The effect of the 3'-terminal monophosphate group on the metal-ion-promoted hydrolysis of the phosphodiester bonds of short oligonucleotides

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The effect of 3'-terminal monophosphate group on the metal-ion-promoted hydrolysis of the phosphodiester bonds of oligonucleotides has been studied. For this purpose, the rate constants for the hydrolysis of the following oligomers in the presence of Zn^{2+} and its 1,5,9-triazacyclododecane chelate, $Zn^{2+}[12]aneN_3$, have been determined: (*i*) ApUpUp(2') and ApUpUp(3'), (*ii*) Up(Tp)$_n$T (n = 0–4 and 7), (*iii*) Up(Tp)$_n$Tp (n = 0–4 and 7). The results obtained are used to propose a mechanism for the Zn^{2+} and $Zn^{2+}[12]aneN_3$ promoted hydrolysis of polynucleotides.

The metal-ion-promoted cleavage of nucleic acids, DNA and RNA, has received considerable attention during the last two decades. One reason for this is the desire to develop metal-ion-based cleaving agents that would be able to recognize a certain base sequence within the target nucleic acid and cleave a given phosphodiester bond. These kinds of artificial nuclease would greatly enlarge the potential of biotechnology, and they might even offer a tool to combat viral infections and hereditary diseases.[1-3] Both hydrolytically[4-6] and oxidatively[7] functioning cleaving agents have been studied. Hydrolytic cleavage, however, has the advantage in that it produces fragments that can be religated by enzymes.
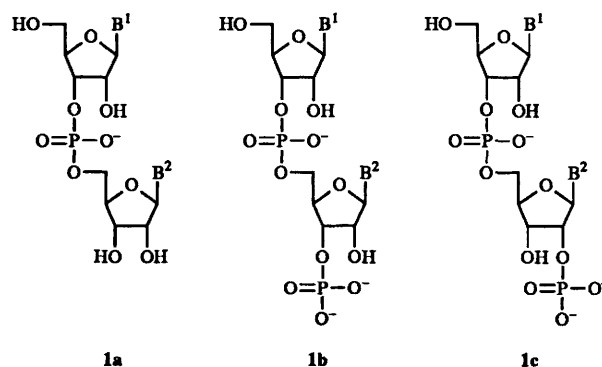
Since RNA is hydrolytically less stable than DNA, numerous studies on the metal-ion-promoted hydrolysis of various RNA mimics have been published. The model compounds employed range from simple diaryl phosphoesters[8-10] to real oligo-nucleotides.[11-14] The mechanism that is widely but not unanimously accepted for the metal-ion-promoted hydrolysis of the phosphodiester bonds of RNA involves coordination of the metal ion to the phosphate group and deprotonation of the attacking nucleophile, *viz.*, the 2'-hydroxy function, by the hydroxo ligand of the metal ion (Scheme 1).[12,14-18]

1a   1b   1c

Scheme 1
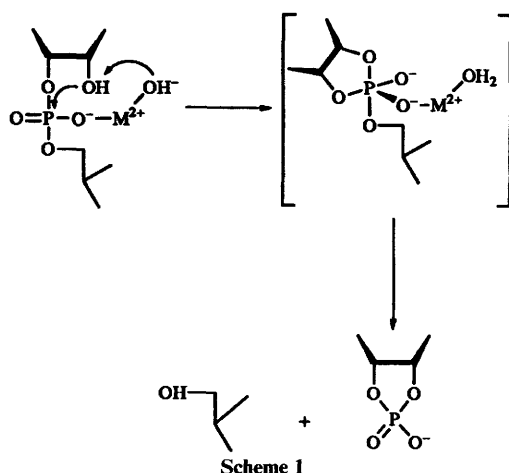
Accordingly, acidic metal ions are generally the most efficient catalysts, even though the coordination geometry of the metal ion also influences the catalytic activity of its chelates.[14-16,18]

Although the mechanism of metal-ion action on the phosphodiester bonds of RNA and on simpler model compounds, such as 3',5'-UpU (**1a**; B^1 = B^2 = U), is likely to be similar,

it is also known that the adjacent phosphate groups may have a large influence on the rate of the metal-ion-promoted hydrolysis.[11,14,18-21] The terminal dianionic 3'-phosphate group, for example, has been shown markedly to accelerate the metal-ion-promoted cleavage of the phosphodiester bond in dinucleoside diphosphates, NpNp (**1b**), whereas the effects of 2'- and 2',3'-cyclic monophosphate groups or acyclic phosphodi-ester groups are modest.[11] This finding suggests that whenever a 3'-phosphate group is produced in the course of metal-ion-promoted hydrolysis of RNA, it efficiently enhances the hydrolysis of the adjacent phosphodiester bond. Our previous results, according to which the phosphodiester bonds of poly-U are cleaved by metal ions up to 100 times faster than that of 3',5'-UpU (**1a**; B^1 = B^2 = U),[14] might hence, *a priori*, be attributed to a rapid stepwise release of 3'-UMP from the 3'-terminus of the polymeric products. However, this does not seem to be the case, since the same studies also showed that the distribution of terminal and non-terminal cleavages is almost statistical when Zn^{2+} aquo ion is used as a catalyst. This result might, at least partly, be accounted for by the observation that the 3'-terminal nucleotides produced by chemical hydrolysis mainly bear a 2',3'-cyclic monophosphate group.[14] The same argument cannot, however, be used to explain the effect of Zn^{2+} (1,5,9-triazacyclododecane) ($Zn^{2+}[12]aneN_3$). This catalyst cleaves 2',3'-cyclic monophosphates more rapidly than does Zn^{2+}, preventing accumulation of 3'-terminal 2',3'-cyclic monophosphate groups. In spite of this, the stepwise de-polymerization from the 3'-end was not preferred.

To resolve these ambiguities we now report a more detailed study on the rate-enhancing influence of a terminal 3'-mono-phosphate group. Firstly, the effect of the position of the

terminal phosphate group was elucidated by studying the reactivity of the two phosphodiester bonds of ribotrimers bearing either a 3'- or 2'-phosphate function (2a and 2b).

2a   2b
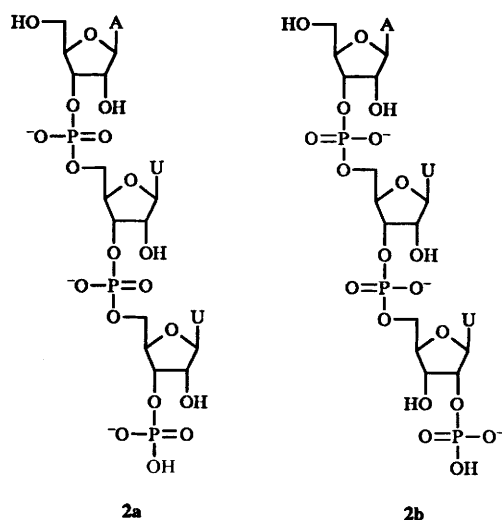
Secondly, the effect of the number of the phosphodiester bonds and the range of the rate-accelerating effect of the 3'-monophosphate group were elucidated by studying the reactions of short oligonucleotides. For this purpose, a series of oligomers that contained only one reactive phosphodiester bond, i.e., one ribonucleoside unit, and several 2'-deoxyribonucleoside units, were prepared. The behaviour of 3'-phosphor-
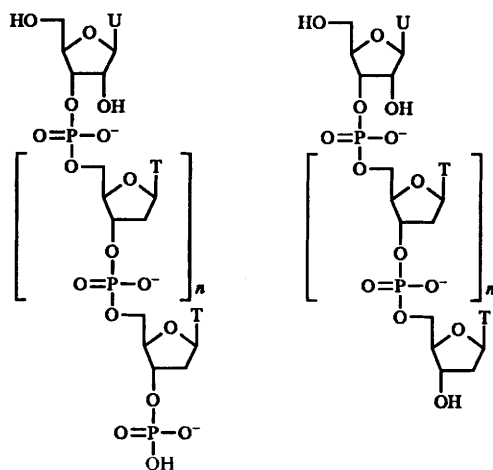
3	n = 0,	UpTp
4	n = 1,	UpTpTp
5	n = 2,	Up(Tp)₂Tp
6	n = 3,	Up(Tp)₃Tp
7	n = 4,	Up(Tp)₄Tp
8	n = 7,	Up(Tp)₇Tp

9	n = 0,	UpT
10	n = 1,	UpTpT
11	n = 2,	Up(Tp)₂T
12	n = 3,	Up(Tp)₃T
13	n = 4,	Up(Tp)₄T
14	n = 7,	Up(Tp)₇T

ylated (3–8) and the corresponding unphosphorylated oligonucleotides (9–14) were compared, and the effect of increasing chain length was studied. The importance of these factors with respect to the metal-ion-promoted hydrolysis of RNA is discussed, and a mechanism for the metal aquo ion and metal-ion chelate promoted hydrolysis of polynucleotides is suggested.

Results and discussion

According to the results of Butzow and Eichhorn,[11] the phosphate group at the 3'-position of the dinucleotide di-

Table 1 The rate constants for the Zn^{2+}-promoted hydrolysis of the phosphodiester bonds of 3'- and 2'-phosphorylated trimers (2a and 2b) at pH 5.5[a] and 333.2 K, $[Zn^{2+}] = 10$ mmol dm^{-3}

| | $k/10^{-5}$ s^{-1} | |
Substrate	ApU bond	UpU bond
ApUpUp(3') (2a)	1.7 ± 0.1[b]	3.3 ± 0.2
ApUpUp(2') (2b)	1.8 ± 0.2	<0.15 ± 0.02
ApU	0.037 ± 0.002	
UpU		0.026 ± 0.001

[a] Adjusted with HEPES buffer; $[HA]/[A^-] = 0.1/0.005$, $I = 0.1$ mol dm^{-3} (NaClO₄). [b] Standard deviation of the mean indicated.

Table 2 The rate constants for the hydrolysis of 3'-phosphorylated and unphosphorylated oligonucleotides in the presence of 10 mmol dm^{-3} Zn^{2+} aquo ion at pH 5.5[a] and at 333.2 K

Substrate	$k/10^{-6}$ s^{-1}	Substrate	$k/10^{-6}$ s^{-1}
UpTp (3)	38 ± 1[b]	UpT (9)	0.2 ± 0.03
UpTpTp (4)	33 ± 1	UpTpT (10)	2.7 ± 0.4
Up(Tp)₂Tp (5)	44 ± 3	Up(Tp)₂T (11)	2.4 ± 0.3
Up(Tp)₃Tp (6)	44 ± 2	Up(Tp)₃T (12)	2.6 ± 0.1
Up(Tp)₄Tp (7)	18 ± 2	Up(Tp)₄T (13)	4.3 ± 0.4
Up(Tp)₇Tp (8)	23 ± 3	Up(Tp)₇T (14)	5.0 ± 0.2

[a] Adjusted with HEPES buffer; $[HA]/[A^-] = 0.1/0.005$, $I = 0.1$ mol dm^{-3} (with NaClO₄). [b] Standard deviation of the mean indicated.

phosphate, ApAp (1b; $B^1 = B^2 = A$), enhances the Zn^{2+}-promoted hydrolysis of the phosphodiester bond by a factor of 100 compared with that of the corresponding dinucleotide monophosphate ApA (1a; $B^1 = B^2 = A$). The rate-promoting effect of the 2'-phosphate function is significantly smaller: the 2'-phosphorylated dimer (1c; $B^1 = B^2 = A$) is 30 times less reactive than the 3'-isomer. The effect of a 2',3'-cyclic phosphate group and an acyclic phosphodiester group is also modest. These results suggest that the position of the phosphate group may have a large influence on the metal-ion-promoted hydrolysis of the neighbouring phosphodiester bond, but they do not give any information about the reactivity of the following bonds in an RNA molecule. Table 1 records the rate constants for the Zn^{2+} aquo ion promoted hydrolysis of the phosphodiester bonds of 3'- and 2'-phosphorylated ribotrimers, ApUpUp (2a and 2b). It can be seen that both ApU and UpU bonds of the 3'-phosphorylated molecule are efficiently hydrolysed, the hydrolysis being approximately 100 times faster than that of the corresponding dinucleoside monophosphates ApU (1a; $B^1 = A$, $B^2 = U$) and UpU (1a; $B^1 = B^2 = U$). The slight difference observed between the stability of the bonds is not significant, but most probably results from the different base moieties.[19] The ApU bond in the 2'-phosphorylated trimer is also rapidly cleaved, while the UpU bond adjacent to the 2'-phosphate function is significantly less reactive. These results give an explanation for the observation according to which the terminal cleavage is not favoured with poly-U. With 2'/3'-phosphorylated oligomers, the terminal 3'-phosphate group enhances not only the hydrolysis of the adjacent phosphodiester bond, but also that of the following bond. Furthermore, the 2'-phosphate group also results in a rate-enhancement, but in this case not on the cleavage of the neighbouring phosphodiester bond, but on the next bond, resulting in the release of a dimeric unit.

To find out how far the rate-accelerating effect of the terminal monophosphate group may range, the reactions of oligomers 3–14 were studied. The results obtained in the presence of Zn^{2+} aquo ion are shown in Table 2. It is seen that different substrates exhibit a significantly different reactivity under similar conditions. The rate constants obtained for the hydrolysis of UpT

(9) is 2×10^{-7} s^{-1} at pH 5.5 and 333.2 K ($[Zn^{2+}]$ = 10 mmol dm^{-3}), whereas a tenfold faster hydrolysis was observed for the UpT bond of UpTpT (10). Accordingly, addition of one phosphate group results in a significant rate enhancement. If this phosphate group is dianionic, as it is in UpTp (3), the hydrolysis is further accelerated by a factor of 17. Additional phosphate groups do not seem to affect the hydrolysis rate of the UpT bond; unphosphorylated compounds from UpTpT (10) to Up(Tp)$_7$T (14) are hydrolysed at approximately equal rates. With 3'-phosphorylated compounds a slight rate retardation is observed on increasing the distance between the 3'-terminal phosphate group and the reacting bond. The rate-enhancement observed is attributed to the metal-ion catalysis: in the absence of metal ions neither Up(Tp)$_7$T (14) nor Up(Tp)$_7$Tp (8) is hydrolysed more efficiently than the corresponding dinucleoside monophosphate 3',5'-UpU.

The results shown in Table 2 can be explained by enhanced complex formation between the substrate and metal ion. Under the experimental conditions employed, less than 10% of UpT (9) binds Zn^{2+}, and is hence in the reactive form (log K_{LM} may be assumed to be less than unity).[22] Addition of one phosphodiester bond provides another oxygen ligand, possibly enabling bidentate binding of the Zn^{2+} cation (structure a).

a   b

c
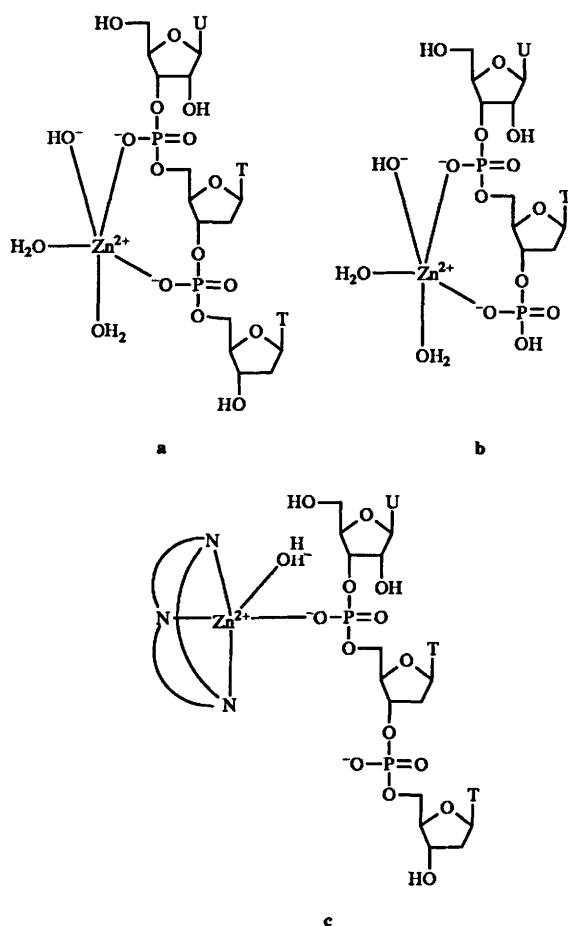
The complex formation is thus enhanced and the hydrolysis is accelerated. The dianionic monophosphate function is obviously an even more favoured coordination site, and the metal ions predominantly bind to it. In addition, the same metal ion appears to be able to coordinate to one of the phosphoryl oxygens of the diester groups (structure b). Since the efficiency of the metal-ion catalysis is not further increased on incorporation of a third phosphodiester bond, it seems probable that the metal ion interacts only with two phosphate groups. These do not

Table 3 The rate constants for the hydrolysis of 3'-phosphorylated and unphosphorylated oligonucleotides in the presence of 1 mmol dm^{-3} Zn^{2+} [12]aneN$_3$ at pH 6.5^a and at 333.2 K

Substrate	$k/10^{-6}$ s^{-1}	Substrate	$k/10^{-6}$ s^{-1}
UpTp (3)	11 ± 1^b	UpT (9)	2.6 ± 0.1
UpTpTp (4)	18 ± 2	UpTpT (10)	2.0 ± 0.5
Up(Tp)$_2$Tp (5)	11 ± 1	Up(Tp)$_2$T (11)	4.5 ± 0.2
Up(Tp)$_3$Tp (6)	8.5 ± 0.5	Up(Tp)$_3$T (12)	3.0 ± 0.2
Up(Tp)$_4$Tp (7)	12 ± 1	Up(Tp)$_4$T (13)	3.8 ± 0.1
Up(Tp)$_7$Tp (8)	3.2 ± 1	Up(Tp)$_7$T (14)	3.8 ± 0.1

^{a} Adjusted with HEPES buffer; [HA]/[A$^-$] = 0.065/0.035; I = 0.1 mol dm^{-3} (NaClO$_4$). ^{b} Standard deviation of the mean indicated.

necessarily have to be adjacent ones, but as the results obtained with the 3'-phosphorylated compounds show, the 3'-phosphate-bound metal ion may exert its catalytic influence over several nucleotide units.

Significantly different results were obtained with Zn^{2+}-[12]aneN$_3$ as a catalyst (Table 3). In the presence of this chelate, a dimer and trimer are hydrolysed at an equal rate, and only a modest rate-enhancement is observed on further increasing the chain length of the substrate. Even the addition of a dianionic monophosphate function does not result in a great rate-acceleration: UpTp (3) is hydrolysed only six times as fast as UpT (9), whereas a 170-fold difference was observed for the Zn^{2+}-promoted hydrolysis. This result supports the concept suggested above. In the Zn^{2+}[12]aneN$_3$ chelate, the Zn^{2+} cation is bound to three nitrogen atoms. Thus, to be able to provide a general-base catalyst, a hydroxo ligand for deprotonation of the attacking nucleophile, Zn^{2+} cannot accept more than one phosphoryl oxygen ligand (structure c). Since 3'-phosphorylation still enhances the rate of the Zn^{2+}[12]aneN$_3$-promoted hydrolysis, one may assume that the chelate does not necessarily have to bind to the phosphate group that is cleaved. In fact, our previous studies with poly-U suggest that the phosphodiester bond adjacent to the dianionic phosphate function might be slightly less readily hydrolysed than the non-terminal bonds. This is not, however, clearly supported by the results shown in Table 3.

It is worth noting that the [12]aneN$_3$ ligand as such does not diminish the catalytic efficiency of Zn^{2+}. If the rate constants obtained for the Zn^{2+} and Zn^{2+}[12]aneN$_3$ promoted hydrolysis of UpT (9) are extrapolated to the same conditions, assuming first-order dependence on both the catalyst and hydroxide ion concentration,[14,16] the chelate is ten times more efficient a catalyst, as expected on the basis of the higher acidity of its aquo ligand. By contrast, UpTpT (10) or the longer oligomers that can provide more than one coordination site for the metal ion, are hydrolysed as efficiently by both catalysts. In other words, a substrate that can offer additional coordination sites for the catalyst, enhances the catalytic activity of the Zn^{2+} aquo, but not that of the Zn^{2+}[12]aneN$_3$ chelate, the ability of which to make use of the additional binding sites is limited by the presence of the triaza ligand. This explains why Zn^{2+} [12]aneN$_3$ accelerates the hydrolysis of poly-U less efficiently than Zn^{2+},[14] although the situation is reversed when the hydrolysis of an isolated phosphodiester bond of dinucleoside monophosphates is concerned.[16]

These results together with those obtained previously for the metal-ion-promoted hydrolysis of poly-U, gave a detailed picture of the metal-ion-promoted hydrolysis of polynucleotides. As an initial process, the Zn^{2+} aquo ion binds to two phosphate groups within the polynucleotide strand and provides a general-base catalyst for the hydrolysis of a phosphodiester bond, which is probably one of those that the metal ion is bound to. The rate constant for the Zn^{2+}-promoted reaction is 2×10^{-6} s^{-1} at pH 5.5 and 333.2 K ($[Zn^{2+}]$ = 10 mmol dm^{-3}). The hydrolysis

produces two fragments, one of which contains a 2′,3′-cyclic monophosphate function at its 3′-terminus. Since this group is monoanionic, it does not enhance the hydrolysis of the neighbouring phosphodiester bonds. Under the conditions mentioned above, the rate constant for the hydrolysis of the 2′,3′-cyclic monophosphate function is 6×10^{-6} s^{-1}. The opening of the cyclic function gives a mixture of 2′- and 3′-phosphorylated oligomers in the ratio 2:1. The dianionic phosphate functions constitute the most favoured coordination site for the catalyst. In addition to binding to the terminal phosphate function, it coordinates to another phosphodiester bond which is then efficiently hydrolysed by the bifunctional catalysis mechanism. Evidently, the catalyst can bridge nucleotides that are several nucleotide units apart. In other words, the rate-enhancing effect of the 3′-terminal 3′- or 2′-monophosphate group may range over several phosphodiester bonds. The rate constant of the hydrolysis promoted by Zn^{2+} that is bound simultaneously to the terminal monophosphate function is approximately 4×10^{-5} s^{-1}. However, the phosphodiester bond adjacent to the 2′-monophosphate function is considerably more stable. For this reason, monomeric 2′-UMP is released from oligomeric products significantly more slowly than 3′-UMP. As a matter of fact, almost no 2′-UMP was detected during the Zn^{2+}-promoted hydrolysis of poly-U.[14] The rate constant observed for the hydrolysis of phosphodiester bonds of poly-U is 2.5×10^{-5} s^{-1}, which is a reasonable mean value for the parallel and consecutive processes involved.

In the presence of the Zn^{2+}[12]aneN$_3$ chelate, the course of the hydrolysis reaction differs from that suggested above. The metal ion binds to the substrate as a monodentate ligand and promotes the hydrolysis of a phosphodiester bond which is not necessarily the same as that to which the metal ion is bound. The rate constant for the initial cleavage is approximately 5×10^{-6} s^{-1} at pH 6.5 and 333.2 K ([Zn^{2+}[12]aneN$_3$] = 1 mmol dm^{-3}). The 2′,3′-cyclic phosphate group produced by the initial cleavage is opened rapidly to give either a 2′- or a 3′-monophosphate function, the rate constant for the latter process being 1×10^{-4} s^{-1}. The terminal monophosphate function is again the most favoured coordination site for the catalyst. The metal ion bound to the monophosphate function promotes the hydrolysis of one of the following phosphodiester bonds by providing a general base to assist the nucleophilic attack, but not by coordinating to the bond. The ligand around the metal ion restricts the action of the catalyst, and the catalytic activity of the chelate can range over only about six nucleotide units. Bonds that are further apart from the terminal phosphate group cannot be reached by the catalyst, and they react similarly to the bonds of an intact molecule. Previous studies on poly-U show that during the Zn^{2+}[12]aneN$_3$-promoted hydrolysis, the ratio of the molar fractions of 2′- and 3′-monophosphate groups at the 3′-terminal position remains constant during the kinetic run, being the same as that produced by the hydrolysis of 2′,3′-cUMP. This suggests that the rate-enhancing effects of the 2′-and 3′-monophosphate functions on the hydrolysis of the adjacent phosphodiester bonds are, in this case, similar.

Experimental

Materials

Long-chain alkylamine controlled pore glass and 5′-O-(4,4′-dimethoxytrityl)thymidine derivatized CPG (type II) were purchased from Sigma. All the reagents for machine-assisted oligonucleotide synthesis were from Chruachem. The oligonucleotides were assembled on an Applied Biosystems 392 DNA/RNA Synthesizer (ABD, Perkin-Elmer Corp.). Adenylyl-(3′,5′)-uridine 3′-monophosphate, uridylyl-(3′,5′)-uridine and
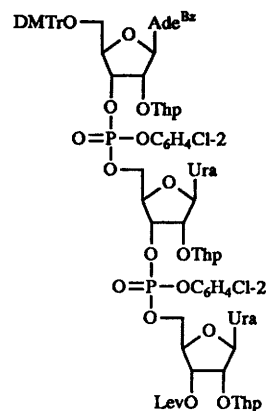
15:    Ade^{Bz} = N^6-Benzoyladenine
       Ura = Uracil
       DMTr = 4,4′-Dimethoxytrityl
       Thp = Tetrahydropyranyl
       Lev = Levulinyl
       C$_6$H$_4$Cl-2 = 2-Chlorophenyl

all nucleosides and their monophosphates were commercial products from Sigma. [12]aneN$_3$ as the trihydrobromide salt was from Aldrich. The buffer constituents and the Zn(NO$_3$)$_2$ salt were of reagent grade.

Trinucleoside triphosphates (2a, b)

The completely protected trinucleoside diphosphate, 15, was prepared by the phosphotriester method in solution using 1-hydroxybenzotriazole as an activator.[23] 3′-O-Levulinyl-2′-O-tetrahydropyranyluridine, used to introduce the 3′-terminal nucleotide, was synthesized by levulinating the 3′-OH of 5′-O-(4,4′-dimethoxytrityl)-2′-O-tetrahydropyranyluridine[23] as described earlier,[24] and removing the dimethoxytrityl group with dichloroacetic acid in dichloromethane (3%, 10 min, room temperature).

Compound 15 (300 mg) was delevulinated with hydrazine hydrate (0.5 mol dm^{-3}) in a mixture of pyridine and acetic acid (4:1, v/v, 20 cm^3). After 20 min at room temperature, the mixture was diluted with dichloromethane (100 cm^3), washed with saturated aqueous potassium dihydrogenphosphate (100 cm^3) and saturated aqueous sodium hydrogencarbonate (100 cm^3), dried over sodium sulfate, and evaporated to dryness. The residue was additionally co-evaporated with dry pyridine (3 × 30 cm^3) and then phosphorylated with phosphorotristriazole in dry acetonitrile.[25] The reaction was stopped by addition of aqueous pyridine. After 3 h at room temperature, the mixture was evaporated to dryness, and the 3′-O-phosphorylated trimer was deblocked as described previously.[23] The deblocked adenylyl-(3′,5′)-uridylyl-(3′,5′)-uridine 3′-monophosphate (2a) was purified by ion-exchange chromatography on Toyopearl DEAE 650 M (HCO$_3^-$-form), using a linear gradient of aqueous ammonium hydrogencarbonate from 0 to 0.3 mol dm^{-3}. Most of the buffer constituents were removed by coevaporations with water. Prolonged treatment in the buffer resulted in partial isomerization of 2a to 2b. The isomeric mixture was first separated by RP-HPLC (Hypersil ODS column, 250 × 10 mm × 5 μm, acetic acid–sodium acetate buffer, pH 4.3, containing 0.1 mol dm^{-3} NH$_4$Cl and 10% acetonitrile), and then desalted on the same column.

The following enzymatic digestions were used to verify the identity of 2a and 2b: (i) RNase A, a pyrimidine specific endonuclease, to cleave the bond between the two uridine moieties, and (ii) alkaline phosphatase to remove the terminal phosphate function. With both compounds, RNase A digestion

gave adenylyl-(3′,5′)-uridine 3′-monophosphate which was identified by spiking the product with an authentic commercial product (Sigma). The other product was 3′-UMP from 2a and 2′-UMP from 2b. Comparison with the calibration samples of known concentrations indicated that 2a and 2b gave ApUp and UMP in a molar ratio of 1:1. Dephosphorylation of both of 2a and 2b gave a single product, obviously ApUpU.

3′-Phosphorylated oligomers (3–8)

A modified solid support was used to obtain 3′-phosphorylated oligonucleotides on a 10 μmol scale. 4,4′-Dimethoxytrityloxyethyl hydroxyethyl sulfone[26] was succinylated[27] and coupled to the long-chain controlled-pore glass by the method described previously.[28] The solid support, 16, prepared showed a loading ranging from 33 to 34 μmol g^{-1} when determined by dimethoxytrityl cation assay.[27]
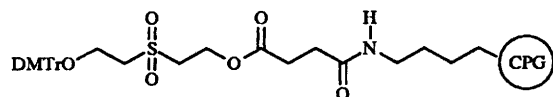
16

To verify the applicability of the solid support, a model oligonucleotide (Tp)$_{10}$ was assembled on a 0.2 μmol scale and, upon standard ammonolytic deprotection, analysed by both ion-exchange and RP HPLC. This experiment showed that the step-by-step coupling yield was normal (> 98.5%). The product, (Tp)$_{10}$, co-eluted with an authentic sample prepared by a method described in the literature.[29] The target oligonucleotides, Up(Tp)$_n$Tp (3–8, $n = 0$–4 and 7), were then assembled on a 10 μmol scale on support 16 using standard protocols (Scheme 2). After the oligothymidylate sequences
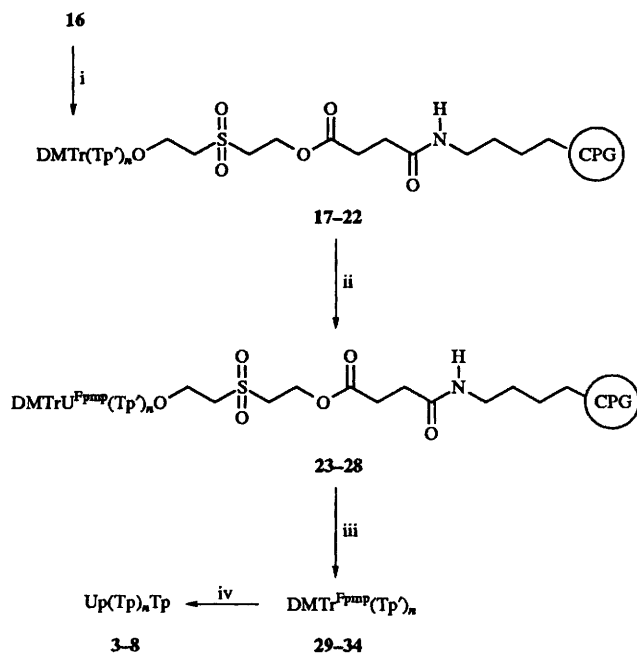
Scheme 2 i, DNA chain assembly; ii, RNA synthesis cycle; iii, NH₃–H₂O, 55 °C, overnight; iv, 0.01 mol dm^{-3} aq. HCl, rt, 20 h. p′ = 2′-cyanoethyl protected internucleosidic phosphate moiety; Fpmp = 2′-O-[1-(2-fluorophenyl)-4-methoxy-4-piperidyl].

17–22 had been assembled by the DNA synthesis mode, the RNA cycle was applied to perform the final coupling of the ribo-U^{Fpmp} building block. The oligonucleotides 23–28 were

treated with concentrated aqueous ammonia overnight at 55 °C. Compounds 29–34 that still bore 5′-O-dimethoxytrityl and 2′-O-Fpmp groups, were isolated by semipreparative RP HPLC (Hypersil ODS described above), using a linear gradient from 40 to 100% B (A = 0.05 mol dm^{-3} triethylammonium acetate, B = triethylammonium acetate in 70% aqueous MeCN). The final removal of the Fpmp-protections with 0.01 mol dm^{-3} aqueous HCl gave 3–8, which were finally purified by RP HPLC chromatography using a mixture of water and acetonitrile (95:5, v/v) as the eluent. To verify the structure of the oligomers, they were cleaved with a mixture of alkaline phosphatase and phosphodiesterase I. The digestion resulted in a mixture of uridine and thymidine. In every case the molar ratio of these was equal to that expected for the starting material employed.

Oligonucleotides 9–14

Oligonucleotides 9–14 were assembled on a 10 μmol scale on a commercial thymidine-derivatized CPG, and purified and characterized as described above for 3–8. The verification of the structure of the oligonucleotides was performed by using the same enzymatic digestion procedure as for 3–8 above.

Kinetic measurements

The principle of the kinetic measurements has been described earlier.[15,30] The aliquots withdrawn from solutions of UT oligomers were analysed as such. The monomeric products were first separated by using the RP-18 HPLC column (250 × 4 mm × 5 μm) with a mixture of acetate buffer (0.1 mol dm^{-3}, pH 4.3, 0.1 mol dm^{-3} NH₄Cl) and acetonitrile (99.75:0.25 v/v). After that, a gradient was applied to elute the dimeric constituents (max. 25% of acetonitrile). The first aliquot of every kinetic run was treated with a mixture of alkaline phosphatase and phosphodiesterase I to obtain a final value for the release of uridine nucleotides. Aliquots containing ribotrimers were analysed by RP HPLC using the gradient program described above.

Calculation of the rate constants

The rate constant for the hydrolysis of compounds 3–14 were obtained by following the concentration of the 5′-terminal uridine nucleotides released by the chemical hydrolysis. The final value for the concentration was obtained by digesting the first aliquot of each kinetic run as described above. The rate constant for the hydrolysis was then calculated by applying the first-order rate-law.

The rate constants for the hydrolysis of the phosphodiester bonds of ribotrimers 2a, b in the presence of Zn^{2+} aquo ion were also determined by following the formation of the products. The initial products formed did not significantly react further. Thus ApUp bearing a 2′,3′-cyclic phosphate function at its 3′-terminus and uridine 2′- or 3′-monophosphate could be attributed to the hydrolysis of the 3′-terminal bond, and 2′,3′-cAMP and UpUp to the hydrolysis of the 5′-terminal bond. In both cases, the final value for the concentration of the products was equal to the initial concentration of the starting material, and the integrated first-order rate equation could be applied.

The dimeric products formed were identified with the aid of the following enzyme digestions. ApUp bearing a 2′,3′-cyclic phosphate function: (i) digestion with phosphodiesterase I and II cleaved the phosphodiester bond yielding monomeric products that were identified by spiking with commercial product; (ii) digestion with RNase A (pyrimidine-specific endonuclease) did not cleave the phosphodiester bond, but opened the cyclic function yielding ApUp(3′) that was assigned by coinjection with a commercial product, and (iii) alkaline phosphatase did not dephosphorylate the product. UpUp(2′/

3'), in turn showed the following behaviour: (*i*) digestion with RNase A gave 3'-UMP from the 3'-phosphorylated molecule and a 1:1 mixture of 2'- and 3'-UMP from its 2'-isomer; (*ii*) alkaline phosphatase dephosphorylated the molecules to 3',5'-UpU, which was identified by spiking with a commercial product (Sigma).

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