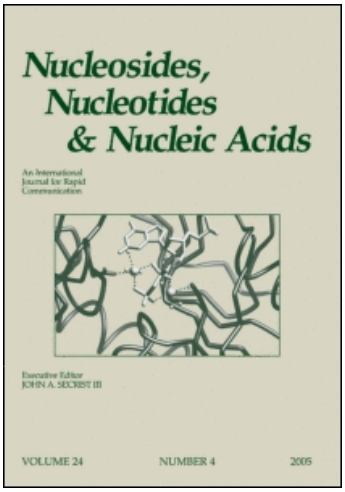

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1675–1692 (2000)

CLEAVAGE OF THE PHOSPHODIESTER BOND OF URIDYLYL-(3′,5′)-8-CARBOXYMETHYLAMINOADENOSINE BY HYDRONIUM, HYDROXIDE AND ZINC(II) IONS: A MODEL STUDY AIMED AT ELUCIDATING THE POTENTIAL OF A CARBOXYLATE FUNCTION AS AN INTRAMOLECULAR CATALYST

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ABSTRACT: Uridylyl-(3′,5′)-8-carboxymethylaminoadenosine has been synthesised, and its transesterification to uridine 2′,3′-cyclic phosphate in the presence and absence of Zn^{2+} ion has been studied. The results show that a carboxylate function in the vicinity of the phosphodiester bond accelerates the metal ion promoted cleavage but not the metal ion independent reaction. Under acidic conditions, the predominant reaction is the cleavage of the side chain, giving the 8-amino derivative.

INTRODUCTION

The kinetics and mechanism of the cleavage of the phosphodiester bonds of RNA have been extensively studied during the last two decades.[1,2] These studies have been motivated by understanding of the mechanisms that protein enzymes and catalytic ribonucleic acids utilise to hydrolyse RNA, and by development of artificial cleaving agents that could mimic the enzyme action and hence enable chemical tailoring of RNA.

The hydrolysis of RNA (*Scheme 1*) is known to be promoted by acids, bases and metal ions.[1-4] Catalysis by general acids and bases is, however, rather modest.[5,6] Metal ions and their chelates that may undergo pre-equilibrium binding to RNA phosphodiester bonds, and hence serve as carriers of intracomplex general acid/base catalysts or

This paper is dedicated to the memory of Professor A.Krayevsky

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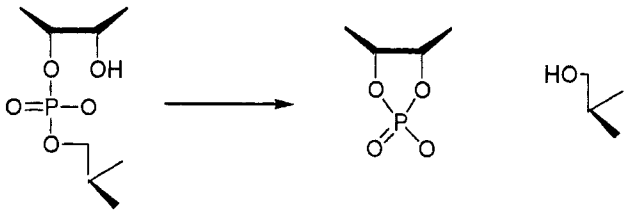
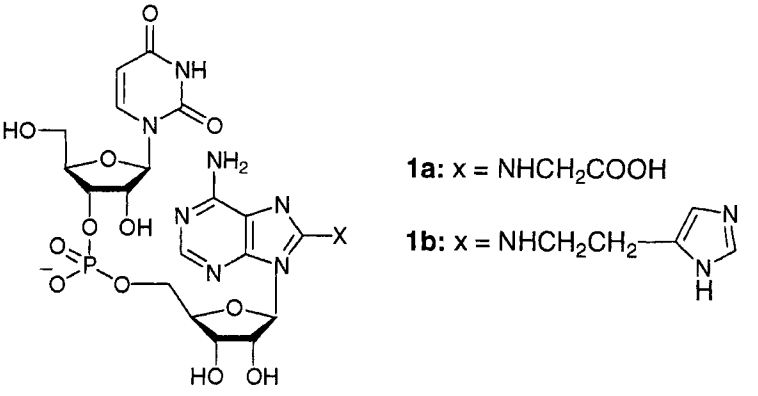
nucleophiles, are generally more efficient catalysts, but the catalytic activity still falls far from that of enzymes.[7-9]

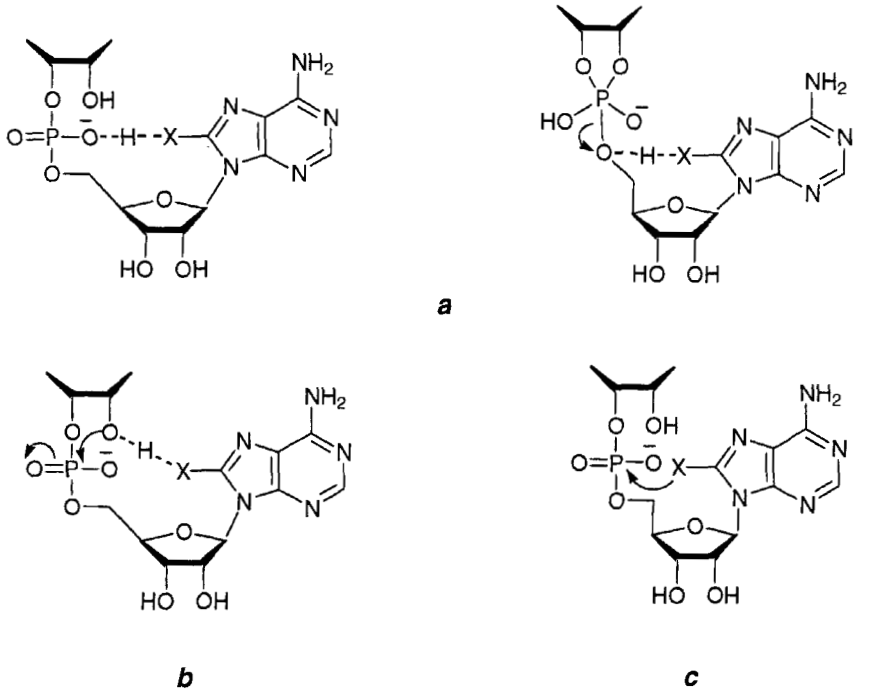


Scheme 1

Tethering of the catalyst to an oligonucleotide that recognises a desired sequence in RNA and brings the catalytic functions in proximity of a given phosphodiester bond, results in more efficient cleavage by reducing the entropy penalty associated with intermolecular catalysis, and makes the reaction sequence selective. In fact, several such conjugates of oligodeoxyribonucleotides have been reported.[10-21] In most cases the catalytic activity is based on a complexed metal ion. The present study is aimed at elucidating how efficient the catalysis could be if only a single catalytically active functional group is brought in the vicinity of the phosphodiester bond to be cleaved. The situation has been modelled by studying the reactions of uridylyl-(3′,5′)-8-carboxymethylaminouridine [3′,5′-Up(8-cmaA);**1a**], a dinucleoside monophosphate having the catalyst covalently attached close to the reaction centre. A similar approach has previously been used by Prakash *et al.*,[22] who have studied the catalytic potential of an histidine function by using **1b** as a model compound.



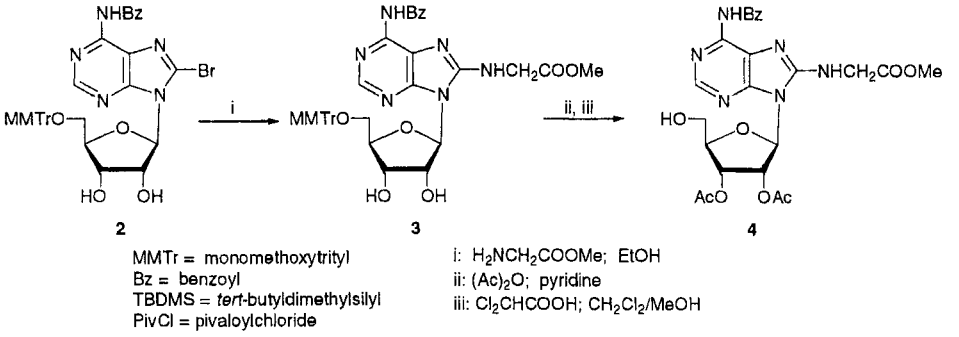
The carboxy group may in principle participate in the cleavage of the phosphodiester bond in several ways: as a general acid (*Scheme 2,* **a**) or as a general base catalyst (*Schemes 2,* **b**) as well as as a nucleophile attacking on the phosphate group (*Scheme 2,* **c**). The intermolecular counterparts of all these mechanisms have been reported.[5,23,24] Furthermore, the potential of carboxylate group as a binding site for a catalytically active metal ion has been elucidated by studying the cleavage of 3′,5′-Up(8-cmaA) in the presence of Zn^{2+} and its 1,5,9-triazacyclododecane chelate (Zn^{2+}[12]aneN$_3$).



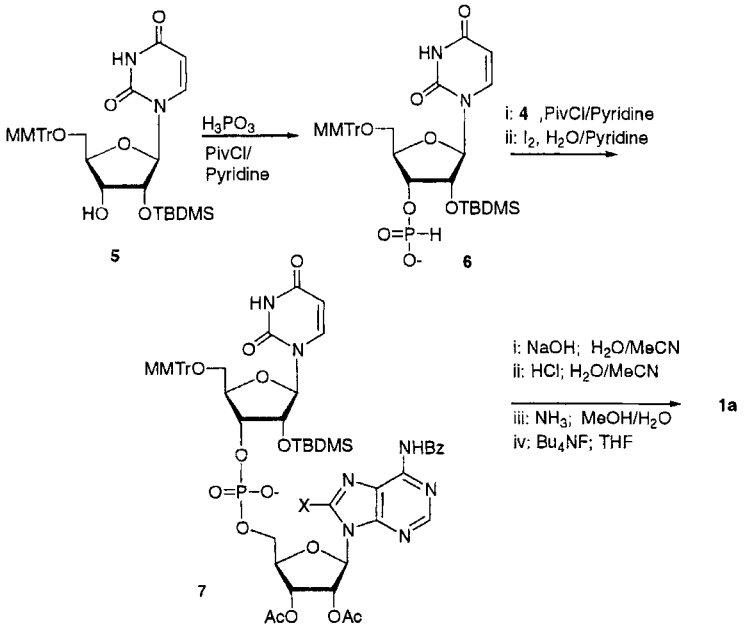
Scheme 2; x = $NHCH_2COOH$

RESULTS AND DISCUSSSION

Synthesis of uridylyl-(3′,5′)-(8-carboxymethylamino)adenosine [3′,5′-Up(8-cmaA); 1a]. The synthesis route for 3′,5′-Up(8-cmaA) (**1a**) is shown in *Scheme 3a,b.* Reaction of **2**[25] with glycine methyl ester in anhydrous ethanol gave the 8-methoxy-carbonylmethylamino nucleoside (**3**), from which the final adenosine building block **4** was prepared by acetylation of the 2′- and 3′-hydroxy functions and subsequent removal of the monomethoxytrityl group (*Scheme 3a*). The internucleosidic linkage was obtained by the hydrogen phosphonate method[26] (*Scheme 3b*).



Scheme 3a

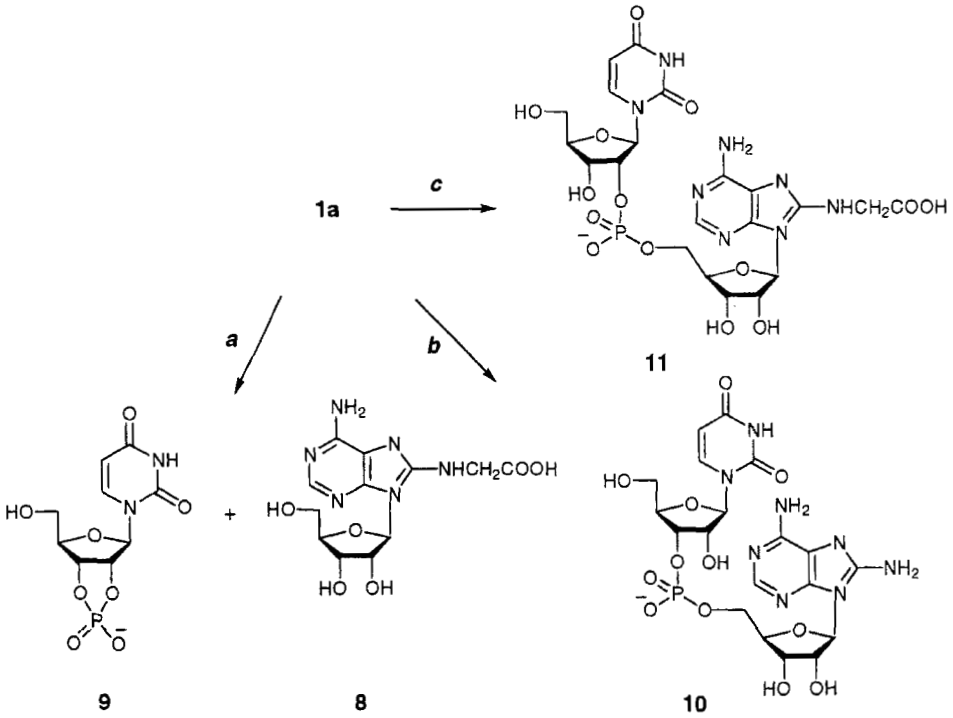


Scheme 3b

Accordingly, 2′-*O*-*tert*-butyldimethylsilyl-5′-*O*-(4-methoxytrityl) uridine **5** was reacted with phosphonic acid (phosphorous acid) in anhydrous pyridine in the presence of pivaloyl chloride. After isolation and purification on a silica gel column, the 3′-hydrogen phosphonate **6** was condensed with the adenosine building block **4**, also in pyridine in the presence of pivaloyl chloride. The hydrogen phosphonate diester formed was not isolated, but it was immediately oxidised to the phosphate diester **7** by adding iodine in the reaction mixture. Deprotection of **7** yielded **1a**, which was purified by

semipreparative RP HPLC, and characterised by ^{1}H, ^{13}C and ^{31}P NMR and mass spectrometry.

Cleavage and isomerisation of 1a in the absence of metal ions. The disappearance of 3′,5′-Up(8-cmaA) (**1a**) was followed by RP HPLC over a wide pH range at 90 °C. All pH values reported in the following refer to this temperature. All products were identified either by spiking with authentic samples and/or with HPLC/ESI-MS analysis as described in the Experimental. Two different cleavage reactions were observed, the proportions of which depended on pH. Above pH 7, the reaction proceeded exclusively by the release of 8-cmaAdo (**8**), giving 2′,3′-cUMP (**9**) as an initial product (*Scheme 4*, route ***a***).

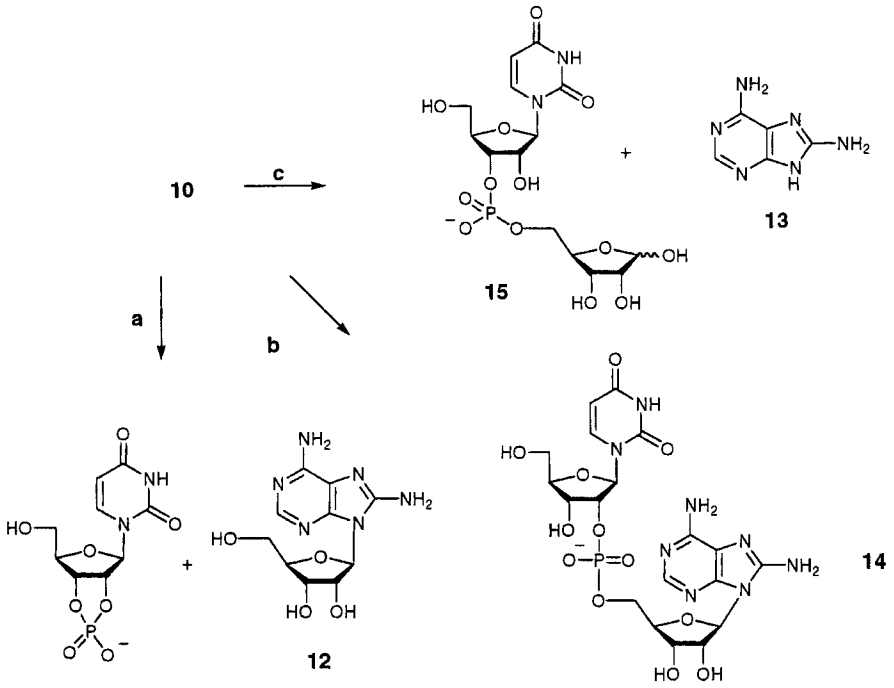


Scheme 4

As the pH was decreased, another cleavage process was observed to compete with the cleavage of the phosphodiester bond. Rather unexpectedly, 3′,5′-Up(8-cmaA) (**1a**) underwent cleavage of the 8-carboxymethyl group, resulting in a formation of uridylyl-

(3′,5′)-8-aminoadenosine [3′,5′-Up(8-NH_2-A); **10**] (*Scheme 4*, route ***b***). In addition to these two reactions, isomerisation of 3′,5′-Up(8-cmaA) to its 2′,5′-counterpart (**11**) took place at pH 3-7 (*Scheme 4*, route ***c***).

The first piece of evidence of the formation of 3′,5′-Up(8-NH_2-A) (**10**) was the appearance of M+1 signal of 589 (*m/z*) in the HPLC/ESI-MS analysis of the reaction mixture. The result was confirmed by positive ion FAB-MS and ^{1}H NMR analysis of the isolated product. Further proof for the structure of 3′,5′-Up(8-NH_2-A) was obtained by studying its subsequent breakdown, and identifying the products formed. Under acidic conditions, 3′,5′-Up(8-NH_2-A) reacted along three different pathways (*Scheme 5*): by cleavage and isomerisation of the phosphodiester bond (routes ***a*** and ***b***, respectively) and by depurination of the 8-aminoadenosine moiety (route ***c***).



Scheme 5

8-Aminoadenosine (8-NH_2-Ado; **12**) and 8-aminoadenine (8-NH_2-Ade; **13**) were identified by spiking with authentic samples of the compounds. The UV-spectrum of 8-NH_2-Ade recorded on an HPLC/diode-array UV detector was also consistent with the

spectral data reported earlier,[27] the UV-maximum being 285 nm. HPLC/ESI-MS analysis of the reaction mixture gave an M+1 signal of 283 (*m/z*) for 8-NH_2-Ado, consistent with the calculated mass of the compound. The 2′,5′-isomer of **10** [2′,5′-Up(8-NH_2-A); **14**] was also identified by HPLC/ESI-MS (M+1= 589). The depurinated dimer **15** was not observed to accumulate. Most likely, **15** undergoes rapid subsequent decomposition, producing several different reaction products.

The reaction pattern related to the decomposition 3′,5′-Up(8-cmaA) (**1a**) is complex, except at pH > 7, where 3′,5′-Up(8-cmaA) is cleaved exclusively *via* transesterification to 2′,3′-cUMP (**9**). At pH < 5.0, the formation of 3′,5′-Up(8-NH_2-A) (**10**) predominates. In fact, the rate constants of the transesterification could not be obtained under these conditions. At pH 5 to 6, **10** is still formed to a significant extent, but an estimate for the rate constant of transesterification of **1a** to 2′,3′-cUMP could be obtained by assuming that the molar absorptivities of 3′,5′-Up(8-cmaA) and 3′,5′-Up(8-NH_2-A) are equal. At pH 5.0, about 75 % of the breakdown of 3′,5′-Up(8-cmaA) may be estimated to proceed *via* formation of 3′,5′-Up(8-NH_2-A), whereas at pH 5.6 breakdown to 3′,5′-Up(8-NH_2-A) and 2′,3′-cUMP takes place approximately as readily. At pH > 6, formation of 3′,5′-Up(8-NH_2-A) becomes an insignificant side reaction.

The rate constants for the cleavage of 3′,5′-Up(8-cmaA) (and its 2′,5′-isomer) to 2′,3′-cUMP and 8-cmaAdo are plotted as a function of pH in FIG. 1, together with the corresponding rate constants of the cleavage of 3′,5′-UpU. The comparison is valid, since previous results[28] show that the second-order rate constants of the hydroxide ion catalysed cleavage of 3′,5′-UpA is only 10 % larger than that of 3′,5′-UpU. The data in FIG. 1 clearly show that the carboxymethylamino side chain does not have any rate-accelerating effect on the transesterification reaction at pH > 5.0. The carboxymethyl-amino group does not have any influence on the isomerisation rate, either. The rate constants for the pH-independent interconversions of 3′,5′-UpA and 3′,5′-Up(8-cmaA) to their 2′,5′-counterparts are 1.4×10^{-6} s^{-1} and 1.0×10^{-6} s^{-1}, respectively. The reactions of 3′,5′-Up(8-NH_2-A) (**10**) were also studied in some detail. In addition to transesterification to 2′,3′-cUMP and 2′,5′-Up(8-NH_2-A), 3′,5′-Up(8-NH_2-A) also undegoes depurination of the 8-aminoadenosine moiety (*Scheme 5*). The depurination is actually the predominant process that **10** undergoes under acidic conditions, consistent

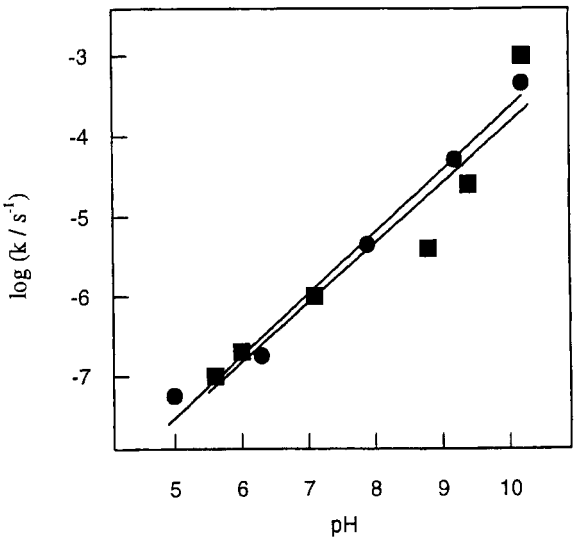


FIGURE 1. Rate constants of the cleavage of the phosphodiester bond of **1a** (■) and **3′,5′-UpU** (●) as a function of pH at 90°C. *I*=0.1 M (NaCl).

with the known instability of the *N*-glycosidic bond of 8-aminoadenosine.[29] Under alkaline conditions, 3′,5′-Up(8-NH_2-A) reacts mainly by transesterification to 2′,3′-cUMP, and similarly to the situation with 3′,5′-Up(8-cmaA), the rate constants seem to be comparable to those of the cleavage of 3′,5′-UpA.

The cleavage of the carboxymethyl group from 3′,5′-Up(8-cmaA) was unexpected, and the mechanism is not clear to us. Evidently, the phosphodiester group does not participate, since the cleavage of the carboxymethyl side chain takes place equally fast with 8-cmaAdo (**8**) and **1a**. The only product observed on cleaving **8** under acidic conditions, was **12**. Consistent with the results obtained with **1a**, **8** appeared to be stable at pH 6.6 and 10.4. The formation of 3′,5′-Up(8-NH_2-A) shows a first-order dependence of rate on hydronium ion concentration under slightly acidic conditions. In carboxylic acid buffers, the rate is, however, two to three orders of magnitude larger than could be expected on the basis of the reaction rate in amine buffers of comparable acidity (cf. acetate and MES buffer). It could be tentatively suggested that the reactive species is the N7 protonated substrate, which bears a partial positive charge on the 8-amino group. The methylene group between the electron deficient nitrogen and the carbonyl group is

electron poor and hence susceptible to nucleophilic attack. In carboxylate buffers, the methylene carbon may be attacked by a carboxylate anion, whereas in a less nucleophilic MES buffer the attacking nucleophile probably is a water molecule, and the reaction is hence slower.

Cleavage of 1a in the presence of Zn^{2+} and Zn^{2+}[12]aneN$_3$. The cleavage of 3′,5′-Up(8-cmaA) was also studied in the presence of Zn^{2+} and its [12]aneN$_3$ complex at pH 5.6. Unlike the situation in their absence, in the presence of metal ion catalysts the transesterification to 2′,3′-cUMP is the only reaction observed. The rate constants obtained are collected in TABLE 1. The cleavage of 3′,5′-UpA was studied for comparative purposes. It is seen, that 10 mM Zn^{2+} promoted the transesterification to 2′,3′-cUMP by a factor of about 1000. Comparison to the kinetic data obtained with 3′,5′-UpA reveals that 3′,5′-Up(8-cmaA) is cleaved by Zn^{2+} 6.3 times as fast as 3′,5′-UpA.

While the carboxymethylamino substituent in 3′,5′-Up(8-cmaA) seems to enhance moderately the Zn^{2+}-promoted cleavage, no such effect was observed on using Zn^{2+}[12]aneN$_3$ as a catalyst. The rate constants of the cleavage of **1a** and 3′,5′-UpA are equal in the presence of the chelate. This difference in the behaviour of Zn^{2+} and Zn^{2+}[12]aneN$_3$ may be accounted for by their different ability to bind to additional donor atoms. Probably the carboxylate group offers an additional binding site for the Zn^{2+} ion. Simultaneous coordination of Zn^{2+} to carboxylate and phosphate groups would stabilise the metal ion - substrate complex, and hence the Zn^{2+}-promoted cleavage is enhanced. An analogous explanation has previously been given for the enhanced cleaving rates of oligonucleotide substrates.[30-34] With Zn^{2+}[12]aneN$_3$, three of the co-ordination sites around the Zn^{2+} ion are already occupied by the nitrogen atoms of the macrocyclic ligand. Accordingly, simultaneous binding to the phosphate and carboxylate functions can make the chelate catalytically inactive by excluding the essential aquo ligand from the inner coordination sphere. Our previous studies[35] clearly show that complexing of Zn^{2+}[12]aneN$_3$ with additional ligands decreases its catalytic efficiency towards ribonucleoside 3'-phosphoesters.

Conclusions. Introduction of a carboxymethylamino group at *C*8 of the adenine ring of 3′,5′-UpA does not accelerate the cleavage of the phosphodiester bond in the absence

TABLE 1. Rate constants of the transesterification of **1a** promoted by metal ion catalysts. 0.1 M HEPES buffer, pH 5.6 at 90 °C ([HA]/[A$^-$] = 0.09/0.01 M), I = 0.1 M (with NaCl)

Substrate	Catalyst	k / 10^{-4} s^{-1}
1b	None	0.001
1b	10 mM $Zn(NO_3)_2$	0.01±0.03
1b	10 mM Zn^{2+}[12]aneN$_3$	0.79±0.03
3′,5′-UpA	10 mM $Zn(NO_3)_2$	0.16±0.01
3′,5′-UpA	10 mM Zn^{2+}[12]aneN$_3$	0.79±0.03

[a] This rate constant is based on an estimation as explained in the text

of metal ions. No indication of the participation of the carboxylate group in the phosphoester reactions either as a general acid/base or a nucleophilic catalyst could be obtained. Unfortunately, cleavage of the carboxymethyl group under slightly acidic conditions prevented the measurements under conditions that ought to be used to observe experimentally the possible intramolecular general acid/base catalysis. The carboxymethylamino group moderately enhanced the Zn^{2+}-promoted cleavage of the phosphodiester bond. Possibly the carboxylate group offers an additional coordination site for Zn^{2+} in the proximity of the scissile bond. Zn^{2+}[12]aneN$_3$, having three co-ordination sites already filled with nitrogen ligands, can not utilise the extra co-ordination site without loosing some of its catalytic activity.

EXPERIMENTAL

General. The NMR spectra were recorded on a JEOL Lambda 400 or JEOL Alpha 500 spectrometer. When required, the assignment of the proton resonances was verified by COSY experiments. The ^{1}H and ^{13}C chemical shifts were referred to internal TMS, and the ^{31}P shifts to external phosphoric acid. The HPLC/ESI-MS analysis was carried out on a Perkin-Elmer API 365 Triple Quadrupole LC/MS/MS mass spectrometer using positive Q1 scan.

N^6-benzoyl-8-bromo-5′-*O*-(4-methoxytrityl)adenosine (2). Compound **2** was prepared by a method described earlier,[22] utilizing conventional methods for bromination,[36] N^6-benzoylation[37] and 5′-*O*-monomethoxytritylation[38] of adenosine. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.62 (s, H2), 8.10 (2 H, d, J = 7.1 Hz, Bz), 7.71 (1 H, t, J = 7.3, Bz), 7.61 (2 H, t, J = 7.3 Hz, Bz), 7.4-6.8 (14 H of MMTr), 6.01 (d, J = 4.6 Hz, H1′), 5.68 (d, J = 5.6 Hz, OH), 5.38 (d, J = 5.9 Hz, OH), 5.36 (m, H2′), 4.59 (m, H3′), 4.18 (ddd, H4′), 3.78 (3 H, s, OCH_3 of MMTr), 3.32 (dd, J_1 = -11, J_2 = 3.9 Hz, H5′a), 3.23 (dd, J_1 = -11, J_2 = 5.6 Hz, H5′b).

N^6-benzoyl-8-methoxycarbonylmethylamino-5′-*O*-(4-methoxytrityl)-adenosine (3). 3.73 g (0.0052 mol) of **2** was suspended in anhydrous ethanol (55 mL), and 1.32 g (0.010 mol) of glycine methylester hydrochloride and 1.12 g (0.011 mol) of triethylamine, dissolved in anhydrous ethanol (25 mL), were added. The mixture was stirred at 70 C for two days, after which TLC analysis showed the reaction completed. The mixture was concentrated and the residue was taken up in dichloromethane. The organic solution was washed with water, dried with Na_2SO_4, and evaporated near to dryness. The product was purified on a silica column, eluted with dichloromethane containing 0 - 8 % of 2-propanol and 0.2 % of triethylamine. The yield was 2.0 g (54 %). – ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ = 8.50 (br s, H2), 8.05 (2 H, d, J = 7.1 Hz, Bz), 7.59 (1 H, t, J = 7.6 Hz, Bz), 7.50 (2 H, t, J = 7.6 Hz, Bz), 7.32-6.78 (14 H, m, MMTr), 6.15 (1 H , br s, H1'), 5.08 (m, H2′), 4.49 (dd, H3′), 4.38 (ddd, H4′), 3.88 (d, J = -17 Hz,NHC*H*a), 3.77 (3 H, s, OCH_3 of MMTr), 3.67 (s, $COOCH_3$)[39], 3.59 (dd, H5′a), 3.50 (d, J = -17 Hz, NHC*H*b), 3.40 (dd, H5′b). FAB$^+$ MS; *m/z* (%): 273 (100) [MMTr$^+$], 731 (9) [M + H$^+$], 753 (5) [M + Na$^+$].

2′,3′-di-*O*-acetyl-N^6-benzoyl-8-methoxycarbonylmethylamino adenosine (4). 2.0 g (0.0028 mol) of **3**, predried by coevaporations with pyridine, was dissolved in 32 mL of anhydrous pyridine, and 2.1 mL (0.022 mol) of acetic anhydride was added. After 18 h stirring at 22 °C, TLC analysis showed that the reaction was complete and 32 mL of water was added. After 30 min, the mixture was extracted with dichloromethane (2 x 65 mL), organic fractions were pooled, dried with Na_2SO_4, filtrated, and evaporated to dryness. The product was purified on a silica gel column eluted with dichloromethane containing 5 → 20 % acetone and 0.2 % triethylamine. The yield was 1.7 g (73 %). ^{1}H

NMR (400 MHz, $CDCl_3$): δ= 8.65 (s, H2), 8.06 (2 H, d, J = 7.1 Hz, Bz), 7.58 (1 H, t, J = 7.1 Hz, Bz), 7.51 (2 H, d, J = 7.5 Hz, Bz), 7.37-6.86 (14 H, m, MMTr), 6.52 (d, J = 8 Hz, H1´), 5.95 (t, J = 8 Hz, H2´), 5.64 (dd, H3´), 4.32 (ddd, H4´), 3.81 (3 H, s, OCH_3 of MMTr), 3.66 (s, $COOCH_3$), 3.72-3.61 (2 H, m, $NHCH_2$), 3.60 (dd, H5´a), 3.20 (dd, H5´b), 2.14 and 2.05 (2 ×3 H, s, 2´- and 3´-OAc). The fully protected nucleoside (1.63 g, 0.002 mol) was dissolved in 9 mL dichloromethane, and 2 mL of methanol was added. In this mixture was added a solution containing 3.6 mL of dichloroacetic acid in 90 mL of dichloromethane. The mixture was kept overnight at 4 °C, after which period the detritylation was completed (TLC). The mixture was diluted with 270 mL of dichloromethane, washed with saturated aqueous $NaHCO_3$ (3 x 100 mL), dried with Na_2SO_4, and evaporated to dryness. The product (**4**) was purified on a silica gel column, eluted with a 97/3 (*v/v*) mixture of CH_2Cl_2/MeOH. ^{1}H NMR (400 MHz, DMSO-d_6; COSY): δ = 10.8 (br, s, N*H*); 8.46 (s, H2), 8.11 (m, 8-N*H*CH_2), 8.0-7.5 (5 H, Bz), 6.32 (d, J = 7.5 Hz, H1´), 6.05 (dd, J_1 = 7.5, J_2 = 5.5 Hz, H2´), 5.92 (m, 5´-O*H*), 5.53 (dd, J_1 = 5.5, J_2 = 2.5 Hz, H3´), 4.27 (ddd, H4´), 4.18-4.10 (2 H, m, 8-NHCH_2), 3.71 (2H, m, 2 x H5´), 3.61 (s, $COOCH_3$), 2.15 (3H, s, OAc), 1.98 (3H, s, OAc). — FAB^+ MS; *m/z* (%): 543 (57) [$M+H^+$], 565 (100) [$M + Na^+$]

2´-*O*-tert-butyldimethylsilyl-5´-*O*-(4-methoxytrityl)uridine 3´-hydrogen phosphonate (6). A mixture of the protected nucleoside **5** (2.6 g; 4.1 mmol, prepared according to a described method[40]) and phosphorous acid (H_3PO_3; 2.0 g, 24 mmol) was dried by coevaporations with anhydrous pyridine, and then suspended in 30 mL of the same solvent. Pivaloyl chloride (1.27 mL, 10 mmol) was added, and the mixture was stirred at 22 °C. After 20 h, another portion (1.78 mL, 14 mmol) of pivaloylchloride was added, and the mixture was stirred for additional 5 h. Triethylammonium acetate buffer (1 M, 20 mL) was added (cooling in water bath), and after 30 min the product was extracted in dichloromethane. The organic solution was dried with Na_2SO_4, evaporated to dryness, and coevaporated twice with toluene. The product was purified on silica gel, eluting with dichloromethane containing 0 → 15 % methanol and 0.2 % (*v/v*) triethylamine. The yield, after evaporation, was 2.4 g (73 %). – ^{1}H NMR (400 MHz, DMSO-d_6; COSY): δ = 11.4 (br s, H-N3), 7.71 (d, J = 8.1 Hz, H6), 7.45-7.20 (12H, m, MMTr), 6.91 (2H, d, J = 9.4 Hz, MMTr), 6.67 (H, d, J = 590 Hz, P*H*), 5.78 (d, J = 5.1 Hz, H1´), 5.26 (d, J =

8.3 Hz, H5), 4.54 (ddd, H3'), 4.31 (t, $J_1 = J_2 = 5.1$ Hz, H2'), 4.14 (m, H4'), 3.77 (3H, s, OCH_3 of MMTr), 3.22 (dd, $J_1 = 10.4$, $J_2 = 2.9$ Hz, H5'; the second H5' shielded by contaminant H_2O at 3.4 ppm), 2.96 (6H, q, NEt_3), 1.25 (9H, t, NEt_3), 0.82 (9H, s, Si-*t*-Bu), 0.04 (6H, Si-Me_2). – ^{31}P NMR (161 MHz): = 0.74 (J_{P-H} = 590 Hz).

Uridylyl-(3',5')-(8-carboxymethylamino)adenosine (1a): 0.5 g (0.92 mmol) of **4** and 0.81 g (1.0 mmol) of **6**, predried by coevaporations with pyridine, were dissolved in 14 mL of anhydrous pyridine. The mixture was concentrated to about 9 mL, and pivaloylchloride (0.68 mL; 5.5 mmol) was added. When the reaction was completed (according to TLC, after ca. 2 h), 0.5 g (2.0 mmol) of iodine (I_2), dissolved in a mixture of pyridine and water, was added. After 45 min stirring, 25 mL of triethylammonium acetate buffer (1 mol L^{-1}) was added. The mixture was extracted with dichloromethane. Aqueous $NaHSO_3$ was added in the organic solution, which turned from dark brown to pale orange. The aqueous layer was separated, the organic fraction was washed with the triethylammonium acetate buffer, and dried overnight with sodium sulfate. Following removal of the salt, the solution was evaporated to dryness, and coevaporated twice with toluene. The product was purified on a silica gel column, eluting with CH_2Cl_2 containing 2-10 % MeOH and 0.2 % triethylamine. The main fraction contained, after evaporation, 0.65 g of protected dimer **7**. – ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.15 (s, H2 Ade), 7.59 (d, J = 8.3 Hz, H6 Ura), 7.5 - 6.8 (19 H, m, MMTr and Bz), 6.17 (d, J = 7.3 Hz, H1' Ado), 5.78 (d, J = 5.6 Hz, H1' Urd), 5.23 (d, H5 Ura), 4.5 - 3.2 (12 H, sugar protons), 3.67 (3H, s, OCH_3 of MMTr), 3.66 (2H, m, NHCH_2), 3.50 (3H, s, $COOCH_3$), 2.05 (3H, s, OAc), 1.86 (3H, s, OAc), 0.76 (9H, s, Si-*t*-Bu), 0.01 (6H, Si-Me_2) - ^{31}P NMR (161.7 MHz, DMSO-d_6): = -2.73 ppm.

The fully protected product **7** (0.65 g) was dissolved in 70 mL of an 80/20 (*v/v*) mixture of acetonitrile/water, and aqueous NaOH (1 mol L^{-1}) was added, to adjust the pH of the solution at 10. After 4 h, the solution was neutralized with aqueous HCl (1 mol L^{-1}), and then made acidic by adding 1 mL of the same acid solution. The pH of the solution was detected to be about 2.5. The monomethoxytrityl group was removed in 3.5 h. Following neutralization with triethylamine, the mixture was evaporated to dryness, and the residue was partitioned between water and dichloromethane. The nucleotidic material remained in aqueous layer, which was washed once with dichloromethane, and then evaporated to

dryness. The detritylated product was dissolved in 15 mL of a mixture of concentrated aqueous ammonia (70 %) and methanol (30 %) and immersed in sealed tubes in a water bath at 50 °C. After 22 h, the mixture was concentrated, and then coevaporated twice with anhydrous tetrahydrofuran. The residue was taken up in 4 mL of tetrahydrofuran containing 0.5 g of tetrabutylammonium fluoride, and kept overnight at 30 - 40 °C. The mixture was partitioned between water and diethylether. The aqueous layer was concentrated, and applied to a cation exchange column (Dowex 50W x 4, 50-100 mesh, Na^+-form), and eluted with water. The UV-absorbing fractions were collected and evaporated to dryness. According to HPLC analysis, about one half of the product had during deblocking decomposed to uridylyl-(3′,5′)-(8-aminoadenosine) (**10,** see below). Compound **1a** was isolated by HPLC on a LiChrospher RP-18 (5 μm) column (250 × 10 mm) eluting with an acetic acid/sodium acetate buffer (pH 4.2) containing 3.5 % (*v/v*) acetonitrile. The buffer salts were subsequently removed on the same column eluting with a 96/4 mixture of water/acetonitrile. - ^{1}H NMR (500 MHz, DMSO-d_6 containing 5 % D_2O): δ = 7.94 (s, H2 Ade), 7.79 (d, J = 8.0 Hz, H6 Ura), 5.91 (d, J = 6.9 Hz, H1′ Ado), 5.79 (d, J = 6.4 Hz, H1' Urd), 5.64 (d, J = 8.1 Hz, H5 Ura), 4.62 (t, J = 6.4 Hz, H2′ Ado), 4.36 (ddd, H3′ Urd), 4.17 (3 H, m, H3′ Ado and NHCH_2COO), 4.08 (dd, J_1 = 6, J_2 = 5 Hz, H2′ Urd), 4.00 (m, H4′ Ado), 3.96 (2 H, m, H4′ Urd and H5′a Ado), 3.90 (m, H5′b Ado), 3.53 (2 H, H5′ Urd). – ^{13}C NMR (125.25 MHz, DMSO-d_6 containing 5 % D_2O): δ = 171.74, 162.91, 152.02, 151.18, 150.68, 150.05, 148.68, 140.48, 116.52, 102.07, 87.31, 86.62, 83.99 (d), 83.66 (d), 73.78, 73.00, 70.32, 70.24, 64.82, 60.95, 43.64. – FAB$^+$ MS; *m/z* (%): 647 (100) [M+H$^+$], 669 (49) [M+Na$^+$], 691 (34) [M+2Na$^+$].

Characterization of the isolated hydrolysis products. *Uridylyl-(3′,5′)-(8-aminoadenosine)* (**10**). The product was isolated from the reaction solution by HPLC under conditions similar to those used in the kinetic measurements (see below). In comparison to the ^{1}H NMR data of **1b**, disappearance of a signal for 2 protons of CH_2 of the glycine side chain at 4.17 ppm is noticed. The corresponding change is also noted in the ^{13}C resonances; in the spectrum of the product, there is no signal corresponding to the starting material carbon resonating at 43.64 ppm. – ^{1}H NMR (500 MHz, DMSO-d_6 containing 5 % D_2O; COSY): δ = 7.89 (s, H2 Ade), 7.82 (d, J = 8.0 Hz, H6 Ura), 5.83

(d, J = 6.8 Hz, H1′ Ado), 5.80 (d, J = 6.3 Hz, H1′ Urd), 5.64 (d, J = 8.0 Hz, H5 Ura), 4.71 (dd, $J_1 = J_2$ = 6.2 Hz, H2′ Ado), 4.38 (m, H3′ Urd), 4.19 (dd, J_1 = 5.5, J_2 = 3 Hz, H3′ Ado), 4.08 (dd, J_1 = 6, J_2 = 5 Hz, H2′ Urd), 3.95 (3 H, m, H4′ Ado and Urd, H5′a Ado), 3.85 (dd, H5′b Ado), 3.50 (2 H, H5′ Urd, shielded by H_2O resonance). ^{13}C NMR (125.25 MHz, DMSO-d_6 containing 5 % D_2O): δ = 163.06, 162.34, 152.10, 151.50, 150.79, 149.61, 148.62, 140.49, 116.84, 102.06, 87.23, 86.55, 84.20 (d), 83.35 (d), 73.76, 73.06, 70.30, 70.08, 65.00, 60.85. – FAB^+ MS; *m/z*: 589 [M + H^+], 611 [M + Na^+], 627 [M + K^+], 633 [M + 2Na^+].

Kinetic measurements. Kinetic runs were carried out in tightly stoppered glass tubes that were immersed in a water bath. The temperature of the bath was adjusted to 90.0±0.1 °C. The reaction solutions were prepared in sterilised water, and their pH was adjusted with an appropriate buffer. The buffers used were sodium formate, sodium acetate, MES (2-(N-morpholino)ethanesulphonic acid, pK_a 6.15 at 25 °C), HEPES (N-[2-hydroxyethyl]piperazine-N′-[ethanesulphonic acid], pK_a 7.50 at 25 °C) and CHES (2-[N-cyclohexylamino]ethanesulphonic acid, pK_a 9.3 at 25 °C). Outside the pH range of the buffers employed, hydrochloric acid and sodium hydroxide solutions were used. The pH of the reaction solutions was measured with a pH meter at 25 °C and adjusted if necessary with a concentrated acid or base solution. The pH values were extrapolated to the temperature of the kinetic measurements by using the known temperature dependencies of the pK_a values. Aliquots were taken at appropriate intervals and they were cooled on an ice bath to quench the reaction.

The aliquots were analysed by RP-HPLC (Hypersil ODS, 250x5 mm, 5 μm particle size) with a mixture of acetic acid buffer (0.05 M, pH 4.3, I = 0.1 M with NH_4Cl) and acetonitrile (3.5 %). The detection wavelength was 260 nm. The equippement used was Perkin Elmer Intergral 4000 with a diode array detector. The calculation of the rate constants was based on the signal areas of the starting material by using the integrated equation of the first-order rate constants.

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39. For an unknown reason, the integrated area of the resonance signal of the glycine methyl ester moiety did not correspond to three protons, rather to only two. To

ascertain the assignement of the proton resonances, a sample of N^6-benzoyl-8-methoxycarbonylmethylamino adenosine was prepared by a method analogous to that used for **6** (data not shown). With this compound having sugar hydroxyls unprotected, a sharp 3-proton singlet was obtained at 3.61 ppm (in DMSO-d_6).

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