order in Zn^{2+} was 1.8 as opposed to 1.5 with **4.**

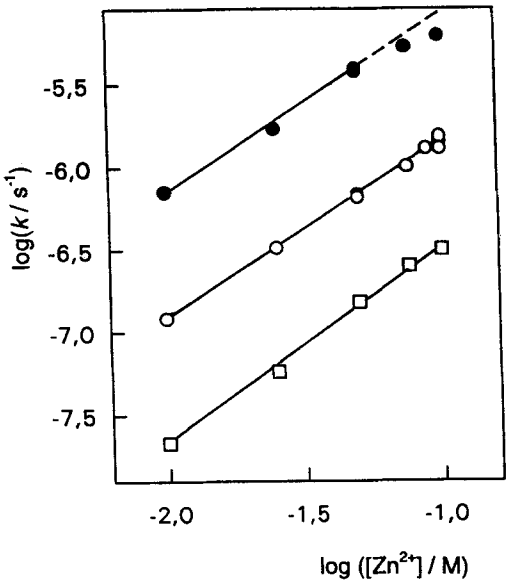

FIG.2. The effect of the $Zn(NO_3)_2$ concentration on the hydrolysis of **1** (open squares), **2** (open circles) and **4** (filled circles) in 0,1 M MES buffers, I=0.5 M, (adjusted with $NaNO_3$), at pH 6.13 and 37 °C.

The effect of metal ion concentration on the cleavage rate was also studied at a higher metal ion concentrations and a lower temperature. As FIG. 2 shows, under these conditions **4** exhibits some inclination to saturation with Zn^{2+}. The reaction order in Zn^{2+} concentration is now only unity, although a higher order dependence has been observed at lower Zn^{2+} concentrations,[16] and the dependence possibly shows some indication of levelling off on approaching the concentration of 100 mM. In contrast, no sign of saturation could be observed with **1** and **2**. Higher metal ion concentrations could not be used, since as the concentration of Zn^{2+} increased, the solubility of the methylphosphonate containing products seemed to decrease, which was a problem especially with **1.** High metal ion concentration also seemed to interfere with the CZE analysis; above Zn^{2+} concentration of 100 mM the results were not quite reproducible.

The finding that a saturation with Zn^{2+} could not be observed more clearly with either **2** or **4** is actually somewhat unexpected. A stability constant (log K) of 2.6 has been reported[18] for the Zn^{2+} complex of uridine 3'-monophosphate dianion. Consequently, the co-ordination of Zn^{2+} to the monophosphate function of **2** and **4** could be expected to be completed in 100 mM Zn^{2+} solution, but as seen in FIG. 2, this is not kinetically visible. Although the terminal monophosphate function is in all likelihood entirely complexed with Zn^{2+}, only a part of the oligonucleotide molecules still form a macrochelate allowing

the monophosphate bound Zn^{2+} to become involved in the cleavage. Evidently at sufficiently high Zn^{2+} concentration the kinetically visible monodentate binding of Zn^{2+} to the scissile bond starts to compete, and the resulting UpU-like cleavage becomes predominant under conditions where the rate of the reaction via a macrochelate structure levels off to a constant value.

The lower reactivity of **2** compared to **4,** suggests that the Zn^{2+} complexes of **2** are weaker than those of **4**, irrespective of the exact structure of the complexes. This is a clear indication of the significance of the intervening phosphodiester bonds as metal ion co-ordination sites and the importance of the extra co-ordination for the stability of the reactive species. The fact that a phosphodiester oligomer **4** rather than a methyl-phosphonate oligomer **2**, exhibits some sign of saturation with Zn^{2+}, suggests that **4** forms macrochelates more easily than **2**, which allows speculations concerning the mechanism of the structural stabilisation by Zn^{2+}. One might expect that the macrochelate formation would be more facile with **2** than with **4**, because there is no electrostatic repulsion between the internucleosidic linkages to overcome. This does not appear to be the case, however, but the Zn^{2+}complexes of **2** are less reactive than those of **4**, and hence the macrochelate structure of **2** appears to be less stable than that of **4.** Accordingly, the metal ions bound to the phosphodiester bonds of oligonucleotides seem to stabilise the macrochelate by docking the two ends of the oligonucleotide on top of each other, not only by neutralising the negative charges.

As a conclusion, the results of this work support our previous suggestion according to which Zn^{2+} ions bind bidentately to oligonucleotides bridging two phosphate groups. With oligonucleotides bearing a 3'-terminal phosphomonoester function, the bidentate binding may result in a back-folding of the oligonucleotide chain forming a macrochelate as shown in *Scheme 2*. The macrochelate can be stabilised by another Zn^{2+} ion, that is bound to the intervening phosphodiester bonds. The role of the second metal ion most likely is to position the two phosphate groups bound to the catalytic metal ion in the vicinity of each other.

EXPERIMENTAL

Materials. The synthesis of the methylphosphonate[17] (**1**,**2**) and phosphodiester[15] oligonucleotides (**3**, **4**) has been reported elsewhere. 3',5'-ApU-3'p was a product of

Sigma, and it was used as recieved. Buffer constituents and metal ion salts were of reagent grade. When working at 37 °C, all the reaction solutions were prepared in sterilised water, and sterilised equipments were used for handling the solutions.

Kinetic measurements. The reactions were perfomed in 500 μL Eppendorf tubes, in a thermostated water bath. The pH of the reaction solutions was checked with a pH meter and adjusted if necessary. The pH values given are those measured at the temperature of kinetic measurements. The total volume of the reaction solution was 350 μL and aliquots of 25 μL were withdrawn at suitable intervals and they were immediately cooled on an ice bath. EDTA solution was added into the aliquots to inactivate the Zn^{2+} catalyst (at least a 2 fold excess compared to Zn^{2+}), except (for the case) with **1**, where addition of EDTA resulted in the precipitation of the oligomers. Aliquots containing **1** were analysed immediately, others were stored in the freezer until the analysis.

Analysis of the aliquots. Aliquots of the oligonucleotide and **6a** reactions were analysed by capillary zone electrophoresis (CZE) and the UpU samples by HPLC, as described before.[19] CZE analysis was performed on a Hewlett Packard ^{3D}CE equipment. A fused silica capillary (50μm i.d., 77 cm total length) was employed. Aliquots containing **1** were analysed using a 0.2 M phosphate buffer, pH 6.0. The low pH was needed to avoid a precipitation of Zn^{2+} in the capillary in the absence of EDTA. The voltage applied in this case was 18 kV. Other aliquots were analysed using a 0.5 M borate buffer, pH 8.5 and the voltage of 30 kV. In all the measurements, UV detection at 260 nm was used.

Using the analysis conditions described above, the migration times of **1** and the oligomeric product were 30 and 20 minutes, respectively. The migration time of 2',3'-cUMP, formed as an initial product, was 35-40 minutes and those of 2'- and 3'-UMP formed by the subsequent hydrolysis of 2',3'-cUMP, 60-70 minutes. In practise, an analysis time of 35 minutes was used, and only **1** and the oligomeric product were detected. Using the borate buffer, the migration times of **2** and its oligomeric product were 15 and 12 minutes, respectively. Again, only the starting material and the oligomeric product were detected.

Calculation of rate constants. The rate constants of cleavage of were calculated for the decrease of the mole fraction of starting material remaining in the solution. The mole fraction of **1-5** was calculated from the areas of the signals of the starting material and the

oligomeric product. The areas were first normalised by dividing the observed area by the migration time. To take into account the number of chromophores present, the normalised area of the product was then multiplied by 1.2. This procedure appears to give correct results: comparisons were made where all the products were detected, and the total area of signal was calculated. The UV spectra of the compounds was also recorded by using the diode array detector of HP ^{3D}CE equipment. No significant difference was observed in the spectra of the substrates and corresponding products formed. The rate constants were calculated by using the integrated rate law for the first-order reaction.

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