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# Self Cleavage of C8-Histamino-r(UpA) Promoted by $ZnCl_2$: Mechanistic Studies on a Designed Ribonuclease Mimic†

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***Abstract***: Studies on self cleavage of dinucleotides (**2-5**) containing conjugated imidazole and ethylenediamine chains indicated that the imidazole conjugated ribodinucleotide **4** hydrolyzed in presence of $ZnCl_2$ whereas other dinucleotides **2**, **3** and **5** were inert. An acceleration of 10-15 times in rate of hydrolysis of **4** was observed as compared to the unmodified dinucleotide **6**. The product analysis by HPLC suggested that the reaction involves cleavage of P-O5' bond with the formation of C8-histamino dA **9** and 2',3'-cUMP **10** which subsequently hydrolyzed to a mixture of 2' and 3' UMP by a slower reaction. The mechanism of hydrolysis of designed model compound **4** is similar to that of the first step of hydrolysis of RNA by RNase A.

## INTRODUCTION

Hydrolytic fission of phosphodiester linkage in nucleic acids[1] is chemically challenging and has recently attracted much attention. Extensive studies on mechanism of ribonuclease A catalysis[2] has led to development of ribonuclease mimics.[3] Model studies have shown that RNA can be cleaved by acid-base catalysis in imidazole buffer via sequential catalysis.[4] Many divalent and trivalent metal ions promote transesterification of RNA.[5] Polycationic polypeptides are known to accelerate the hydrolysis of oligonucleotides.[6] Simple metal complexes and amines efficiently cleave activated phosphodiesters, but often require elevated concentration and temperature.[7] Although amino acids lysine and imidazole have been covalently linked to oligonucleotides at suitable positions, no hydrolysis of phosphate have been observed using the modified oligonucleotides.[8]

In biological systems, enzymes achieve high rates for reactions by pre-organization of functional groups catalytically involved in a particular reaction.[9] In ribonuclease A, two histidines and one lysine converge around the substrate phosphate and co-ordinate in the acid-base catalysis[2] in active site 1 In an attempt to mimic this situation, we designed the dinucleotides (**2-5**) in which the catalytic groups are covalently anchored in various combinations to the dinucleotide substrate[10,11]. In this paper, by employing time dependent HPLC analysis, we demonstrate that the ribonucleotide **4** specifically undergoes cleavage in presence of $ZnCl_2$, at a rate 10-15 times faster than the unmodified dinucleotide **6**. Further, the reaction goes through the same steps as the enzymatic reaction with initial formation of 2',3'-cUMP which is subsequently hydrolyzed to 2',3'-monophosphate.

## RESULTS AND DISCUSSION

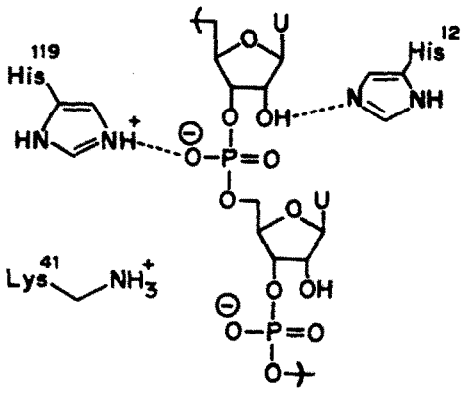
The dinucleotides (**2-6**) were prepared by (i) condensation of appropriate 5'-O-DMT-3'-O-(o-chlorophenyl)phosphodiesters **7**, with 5'-hydroxyl-dA component **8** in presence of MSNT, (ii) 5'-O deprotection with 3% dichloroacetic acid in $CH_2Cl_2$ and (iii) N- deprotection with MeOH-$NH_3$. In case of diribonucleotides **4** and **6** this was followed by treatment with TBAF to achieve 2'-O-desilylation. The fully deprotected dinucleotides were purified by ion-exchange chromatography over DEAE Sephadex A-25 using gradient elution with volatile TEAB buffer. The purity was checked by HPLC and compounds characterised by $^1H$ and $^{31}P$ NMR as reported earlier.[10,11]

*Hydrolysis of 2-6 with $ZnCl_2$*

The dinucleotides **2-6** were subjected to reactions separately with $CuCl_2$, $NiCl_2$, $CoCl_2$, $MgCl_2$ and $ZnCl_2$ (all 1 mM) without addition of any imidazole. The substrates were incubated individually with the above salts at pH 7.0 at different concentrations and at different temperatures (30-80°C) for 24-72 h. and the reactions were monitored by HPLC. Except **4** and **6** all other dinucleotides showed complete absence of reaction under the above conditions even at elevated temperatures of 70°C. Compounds **4** and **6** exhibited cleavage only in presence of $ZnCl_2$ whereas in presence of other metal ions no fission was seen. The reaction of **4** with $ZnCl_2$ showed complete cleavage even at 40°C in 207 h (Figure 1b) while at 70°C the reaction was complete within 10-12 h as seen by disappearance of starting materials (Figure 1d). Both reactions led to formation of two products. The peak eluting at 10.35 min was identified as the 5'-OH nucleoside **9** by comparison and coinjection with an authentic sample of **9** available by prior work.[10] This result indicated that the cleavage is taking place at P-O5' bond rather than O3'-P bond, similar to the enzymatic cleavage of RNA that leads to the formation of 5'-OH component and 3'-phosphate residue.

The unmodified ribodinucleotide **6** also behaved similarly in $ZnCl_2$ reactions with or without addition of imidazole.[12] It yielded the corresponding 5'-OH and 3'-phosphate components. Thus C8 modification does not lead to any change in the cleavage pattern in $ZnCl_2$ reaction and the hydrolysis occurs with cleavage at P-O5' site. The reaction was not seen with metal-ions other than zinc, implying a specific involvement of $Zn^{2+}$. The non-observance of this reaction with 2'-deoxy analogues **2** and **3** suggested the prime requirement of the 2'-hydroxyl group which is involved in an intramolecular attack on phosphate diester to generate 2'3'-cyclic phosphate, accompanied by cleavage of P-O5' bond to form the 5'-hydroxy component **9**.

However when hydrolysis experiment is done on **4** in presence of $ZnCl_2$ at 70°C, the reaction was almost complete in 13 h as seen from disappearance of 10.92 min peak due to **4** and at the end of 49 h, the peak at 1.99 min was totally transformed into a broad peak at 2.64 min. The intermediate peak at 1.99 min was identified as 2',3'-cUMP (**10**) by comparison with authentic standard (HPLC coinjection and $^{31}P$ NMR, δ 20.27 ppm), and that at 2.64 min. as a mixture of 2' and 3'-UMP. Thus, $ZnCl_2$ reaction of **4** and **6** at 40°C led to formation of 2',3'-cUMP, while at 70°C, the initial product cUMP is hydrolyzed to 3' (**13**) and 2' (**14**) O-phosphates. It is known from the literature that $ZnCl_2$ hydrolyses 2',3'-cyclic phosphates to a mixture of 2'/3'-monophosphates[5d]. It is well known that the enzymatic hydrolysis of RNA leads to formation of only 3'-monophosphate while alkali hydrolysis would lead to a mixture of 2' and 3'-phosphates. In both cases, it is difficult to detect the intermediate 2',3'-cUMP.



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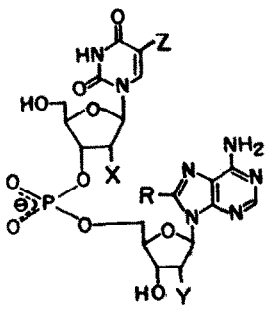
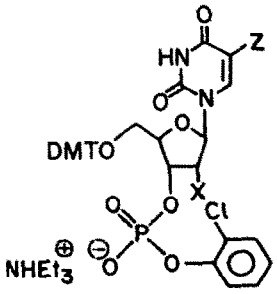


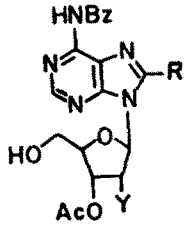
| | X | Y | Z | R* |
|---|---|---|---|---|
| **2** | H | H | Me | His |
| **3** | H | OH | Me | His |
| **4** | OH | OH | H | His |
| **5** | H | H | Me | Eda |
| **6** | OH | OH | H | H |

* His = 1H-Imidazole-4-ethanamino.
Eda = Ethanediamino.



**7**

(a) X = H, Z = Me.
(b) X = OTBDMS, Z = H.



**8**

Y = H/OAc.
R = $CF_3CONH(CH_2)_2NH$ or 1-acetyl-imidazole -4-etheneamino.

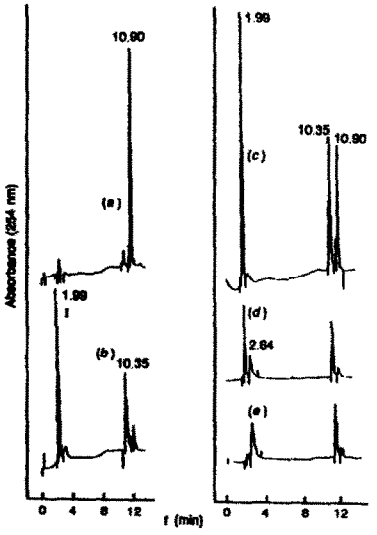



Figure 1: HPLC analysis of cleavage 4
(a) 40°C,0 h, (b) 40°C,207 h. (c) 70°C,4 h.
(d) 70°C,17 h. and (e) 79°C,49 h.

*Reaction kinetics.*

In order to evaluate the catalytic efficiency imparted by the anchored imidazole, the kinetics of $ZnCl_2$ reactions with **4** and **6** were investigated using quantitative HPLC to follow the reaction progress. Kinetic constants were obtained by measuring the rate of disappearance of starting dinucleotide from HPLC peak integration and were reproducible to an accuracy of +8%. The reaction obeyed pseudo first order kinetics as seen from the linearity of the concentration-time plots and the rate constants were derived from the observed slopes. The experiment was repeated for both **4** and **6** at three different temperatures 40°, 55°, and 70°C and both compounds showed increase in rates at higher temperatures. It is seen from Table I that hydrolysis of designed r(UpA)-His **4** is 10-15 times faster than that of unmodified r(UpA) **6** at the range of temperatures investigated.

Table I: Pseudo first order rate constant $(k_1)^*$ for the hydrolysis of **4** and **6**.

| Entry | Temperature °C | $k_1$ (4) | $k_1$ (6)† | $k_1$ (4)/$k_1$ (6) |
|---|---|---|---|---|
| 1 | 40 | 12.90±0.65 | 0.89±0.05 | 14.50 |
| 2 | 55 | 70.60±1.24 | 7.24±0.22 | 9.60 |
| 3 | 70 | 316.60±21.5 | 30.37±2.5 | 10.10 |
| 4 | 55** | 74.0±1.2 | 1.2±0.06 | 61.70 |

* rate constants x $10^3$ $h^{-1}$, buffer pH 7.0, 10 mM HEPES, $ZnCl_2$ (1 mM).
†Reaction with **6** same as above, but in presence of imidazole (0.5 mM).
** At $ZnCl_2$ (10 mM), rest as above.

The data obtained from the Figure 1 also lead to calculation of kinetic rate constant for hydrolysis of 2',3'-cUMP, following the rate of disappearance of peak at 1.99 min. The $k_1$ thus obtained was 12 times slower than $k_1$ for the first step (transphosphorylation).

*pH-Rate profile of $ZnCl_2$ reaction.*

The observed catalysis in $ZnCl_2$ reaction, can also arise due to metal bound hydroxide which may be involved in a direct nucleophilic attack on the phosphate or deprotonation of the 2'-OH to initiate this process. To distinguish this mechanism from that involving the imidazole of histidine, the effect of pH on $ZnCl_2$ reaction was studied. The reaction of **4** in buffers at various pH's (6.2, 6.52, 7.0, 7.42 and 7.9) containing 1 mM $ZnCl_2$ was followed by HPLC analysis. A control reaction of **4** in above buffers but without any $ZnCl_2$ indicated that hydrolysis of **4** was negligible, with no pH dependent background water reaction. The pH-rate profile (Figure 2) obtained in presence of $ZnCl_2$ corresponded to a characteristic bell shape, with a maximum at pH 7.0. This is typical of bifunctional acid-base catalysis, with optimal activity when one function is basic and the other is acidic. It is known[2b] that unmodified ribodinucleotides (such as **6**) also exhibit a bell-shape in pH-rate profile, with maxima around pH 7.0 in imidazole-$ZnCl_2$ reaction.

*Effect of $ZnCl_2$ concentration on $k_{obs}$.*

The concentration of $ZnCl_2$ used in the above reactions (1 mM) corresponds to approximately 100 times that of the substrates (**4** and **6**) and the control hydrolysis with **6** contained imidazole at 30 times the concentration of **6**. These amounts of $ZnCl_2$ and imidazole were minimally essential to observe measurable kinetics for

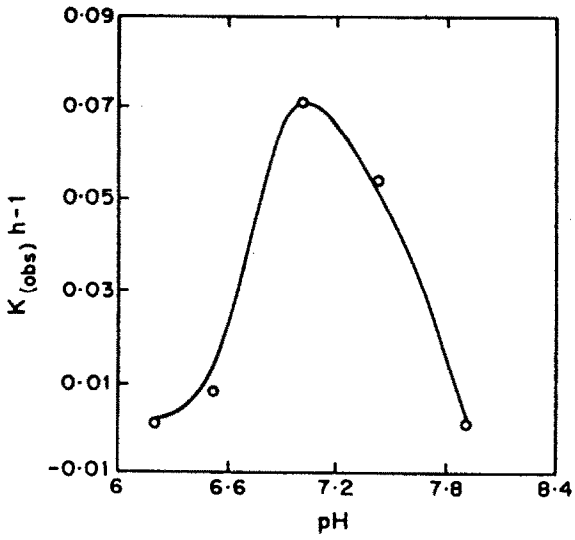


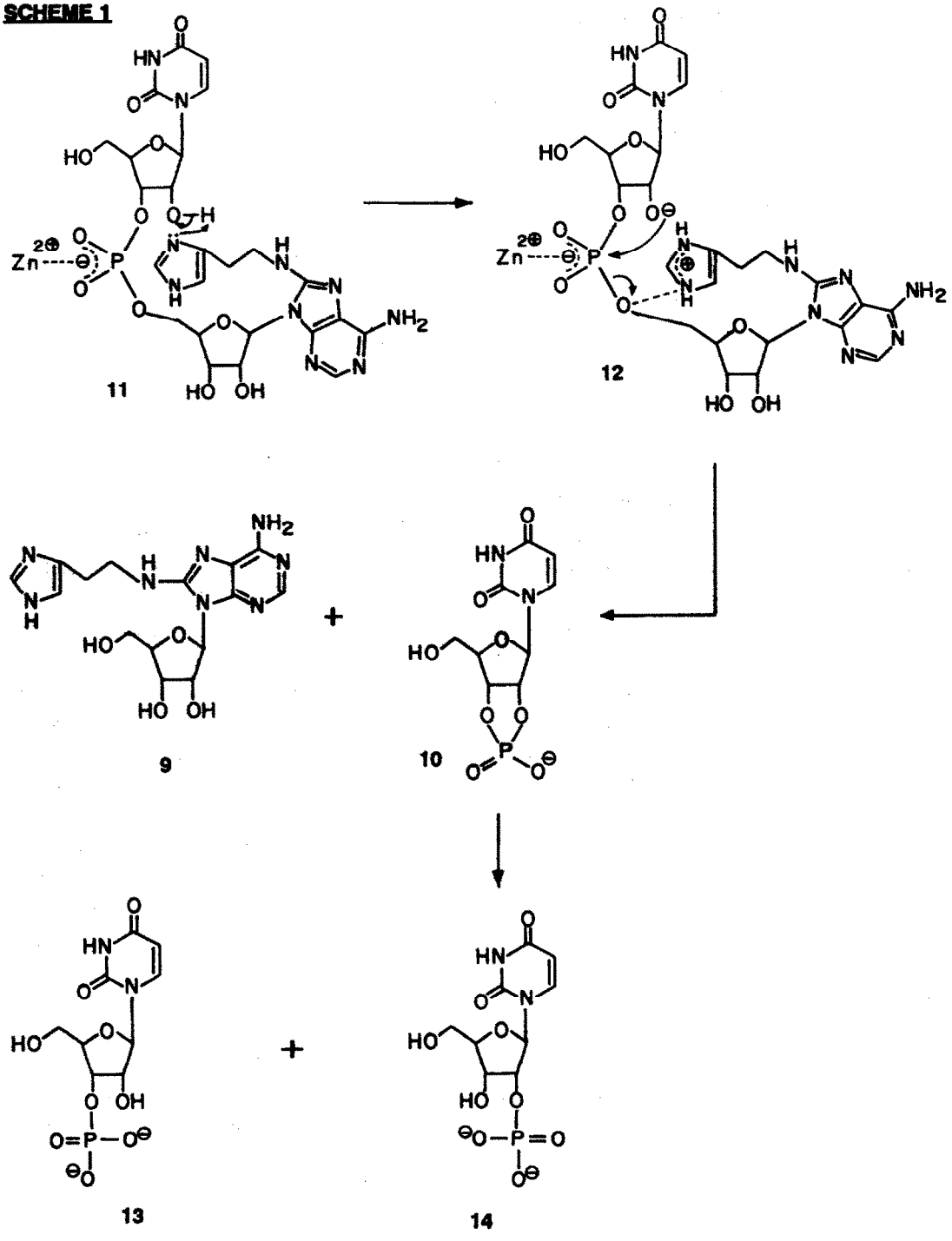

Figure 2: pH-rate profile for cleavage of **4**

hydrolysis of **6**. No extraneous imidazole was used in reactions with the **4**. Increasing the concentration of $ZnCl_2$ from 1 mM to 10 mM had no effect on the observed kinetics of the designed mimic **4** while that of the unmodified control **6** was considerably decreased (55°C, entry 4 vs entry 2 in Table 1). Such effects of decreasing rates with increasing $ZnCl_2$ concentration has been well recognized in reactions of unmodified ribodinucleotides with $ZnCl_2$-imidazole[2b] and Zn-polyazamacrocycle complexes.[5a] In the former case this was attributed to inactivation of catalytic imidazole by Zn complexation, while in the latter, formation of less active, higher order $Zn^{2+}$-phosphate complexes was thought to slow the reaction rates. In contrast to these observations, the designed mimic **4** did not exhibit any rate dependence on $ZnCl_2$ concentration in the range 1mM to 10 mM. This may perhaps be ascribed to absence of $Zn^{2+}$ complexation by the linked imidazole in **4**. Any such complexation would also inhibit the ability of imidazole to act as a base in the first step. The presence of conjugated imidazole in **4** therefore does not inherently affect the affinity of phosphate to $Zn^{2+}$ by simultaneous complexation and thus, the observed difference in rate behaviours of **4** and **6** is not due to different degrees of saturation by metal. $ZnCl_2$ is also known to suppress the 3'-5' to 2'-5' isomerization which is prevalent in imidazole catalysis.[2b] A larger rate enhancement in hydrolysis of **4** over **6** at higher $ZnCl_2$ concentration arises from a decreased rate of control reaction with **6**.

*Mechanistic discussion of the reactions.*

Based on the above data the probable chemical steps involved in $ZnCl_2$-promoted hydrolysis of **4** may be summarized as in Scheme 1. The first step involves electrophilic binding of phosphate by $Zn^{2+}$, accompanied by a rapid deprotonation of 2'-OH by the basic N of imidazole (**11**). Intramolecular attack by 2'-$O^-$ on phosphate is followed by cleavage of P-O5' bond (**12**). The scission of P-O5' bond with alcoholate as a leaving group is perhaps aided by either $Zn^{2+}$ complexation or by intramolecular protonation with acidic nitrogen of C8 anchored imidazole. The mechanism is supported by unambiguous identification of 2',3'-cUMP as the intermediate.

**SCHEME 1**



The rationale behind design of ribodinucleotide r(UpA) (4) containing histamino side chain at C8 was that the spatial proximity of imidazole would accelerate the self-hydrolysis of 4. The kinetic data clearly indicated a rate enhancement of 10-15 in the hydrolysis of 4 with $ZnCl_2$ as compared to that of 6 under similar conditions. This corresponds to an effective molar concentration of 10-15 M for the catalytic imidazole in 4 and is a very reasonable value for local concentration effects[13] in intramolecular nucleophilic attack of 2'-$O^-$ to generate cyclic phosphate. The rate enhancement is not that dramatic as seen in intramolecular reactions involving a direct attack of nucleophile on phosphates.

Metal-bound hydroxyl ions are potent nucleophiles;[5f,7a] for e g., water bound to zinc complexes ionize with a $pK_a$ in the range 7.0-8.0, in contrast to free water with a $pK_a$ of 14. This is true when $Zn^{2+}$ is 3 or 4 co-ordinate but for normal 6 co-ordinate $Zn^{2+}$, the pKa of bound water is higher (= 10). The observed pH-rate profile with a bell shape maximum at pH 7 in $ZnCl_2$ promoted hydrolysis of 4 and 6 corresponds to involvment of two functionalities in catalysis. The acidic component would be either $Zn^{2+}$ or imidazolium species while the basic component would be either Zn-bound hydroxide or imidazole. It is unlikely that $H_2O$ bound to $Zn^{2+}$ co-ordinated to anionic phosphate would have such a low $pK_a$ as 7 and so hydroxide bound to $Zn^{2+}$-phosphate may not be either an effective base in transesterification or a nucleophile in the hydrolytic step. The role of $Zn^{2+}$ may be either of the following: (i) it can act as an electrophilic catalyst by neutralization of anionic charge (P-$O^-$) of phosphate and thereby enhance the intramolecular nucleophilic attack of 2'-$O^-$ (ii) it may enhance the leaving group tendency of 5'-$O^-$ by stabilization via co-ordination. The bell shape in pH-rate profile is a combined result of the electrophilic catalytic role of $Zn^{2+}$ in promoting attack on phosphate and general base role for imidazole. At pH >7-7.2, $Zn^{2+}$ is removed from complexation with phosphate by formation of $Zn^{2+}(OH^-)_x$. It is also possible that $Zn^{2+}$ may simultaneously complex with phosphate and imidazole to activate phosphate for receiving a nucleophilic attack. In such a case the pendant imidazole would not be able to act as a base in the transesterification step. These facts together support the mechanism proposed in Scheme 1 in which, the transesterification-hydrolysis proceeds through a bifunctional catalysis in which $Zn^{2+}$ performs the role equivalent to that of imidazolium in enzymatic catalysis. $ZnCl_2$ is also known to suppress the 3'-5' -> 2'-5' isomerization which is prevalent in imidazole catalysis.[2b]

For intramolecular catalysis although proximity of a group is of overriding importance, the requirement of correct orientation for facile catalysis should also be considered. The catalyst must not only have large effective concentrations, but also possess a correct stereochemical orientation.[6] The imidazole attached to C8, although causes a higher local concentration, may not have a proper orientation to enhance the catalysis dramatically. The spatial pre-disposition of side chain in 4 is perhaps influenced by the rigid dinucleotide structure with preferred sugar conformation and glycosyl distortions.[10,11]

*Relevance to RNA mechanism*

The sequence of events taking place in the depolymerization of RNA by RNase[2,15] (i) involves trans-phosphorylation reaction from the 5'-position of one nucleotide to the 2'-position of the adjacent nucleotide leading to formation of a 2',3'-cyclic phosphodiester and (ii) hydrolysis of the 2',3'-cyclic phosphodiester to a 3'-nucleotide. Although formation of 2',3'-cyclic phosphate is mandatory[16] to the reaction mechanism, considerable ambigiuty exists as to whether the reaction is taking place sequentially on the enzyme or the cyclic phosphate is actually released to the medium as a true product. In this case, no hydrolysis takes place until all susceptible 3',5'-phosphodiester bonds have been cyclised. A recent[17] report has given evidence for a time

lag in the formation of 3'-mononucleotide. The kinetic parameters have indicated that the ratio of rate of transphosphorylation to the rate of hydrolysis of cyclic phosphodiester[18] at pH 7.0 and 25°C is about 1800.

The $ZnCl_2$ catalysed self cleavage of **4** presented in this paper can be termed as a conceptual mechanistic mimic of ribonuclease enzymatic reaction. As discussed, it goes through same steps as the enzyme till the formation of cyclic phosphodiester. The only difference is that $Zn^{2+}$ performs the role of imidazolium (His 119) species while the C-8 linked imidazole is like His 12. The primary product of the chemical reaction has been unambiguosly identified as the cyclic phosphodiester just as in case of ribonuclease. The next step of hydrolysis is slower than the first step. In the chemical reaction at 70°C, it was found that the hydrolysis of 2',3'-cUMP by $ZnCl_2$ is about 12 times slower than the first transphosphorylation step.

In summary, metal ion mediated hydrolytic cleavage of modified dinucleotides **2-6** shows that the imidazole conjugated ribodinucleotide **4** was hydrolyzed in presence of $ZnCl_2$ whereas other dinucleotides **2**, **3** and **5** were passive. The rate of hydrolysis of **4** in presence of $ZnCl_2$ was 10-15 times higher than the unmodified dinucleotide **6**. The product analysis by HPLC suggested that the reaction involves cleavage of P-O5' bond with the formation of C8-histamino dA and 2'3'-cUMP in the first step. The latter subsequently got hydrolysed by a slower reaction to a mixture of 2' and 3' uridine monophosphates. Thus the designed model compound **4** is a true mechanistic mimic of the first step of the hydrolysis of RNA by RNase A. Future efforts are focussed on further tuning of catalysis by variation in spacer chain, introduction of electrophilic cations (eg. guanidinium[19]) and design of appropriate tethers to achieve efficient hydrolysis of complementary strand.

## EXPERIMENTAL SECTION

The metal salts $ZnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$ and imidazole were of highest purity available. Reagent grade HEPES (N-(2-hydroxyethyl)piperazine- N'-ethanesulphonic acid ), MOPS (4-Morpholinepropanesulphonic acid), ACES {2-[(2-amino-2-oxoethyl)-amino] ethanesulphonic acid} and Tris {2-amino-2-(hydroxymethyl)-1,3-propanediol} were procured from Sigma (USA). The concentration of Zinc (II) was determined by titration against EDTA with Eriochrom black T as indicator. All solutions were made with Milli-Q purified water after autoclaving. Compounds **2-6** were synthesised as reported.[11]

HPLC system (Waters) equipped with M440 absorbance UV detector set at 254 nm, M 6000A dual pumps, U6K injector and HP3380 integrator was used for the analysis of the reaction products..All kinetic analysis were done on Novapak C18 column (3.9 mm x 15 cm). *HPLC Conditions.* Buffer A, 0.1 M Triethylamine (TEAA); Buffer B, 0.1 M TEAA containing 30% acetonitrile; A to B in 20 min; Flow rate 2 ml/min; UV at 254 nm. A digital ion analyzer equipped with temperature selector probe was used for pH adjustments. The following buffers (10 mM) were used for reactions at different pH's. pH 6.2 (ACES); 6.5 and 7 (HEPES); 7.4 and 7.9 (MOPS). The concentration of dinucleotides **2**, **3**, **4** and **5** adjusted to $1.7 \times 10^{-5}$ M as by UV absorption using following extinction coefficient (**2**, $\varepsilon = 6.07 \times 10^4\ M^{-1}cm^{-1}$, **3** $\varepsilon = 10.89 \times 10^4\ M^{-1}cm^{-1}$, **4** $\varepsilon = 7.82 \times 10^4\ M^{-1}cm^{-1}$, **5** $\varepsilon = 5.52 \times 10^4\ M^{-1}cm^{-1}$).

*Kinetic analysis.*

Compound **4** ($1.7 \times 10^{-5}$ M, 440 µl) in HEPES buffer (pH 7, 10 mM) containing $ZnCl_2$ (1 mM) in an eppendorf was heated at 55°C. At different intervals, aliquots of 40 µl were drawn out and quenched either by

keeping at 0°C (deep freeze) or by adding EDTA (1 mM). 5 μl of the solution was injected into HPLC and the peak area was used for kinetic analysis. A plot of logarithm of the area percentage against time gave a straight line and from the slope of the line, pseudo first order rate constants were calculated. A control experiment in which all components were present except $ZnCl_2$ was carried out to check the stability of compounds for prolonged times at 55°C. The reactions were repeated at 40°C and 70°C. The analysis at each temperature was repeated 3 times to obtain consistent rate constants.

Kinetic analysis of hydrolysis of **6** ($1.7 \times 10^{-5}$ M) was also done similarly with 1 mM $ZnCl_2$ in 10 mM HEPES buffer containing 0.5 mM (30 eq.) imidazole at pH 7, to keep the reaction under first order conditions.

*pH-Rate profile.*

The pseudo first order rate constants for hydrolysis of compound **4** was determined at different pH at 55°C. Solutions of different buffers (10 mM) corresponding to pH's of 6.2, 6.52, 7, 7.42, 7.9 and containing $ZnCl_2$ (1 mM) were used for reactions. In all experiments, the corresponding controls were run in only buffer and **4** without $ZnCl_2$.

*RNase A digestion of 4 and 6.*

Compounds **4** and **6** (1 μl = 0.1 OD) were treated separately with RNase A (10.8 units) in Tris HCl (pH 7.5, 10 mM) and NaCl (15 mM). The mixture was incubated at 37°C for 1 h and the product was analyzed by HPLC.

*Alkali digestion of compounds 4 and 6.*

Compounds **4** and **6** (1 μl, 0.1 OD) were dissolved individually in 1 M NaOH (20 μl) and heated at 37°C for 22 h. The products were analyzed by HPLC.

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