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The first example of a model compound of RNase U2 and its intermediate with CPP directly monitored by ESI-MS†

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A Tb(III) complex of THP [1,4,7,10-tetrakis(2-hydroxypropyl)-cyclen] (THPTb) cleaved RNA phosphate ester bonds at A-specific sites to mimic RNase U2. The intermediates of the adducts of $THPTb^{2+}$ and CPP^{-} (cyclic propylene phosphate) or $HPNP^{-}$ (2-hydroxy propyl-*p*-nitrophenylphosphate) in the hydrolysis of HPNP were directly monitored by electrospray ionization mass spectrometry (ESI-MS).

Ribonuclease (RNase), which can be divided into endoribonuclease and exoribonuclease, is a type of nuclease that catalyzes the degradation of ribonucleic acid (RNA) into smaller components.[1] Similar to restriction endonucleases, which cleave highly specific sequences of double-stranded deoxyribonucleic acid (DNA), some endoribonucleases can also cleave specific sequences of single-stranded RNA. For example, RNase A (ref. 2) and RNase PhyM (ref. 3) are specific for single-stranded RNAs and cleave the 3′-end of unpaired U and C or A residues, respectively. This forms a 3′- phosphorylated product *via* a 2′, 3′-cyclic monophosphate intermediate. The RNase T1 (ref. 4) and RNase U2 (ref. 5) in particular can recognize single nucleotide sites and cleave the 3′-end of unpaired G and A residues, respectively. Many research groups have sought to develop metal complexes to serve as artificial ribonucleases that can cleave biologically important phosphate esters.[6] Several complexes can hydrolyze the dinucleoside of ApA and/or UpU,[7] but they did not show the ability to catalyze the cleavage of RNA at A-specific sites to mimic Rnase U2. Moreover marked efforts have been made in understanding the enzymatic reaction mechanism *via* ultraviolet visible spectra (UV-Vis), nuclear magnetic resonance spectra (NMR) and gel electrophoresis.[8] Nevertheless, there is still no direct evidence for the production of 2′, 3′-cyclic monophosphate intermediates, especially for the intermediate of metal complexes with the 2′, 3′-cyclic monophosphate.

Cyclen-based macrocyclic ligands[9] and four oxygen pendent groups form octadentates by chelating to an ennea-coordinated metal ion such as Tb(III), La(III), *etc.* to leave one coordination site open to interact with the phosphate ester.[9c] We carefully designed two Tb(III) complexes of THP [1,4,7,10-tetrakis(2-hydroxypropyl) cyclen] (THPTb) and DOTAM [1,4,7,10-tetrakis(carbamoylmethyl)-cyclen] (DOTAMTb) to mimic RNase U2 in cleaving phosphate ester bonds of RNA at A-specific sites. The reaction of THPTb-promoted RNA cleavage was initially studied using dinucleotides containing only one reactive ribonucleoside unit (ApA, CpC, GpG, UpU) as the nucleic acid mimics. The NpN (0.10 mM) and THPTb (0.05 mM) were dissolved in diethylpyrocarbonate (DEPC)-treated water (1 : 1) and analyzed by ESI-MS after incubation for 16 h at 37 °C. Only the ApA reaction offered useful intermediate data (Fig. S1,† associated ion of 2′, 3′-cAMP and THPTb). In contrast, the DOTAMTb failed to promote the cleavage of the four diribonucleotides. Then three oligoribonucleotides R1-3 with all 16 possible diribonucleotide combinations were chosen to screen the efficiency and selectivity of cleavage sites because THPTb can selectively cleave dinucleotide ApA. The oligoribonucleotide cleavage was followed by gel electrophoresis and MALDI-TOF-MS (matrix-assisted laser desorption ionization time of flight mass spectrometry). A fluorescently-labeled FAM was introduced into the 5′-end position to detect the substrate and cleavage products. Only the 5′-FAM-labeled sections were detectable in gel electrophoresis. The cleavage products were also identified by MALDI-TOF-MS using linear mode.

The oligoribonucleotides R1-3 were all cleaved by THPTb at A-specific sites in a concentration-dependent mode (Fig. S2†). The R1 exhibits a peak at *m*/*z* 2704.412 (Fig. 1a), and a new fragment ion peak at *m*/*z* 868.966 assigned to the cleavage product of 5′-FAM-A of R1 was formed and increased during the

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† Electronic supplementary information (ESI) available: Detailed experimental description and single-crystal structure refinement data for $[Tb(THP)](NO_3)_3 \cdot H_2O$ and $[Tb(DOTAM)](NO_3)_3 \cdot H_2O$. CCDC 980345 and 824496. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c4ra07950g

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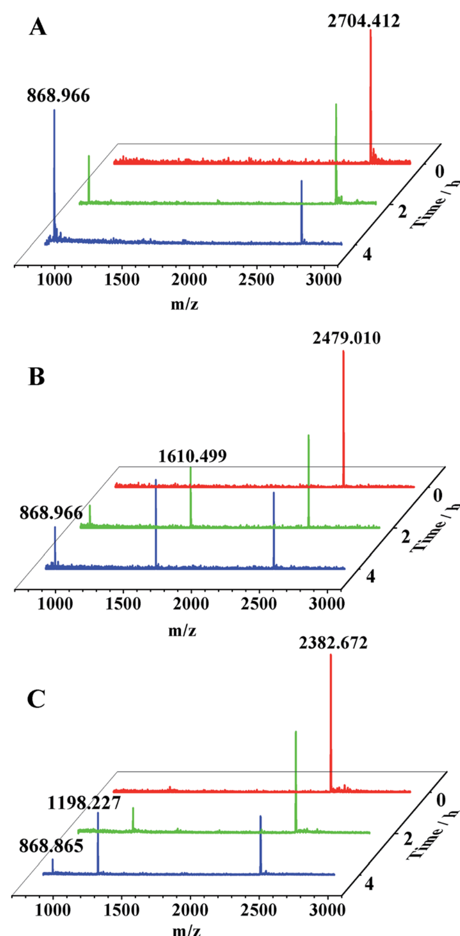


Fig. 1 Mass spectra of the cleavage of oligoribonucleotides R1-3 promoted by 50 μM THPTb. (a) Oligoribonucleotide R1 5′-(FAM)ACGCUUA-3′ (m/z 2704.412), 5′-(FAM)A (m/z 868.966), (b) oligoribonucleotide R2 5′-(FAM)AGGUGA-3′ (m/z 2479.010), 5′-(FAM)A (m/z 868.966) and GGUGA-3′ (m/z 1610.499), (c) oligoribonucleotide R3 5′-(FAM)AAUCCA-3′ (m/z 2382.672), 5′-(FAM)A (m/z 868.865) and 5′-(FAM)AA (m/z 1198.227).

hydrolysis of the ApC site. Particularly, a new peak at m/z 1610.499 corresponding to the unlabeled fragment complementary to the cleavage product of 5′-FAM-A was observed in the cleavage of the ApG site of R2 (Fig. 1b). Unlike the mass spectra of R1 and R2 after 2 h of reaction, there was a new peak of R3 (Fig. 1c) at m/z 1198.227 representing the cleavage product of 5′-FAM-AA. Up to 4 h of reaction, a peak at m/z 868.865 was noticed, although it was much weaker than that at m/z 1198.227. R3 had two cleavage sites, *i.e.* ApA and ApU. We also investigated the cleavage of ApA and ApU at different positions in R4 (Fig. S3†). The peaks at m/z 598.231 and 1467.726 can be attributed to the cleavage of ApU and ApA respectively. Cleavage fragments of oligoribonucleotide R1–R3 by THPTb were shown in Table 1. Interestingly, the observed ApN selectivity, which resembles that shown by natural RNases U2, was obtained in the THPTb-promoted RNA cleavage experiment. This selectivity is different from the earlier reports in the cleavage of RNA, where a preference for the cleavage of CpA, UpA, ApA and/or UpU sequences was observed.[6d,7d,f,g] THPTb could act as A-specific sites of RNA recognition and cleaving agents.

Table 1 Cleavage fragments of oligoribonucleotide R1–R3 by THPTb

Sequence		Cal mass	Expt mass (linear mode, M + H$^+$)
R1	5′-(FAM)ACGCUUA-3′	2701.80	2704.412
	5′-(FAM)A	868.69	868.966
	CGCUUA-3′	1833.11	ND[a]
R2	5′-(FAM)AGGUGA-3′	2475.70	2479.010
	5′-(FAM)A	868.69	868.966
	GGUGA-3′	1607.01	1610.499
R3	5′-(FAM)AAUCCA-3′	2379.70	2382.672
	5′-(FAM)A	868.69	868.865
	AUCCA-3′	1511.01	ND
	5′-(FAM)AA	1197.96	1198.227
	UCCA-3′	1181.74	ND

[a] ND (not detected).

The cleavage sites of the oligoribonucleotides R1-3 were also confirmed by qualitative analyses. The results of gel electrophoresis are in accord with those from mass spectrometry. Fig. 2a and b show only one band after cleavage of R1 or R2, which can be assigned to the cleavage product of 5′-FAM-A. For R3, the ApU was cleaved much faster than the ApA. Hence, we identified the cleavage band as 5′-FAM-AA and failed to discern the 5′-FAM-A band (Fig. 2c). Meanwhile, R1, R2 and R3 were cleaved time dependently. With prolonged cleavage, the visible bands became more evident but at different levels. Notably, the cleavage reactions are selective at A-specific sites, and the reactivity follow a descending order of ApU > ApC > ApG > ApA. Since no reaction of GpN was observed, adenine other than guanine was probably targeted because the adenine base facilitates the reaction by stabilizing the transition state of RNA cleavage as a hydrogen bond donor in interactions.[10] The base in the 3′-position can either be a pyrimidine or a purine base, but pyrimidine is clearly preferred because of the high reactivities of ApU and ApC phosphodiester bonds. Phosphodiester bonds between pyrimidine and adenosine have frequently been suggested to be particularly reactive.[11]

The cleavage mechanism of THPTb against phosphate ester bond was further studied by the well-studied and activated

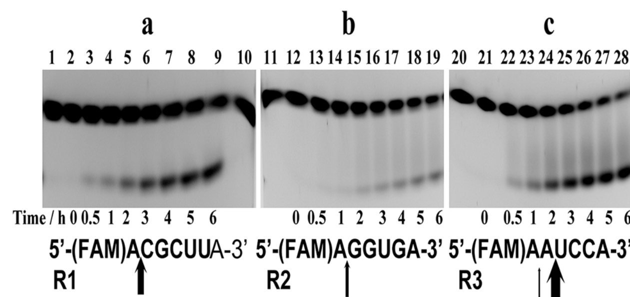


Fig. 2 Gel electrophoretograms of the cleavage of oligoribonucleotides R1-3 promoted by 50 μM THPTb. Lanes 1, 11 and 20 show the cleavage in 50 μM THP. Lane 10 RNA only in DEPC-treated water, 6 h. Arrows indicate cleavage site and intensity.

phosphate diester: 2-hydroxy propyl-*p*-nitrophenylphosphate (HPNP). Fig. S4† shows that the activity of the cleavage reaction is affected from pH 6.0 to 9.0. The THPTb clearly accelerated HPNP cleavage at pH 7.2 when a 200 μM complex yielded an observed rate constant k_{obs} of 1.01×10^{-2} min^{-1} at 25 °C, compared to 4.74×10^{-5} min^{-1} measured in the absence of THPTb (Fig. S5†).

Moreover, hydrolysis depends on the pH in a sophisticated pattern.[12] The greatest extent of hydrolysis of HPNP with THPTb at pH 7.2 suggests that $[HNPP^- + THPTb^{2+}]$ has the best stability at that pH value. The addition of H^+ did not favor protonation under acidic conditions, whereas OH^- negatively affected the stability of neutral intermediates under alkaline conditions. The electronic configuration changes indicate that the negative charge is neutralized by metal ions and the phosphorus atom becomes more electrophilic upon activation.

ESI-MS was also used to monitor HPNP hydrolysis by capturing ionic intermediates and products and thus studied the mechanism. In the initial period of HPNP hydrolysis by THPTb in pH 7.2 aqueous solution at 25 °C, an adduct ion of $HPNP^-$ and $THPTb^{2+}$ at *m*/*z* 838.25 was formed (Fig. S6a†). During the dissociation of HPNP, the adduct ion of $HPNP^-$ and $THPTb^{2+}$ decreased gradually, but a new peak at *m*/*z* 699.25 comprising $THPTb^{2+}$ and CPP^- (cyclic propylene phosphate) increased dramatically (Fig. 3a and S6b†). On the other hand, the ESI-MS negative ion mode (Fig. 3b, S6c and d†) showed that the $HPNP^-$ (*m*/*z* 276.08) dissociated into CPP^- (*m*/*z* 137.08) by degrees.

X-ray crystallography shows that THPTb and DOTAMTb complexes are structurally similar. Each of them contains an independent $[TbL]^{3+}$ monomer, three uncoordinated nitrates and a water molecule, but the conformations of the macrocyclic rings in them are Δ(δδδδ) and Λ(λλλλ) respectively. THPTb adopts a typical mono-capped square antiprism geometry, in

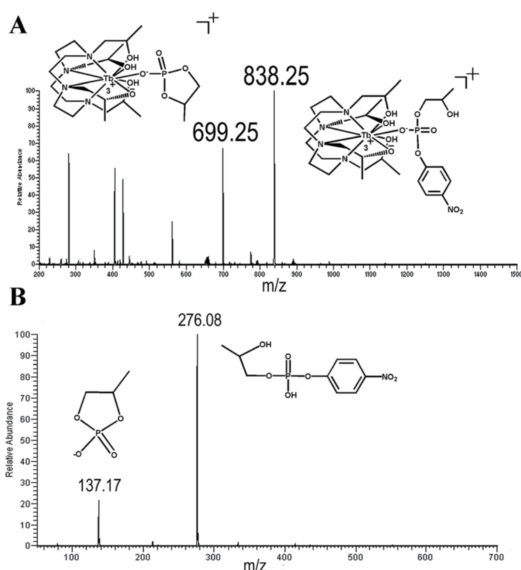
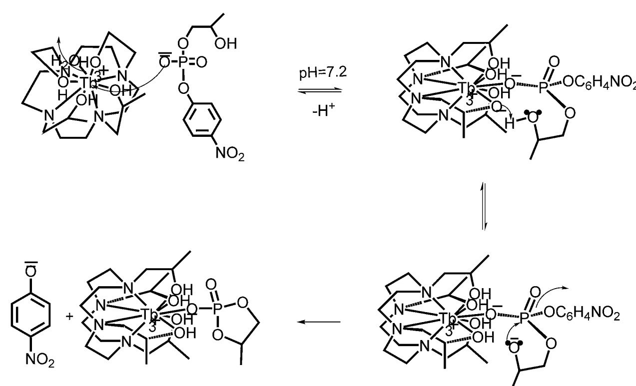


Fig. 3 ESI-MS of HPNP cleaved by THPTb. (a) Positive-ion mode spectra for 12 h; (b) negative-ion mode spectra for 12 h.



Scheme 1 Mechanism of HPNP cleavage by THPTb.

which the central Tb(III) ion coordinates to four amino moieties of cyclen and four oxygen atoms from the hydroxyl groups of pendant arms. The ninth coordination site is occupied by a water molecule (Fig. S7†). In other words, the THP ligand contains –OH group that can remain protonated as an alcohol or can be deprotonated as an alkoxide during complexation. Instead of –OH groups, four amide groups from the pendant arms in DOTAMTb provide oxygen atoms for coordination. Therefore, DOTAMTb lacks potent nucleophilicity and does not form hydroxide species. In fact, the ESI-positive mode MS demonstrated the nuclearity of THPTb in CH_3OH. Meanwhile, trivalent metal ions, when one or two –OH groups in THP were deprotonated (*i.e.* H_4L changed into H_3L^- and H_2L^{2-}), became tetravalent ones.

A possible mechanism was proposed *via* the kinetics and ESI-MS data (Scheme1). The THPTb-promoted HPNP cleavage involves an intracomplexnucleophilic attack of the metal-bound alkoxide on a metal-coordinated 2′-OH group. The reactions result from the nucleophilic attack of phosphorus by an activated protonated hydroxyl nucleophile (2′-O–) and may be ascribed to transesterification. Nucleophiles are generally attracted by metal complexes in HPNP hydrolysis and are provided by the 2′-OH group of the substrate. The hydrolysis reaction yields a cyclicphosphodiester as an intramolecular adduct.[8a,9a]

Conclusion

THPTb was the first example of model compound of RNase U2, which cleaved RNA phosphate ester bonds at A-specific sites. Surprisingly, this selectivity is completely different from the previous reports in the cleavage of diribonucleoside monophosphates, where a high preference for the cleavage of ApA and/or UpU sequences was observed. THPTb may be generally employed to develop site-directed RNA cleaving agents. The intermediates of adducts of $THPTb^{2+}$ and CPP^- or $HPNP^-$ in the hydrolysis of HPNP were directly monitored by ESI-MS. Based on these intermediates and the crystal structure of THPTb, a possible THPTb-promoted RNA cleavage mechanism was proposed. Obviously this model compound may be potentially applied in RNA manipulation.

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