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Cleavage of nucleotides by Ce^{4+} and the lanthanide metal complexes

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The cleavage of adenosine-5′-monophosphate (5′-AMP) and guanosine-5′-monophosphate (5′-GMP) by Ce^{4+} and lanthanide complex of 2-carboxyethylgermanium sesquioxide (Ge-132) in acidic and near neutral conditions was investigated by NMR , HPLC and measuring the liberated inorganic phosphate at 37°C and 50°C. The results showed that 5′-GMP and 5′-AMP was converted to guanine (G), 5′-monophosphate (depurination of 5′-GMP), ribose (depurination and dephosphorylation of 5′-GMP), phosphate and adenine (A), 5′-monophosphate (depurination of 5′-AMP), ribose (depurination and dephosphorylation of 5′-AMP), phosphate respectively by Ce^{4+}. In presence of lanthanide complexes, 5′-GMP and 5′-AMP were converted to guanosine (Guo) and phosphate and adenosine (Ado) and phosphate respectively. The mechanism of cleaving 5′-GMP and 5′-AMP is hydrolytic scission.

Keywords: adenosine-5′-monophosphate and guanosine-5′-monophosphate cleavage, $Ce(NH_4)_2(NO_3)_6$ and $Ce(SO_4)_2$, lanthanide ions, lanthanide complexes

Introduction

Selective scission of nucleic acids is one of the most challenging topics, and many elegant artificial nucleases have been reported. However, most of them take advantage of oxidative cleavage of deoxyribose at the target site(Schultz *et al*. 1982; Taylor *et al*. 1984; Chu & Orgel 1985; Stern *et al*. 1990; Mack & Dervan 1990). Hydrolytic scission is preferable for most applications since the resultant fragments can be manipulated directly by means of the techniques employed in current molecular biology. However, the greatest obstacle is the lack of appropriate catalytic residue for the purpose. The surprisingly high activities of lanthanide ions in the hydrolysis of compounds of biological interest have recently attracted a great deal of attention (Bamann *et al*. 1954; Eichhorn & Butzow 1965; Breslow & Huang 1991; Yohannes & Kristin 1993; Morrow *et al*. 1992). Recently Makato Komiyma *et al.* succeeded in the first nonenzymatic hydrolysis of linear DNAs by using of lanthanides. The catalytic activity of $CeCl_3$ is especially high, which is ascribed to the Ce^{4+}-bound hydroxide ions formed in the reaction mixture (Sumaoka *et al*. 1992; Komiyama *et al*. 1994). Takasaki and Chin suggest that oxidative cleavage of dApdA phosphate diester bond was achieved with Ce(III) and molecular oxygen (Takasaki *et al*. 1994). However, lanthanide ions precipitate readily under basic conditions due to the formation of insoluble hydroxide gels which is the major obstacle to their use in aqueous solutions. Thus, it is important to find a homogeneous hydrolytic system which is stable enough to prevent the formation of hydroxide gels and is more active. Here we report that the hydrolysis of 5′-AMP and 5′-GMP by Ce^{4+} under acidic condition and the cleavage of 5′-AMP and 5′-GMP by lanthanide complexes of Ge-132 at pH 6 as well as the mechanism of their cleavage.

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Materials and methods

Material: 5′-AMP and 5′-GMP were obtained from Sigma Company, Aqueous Solution of $Ce(NH_4)_2(NO_3)_6$ was prepared from $Ce(NH_4)_2(NO_3)_6$. Solution of $Ce(SO_4)_2$ was prepared by dissolving CeO_2 in H_2SO_4(18M). The concentration of Ce^{4+} was determined by titration with $Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$. Lanthanide chlorides were prepared from their oxides(purity 99.99%). The concentration was determined by EDTA. All other reagents were of analytical grade.

Synthesis of the complex of Ln^{3+}-Ge-132: To a rare earth chloride solution($LnCl_3$) (pH 2) was added salt solution of Ge-132. pH of the solution was adjusted to 3~4, and the mixture was heated and stirred for 1 hr at 60~70°C. After filtration, a little insoluble lanthanides hydroxide gel was removed. The solution was continuously stirred 3hr at room temperature. After addition of the same volume of alcohol, the solid complex of Ln^{3+}-Ge-132 was filtered off and washed by 1 : 1 alcohol. The molecular formula of Pr^{3+}-Ge-132 is $[(OH)_3Ge\text{-}CH_2{}^{\alpha}\text{-}\ CH_2{}^{\beta}\text{-}COO)]_3 \cdot Pr \cdot 2H_2O$(Found: C, 13.66; H, 2.88; Ge, 28.16; Pr, 18.35. $[(OH)_3Ge\text{-}CH_2\text{-}CH_2\text{-}COO)]_3 \cdot Pr \cdot 2H_2O$ requires C, 14.12; H, 3.27; Ge, 28.47, Pr, 18.42. ^{1}H n. m. r. δ (D_2O 400MHz) 2.87^{α}, t, 1.74^{β}, t, J, 8Hz. Infrared data (cm^{-1}): 1571, 1447, 880, 790. The molecular formula of Nd^{3+}-Ge-132 is $[(OH)_3Ge\text{-}CH_2{}^{\alpha}\text{-}CH_2{}^{\beta}\ COO)]_3 \cdot Nd \cdot 2H_2O$(Found: C, 13.48; H, 2.76; Ge, 28.14; Nd, 18.63. $[\ (OH)_3Ge\text{-}CH_2\text{-}CH_2\text{-}COO)]_3 \cdot Nd \cdot 2H_2O$ requires C, 14.06; H, 3.25; Ge, 28.35, Nd, 18.78. ^{1}H n. m. r. δ (D_2O 400MHz) 2.85^{α}, t, 1.73^{β}, t, J, 8Hz. Infrared data (cm^{-1}): 1571, 1447, 880, 790.

Apparatus: ^{1}H NMR and ^{31}P NMR spectra were obtained on a Varian UNITY 400 NMR spectrometer at 37°C, and ^{31}P NMR spectra was taken at 161.9 MHz. 2, 2-dimethyl-2-silapentane-5-sulfonate(DSS) and 85% phosphoric acid were used as internal reference and external reference respectively. HPLC was performed on a HITACHI 638-50 (detector wavelengths: 254 nm and 260.5 nm). A Spherisorb C_{18} column was used, The mobile phase is consisted of 5% CH_3OH, 20 mM HAc-Ac^-(pH 5) and 1 mM EDTA, fluid rate was chosen as 0.7 ml/min. Pure nucleotides were used as standards. Products were identified by comparison of their retention times to those of standards.

Measurement of nucleotides hydrolysis: The dephosphorylation of 5′-AMP and 5′-GMP by Ce^{4+} was carried out at 37°C and the dephosphorylation of 5′-AMP and 5′-GMP by lanthanide complexes was carried out at 50°C. The concentrations of inorganic phosphate $[PO_4]_t$ at various reaction times in the reaction mixtures were determined by use of the molybdenum blue method. The degree of hydrolysis was calculated on the basis of phosphate ester bond in 5′-AMP and 5′-GMP. A blank was run using parallel concentrations of different acids. The free phosphate initially present(1–2%) was taken into account.

Results and discussion

Structures of 5′-AMP and 5′-GMP are shown in Fig 1. It has been known that the N-glycosidic bond of nucleotides is not stable in acidic conditions (Oivanen *et al.* 1988; Oivanen & Lönnberg 1989). Hydrolysis of the phosphomonoester bond and glycosidic bond of 5′-GMP was promoted by $Ce(SO_4)_2$ at 37°C and pH 0. The effects of Ce^{4+} and H_2SO_4 (1M) on the hydrolysis of 5′-GMP were clearly displayed in the ^{1}H and ^{31}P NMR (Fig. 2 and Fig. 3). The ^{1}H NMR spectra of 5′–GMP are featured by the chemical shifts of 5.94ppm and 8.17ppm, which were assigned to be the C_1'–H and H-8 of 5′-GMP (Pinnavaia *et al.* 1975). In presence of H_2SO_4 (1M), the hydrolysis of the C-N bond of 5′-GMP was evidenced by the appearance of the H-8 resonance of guanine(G) (8.24ppm) and the C_1'–H resonance of 5′-monophosphate(5′-MP) (5.76 ppm), no other new peaks appeared even up to 16hr. It indicated that the C-N bond of 5′-GMP was cleaved by H_2SO_4, no effect on the P-O bond of 5′-GMP. However, in presence of $Ce(SO_4)_2$, beside the peaks for H-8 of G and C_1'–H of 5′-MP, a new peak at 5.65ppm appeared after the reaction was started, which was due to the C_1'–H resonance of ribose (R). A longer reaction time led to an increase in intensity of signal of the C_1'–H of R with no other new peaks appearing. This result indicated that the C-N bond of 5′-GMP was cleaved completely within the reaction time and the concentration of ribose increased with increasing the reaction time.

To verify the result of ^{1}H NMR, the reaction was further studied by comparing the ^{31}P NMR spectra of 5′-GMP in solution of $Ce(SO_4)_2$ and at different reaction times. As shown in Fig. 3, the chemical shift of phosphates of 5′-GMP is 0.47 ppm. In presence

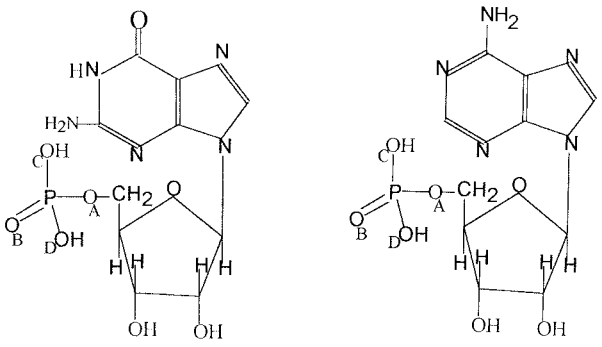


Figure 1. Molecular structures of 5′-AMP and 5′-GMP

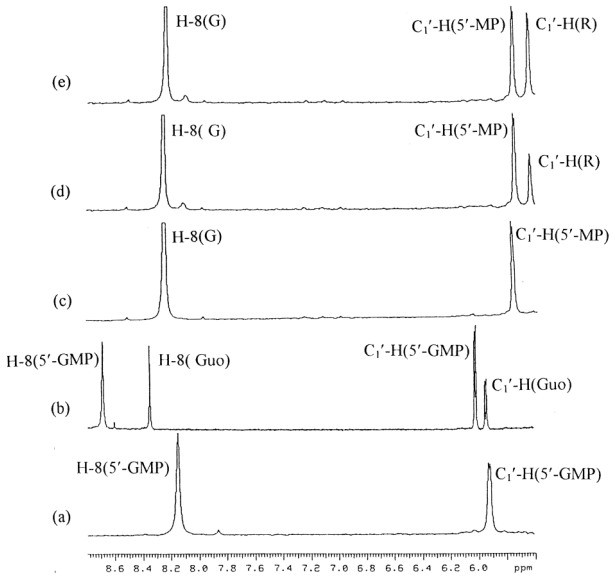


Figure 2. I: ^{1}H NMR spectrum for the cleavage of 5′-GMP(18mM) by $Ce(SO_4)_2$(241mM) at pD 0 and 37°C. (a) pure sample of 5′-GMP, (b) mix guanosine(Guo) to the solution of 5′-GMP, (c) incubation of the solution of 5′-GMP for 16hr in presence of D_2SO_4(1M), (d) incubation of the solution of 5′-GMP for 4hr in presence of $Ce(SO_4)_2$, (e) incubation for 16 hours

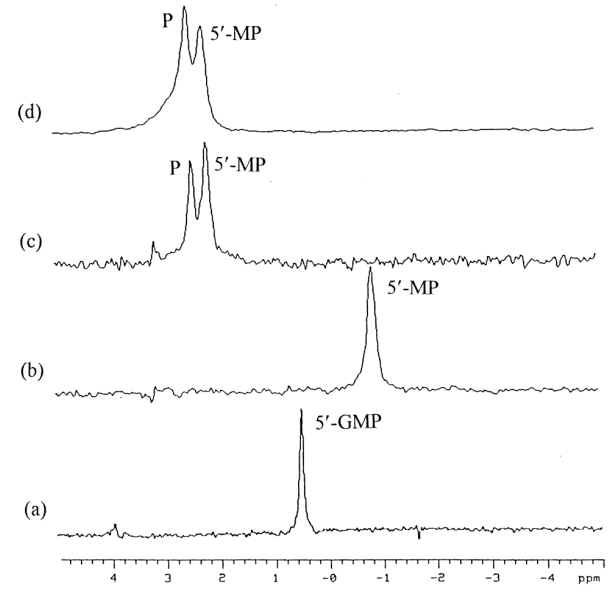


Figure 3. ^{31}P NMR spectrum (161.9MHZ) for the cleavage of 5′-GMP(18mM) by $Ce(SO_4)_2$ (241mM) at pH 0 and 37°C. (a) pure sample of 5′-GMP, (b) pure sample of 5′-GMP in presence of H_2SO_4(1M) for 16 h, (c) incubation of the solution of 5′-GMP for 16 h in presence of $Ce(SO_4)_2$, (d) incubation for 30 h

of H_2SO_4 (1M), a new peak at –0.88 ppm appeared, which was due to the phosphate group of 5′-monophosphate (5′-MP). No other new peaks appeared within 16hr. This indicated that only the C-N bond of 5′-GMP was cleaved by H_2SO_4 (1M) and there was no effect on the phosphate group of 5′-GMP. However, in the presence of Ce^{4+}, two new peaks appeared, a peak at 2.17 ppm, which was assigned to be the phosphate of 5′-MP and a peak at 2.45 ppm, the chemical shift of which indicated that it was inorganic phosphate due to the P-O bond cleavage of 5′-MP. The signal of the inorganic phosphate enhanced with increasing the reaction time of hydrolysis. It indicated that the concentration of inorganic free phosphate increases with increasing the reaction times. Thus, ^{31}P NMR studies of solutions of 5′-GMP in the presence of $Ce(SO_4)_2$ and H_2SO_4 (1M) further verified the results of ^{1}H NMR.

As mentioned above, guanine, 5′-monophosphate, ribose and inorganic phosphate are the main products of the hydrolysis of 5′-GMP. In addition, we studied the hydrolysis of 5′-AMP in the presence of Ce^{4+} by ^{1}H and ^{31}P NMR spectroscopy. The results indicated that Ce^{4+} hydrolyze 5′-AMP to adenine(A), 5′-monophosphate, ribose and inorganic phosphate. The inability of sulfuric acid to hydrolyze the P-O bond of 5′-GMP and 5′-AMP rules out the possibility of acid hydrolysis. Hydrolytic scission is the mechanism of cleavage of 5′-GMP and 5′-AMP with no trace of products due to cleavage of the sugar.

Since the sulfate complexes Ce^{4+} are known to be quite stable (Sillen & Martell 1971), the addition of sodium sulfate leads to a decrease in the concentration of aquocerium(IV). The degree of dephosphorylation of 5′-AMP decreases when increasing the concentration of Na_2SO_4 (Fig. 4). It is obvious that aquocerium(IV) in solution is responsible for the dephosphorylation of 5′-AMP.

Furthermore, we expected that the influence of increasing concentration of sulfuric acid on $Ce(SO_4)_2$ promoted 5′-AMP dephosphorylation is different from that with HCl and $HClO_4$. The results (Fig. 5) show that with increasing H_2SO_4 concentration, the increased concentration of SO_4^{2-} will cause a lowering of the concentration of aquocerium(IV) and also of dephosphorylation. On the other hand, the increasing concentration of HCl and $HClO_4$ promoted the dephosphorylation further due to increased protonation of SO_4^{2-} and increased Ce^{4+} concentration. The 5′-AMP dephosphorylation promoted simply by acids was not measurable. Thus, we speculate that the concentration of aquocerium(IV) is the major factor determining 5′-AMP dephosphorylation in the presence of $Ce(SO_4)_2$.

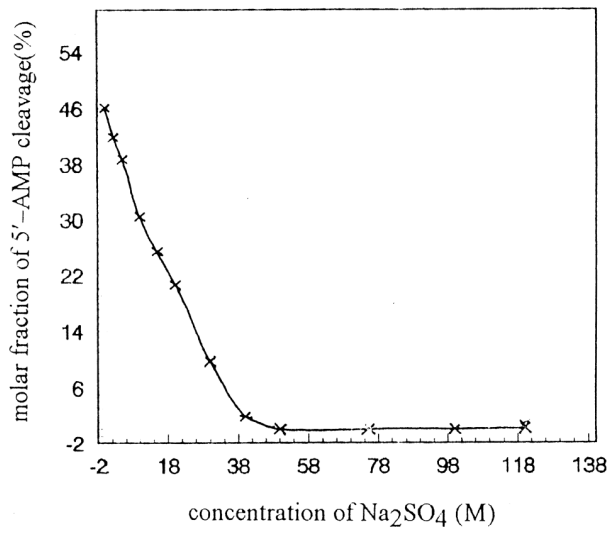


Figure 4. The influence of Na_2SO_4 on dephosphorylation of 5′-AMP(0.2mM) by $Ce(SO_4)_2$ (9.7mM) at pH 1 and 37°C, 10 h.

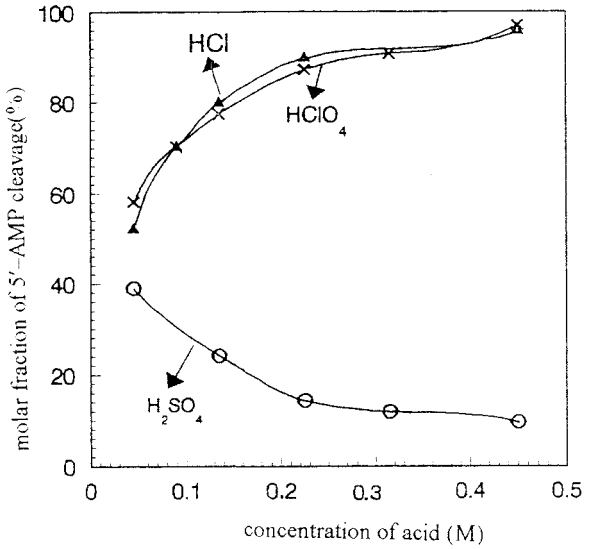


Figure 5. The influence of concentration of different acid on dephosphorylation of 5′-AMP(0.2mM) by $Ce(SO_4)_2$ (9.7mM) at 37°C, 10 h.

In addition, we studied the difference between $(NH_4)_2Ce(NO_3)_6$ and $Ce(SO_4)_2$ under acidic condition. As shown in Table 1, the dephosphorylation was accelerated more significantly by $Ce(NH_4)_2(NO_3)_6$ than by $Ce(SO_4)_2$. Due to the higher stability constant of the sulphato complex of Ce^{4+}, the concentration of aquocerium(IV) in $Ce(NH_4)_2(NO_3)_6$ solution is higher than that of $Ce(SO_4)_2$. The results are also in support of the postulation that the aquocerium(IV) is the active species in acidic conditions.

As mentioned above, the aquocerium(IV) in solution of $Ce(SO_4)_2$ is the actual reactive species for dephosphorizing mononucleotides. The positive charge of Ce^{4+} neutralizes the negative charge of oxygen of the PO_4^-, thus leading to the weakening of the P-O bond. It results in the cleavage of the phosphate monoester linkage of mononucleotides.

The dephosphorylation of 5′-AMP and 5′-GMP by $Ce(SO_4)_2$ at pH 9 and acidic conditions was monitored by measuring the liberated inorganic phosphate. The degree for the dephosphorylation of 5′-AMP and 5′-GMP is shown in Table 2. The degree of dephosphorylation of 5′-AMP is larger than that of 5′-GMP at pH 9, 37°C. Interestingly, the results

Table 1. The degree of dephosphorylation of 5′-Amp by $Ce(NH_4)_2(NO_3)_6$ and $Ce(SO_4)_2$ at 37°C and pH 0.2. $[Ce(NH_4)_2(NO_3)_6]_0 = [Ce(SO_4)_2]_0 = 9.6mM$, $[5'\text{-AMP}]_0 = 0.2mM$, $[\ ]_0$-initial concentration.

	% of 5′-AMP dephosphorized[a]	
reaction time (hr)	$Ce(NH_4)_2(NO_3)_6$	$Ce(SO_4)_2$
0.5	6.7 ± 0.3	0
1	26.7 ± 0.3	0
4	55.2 ± 0.3	0.4 ± 0.1
7	84.8 ± 0.4	2.4 ± 0.1
10	95.7 ± 0.4	13.3 ± 0.2

(a) Values are mean ± SD.

Table 2. The degree of dephosphorylation of nucleotides(0.2mM) BY $Ce(SO_4)_2$(9.6mM) AT 37°C

	% of 5'-AMP dephosphorized[a]		% of 5'-GMP dephosphorized[a]	
reaction time(hr)	pH 9	pH 0.2	pH 9	pH 0.2
0.5	5.4 ± 0.2	0	2.3 ± 0.3	2.3 ± 0.1
1	21.6 ± 0.3	0	13.3 ± 0.2	7.5 ± 0.1
4	65.4 ± 0.3	0.4 ± 0.1	52.0 ± 0.4	19.4 ± 0.2
5	72.5 ± 0.4	0.8 ± 0.1	58.2 ± 0.4	25.4 ± 0.3
7	82.4 ± 0.4	2.4 ± 0.1	71.6 ± 0.4	33.3 ± 0.2
10	97.8 ± 0.4	13.3 ± 0.1	88.0 ± 0.4	45.5 ± 0.3

([a])Values are mean ± SD.

were reversed for the dephosphorylation of 5′-AMP and 5′-GMP by Ce^{4+} in acidic conditions. The activity for dephosphorizing 5′-GMP was greater than that of 5′-AMP. The net charge of each atom in PO_4^{3-} of 5′-AMP and 5′-GMP were calculated by the Gast-Hück method (Gasteiger & Marsili 1980, Purcell & Singer 1967) (Table 3). The net charge of phosphate of 5′-AMP is more positive than that of 5′-GMP , so it was more effective for 5′-AMP that hydroxide ion bound to the Ce^{4+} functions as the nucleophile and intramolecularly attacks the phosphate at pH 9, 37°C. As a result, the degree of dephosphorylation of 5′-AMP is greater than that of 5′-GMP. However, the degree of dephosphorylation of 5′-GMP by Ce^{4+} is greater than that of 5′-AMP in acidic conditions, because of more effective neutralizing functions produced by Ce^{4+} for 5′-GMP due to the charge of O_A O_B O_C O_D of 5′-GMP which is more negative than that of 5′-AMP.

It was reported by Bamann *et al.* that lanthanide ions can cleave 5′-AMP only under basic conditions, and that under mild conditions, this cleavage never occurred (Bamann *et al.* 1954). However, lanthanide ions readily precipitate under basic condition due to the formation of insoluble hydroxide gels, hence, their use is difficult in aqueous solution and in vivo. The degree of hydrolysis of 5′-AMP and 5′-GMP by lanthanide metal complexes of Ge-132, Ge-132 and lanthanide ion at pH 6.0 and 50°C is shown in the Table 4. The effect of various active species on the cleavage of 5′-AMP and 5′-GMP is different, the most active species for cleaving 5′-AMP and 5′-GMP being lanthanide metal complexes, and among these the more active species is ligand (Ge-132), while lanthanide metal ions show no activity at all.

The cleavage of 5′-AMP to adenosine (Ado) and inorganic phosphates by Pr^{3+} complex were clearly evidenced by 400 MHz ^{1}H NMR and 161.9 MHz ^{31}P NMR at pD 6 and 50°C, as shown in Figs. 6 and 7, by monitoring the signals due to the C_1'-H protons and the signal of phosphate of 5′-AMP. As previously described, the chemical shift of C_1'-H of 5′-AMP is 6.14 and 6.12 ppm (Berger & Eichhorn 1971), the new peak at 5.3ppm, arising from the C_1'-H peak of adenosine (Ado), appeared 25 h after the beginning of the reaction. Since then the C_1'-H peak of Ado increases gradually, showing that the concentration of Ado increases by increasing the reaction time. The chemical shift of the phosphate of 5′-AMP was -0.88 ppm. However, a new peak at 15.3 ppm was detected in the presence of the Pr^{3+} complex. This is the the peak of free phosphate indicating that the concentration of free phosphate increases by increasing the reaction time.

The results of ^{1}H NMR and ^{31}P NMR indicate that the Pr^{3+} complex of Ge-132 hydrolyze 5′-AMP to Ado and free phosphate. We also studied the hydrolysis of 5′-GMP in the presence of Pr^{3+} complex of Ge-132 by ^{1}H NMR and ^{31}P NMR spectrum. The results indicate

Table 3. Atomic charge of 5'-Amp and 5'-Amp

Molecule	5'-AMP	5'-GMP
Method	Gast-Hück	Gast-Hück
P	0.479	0.187
O_A	–0.290	–0.44
O_B	–0.252	–0.32
O_C	–0.309	–0.46
O_D	–0.309	–0.46

Table 4. Hydrolytic cleavage of 5′-Amp and 5′-Gmp by lanthanide complexes of Ge-132, Ge-132 and lanthanide(iii) metal ions at pH 6, 50°c. 25h. $[Pr^{3+}\text{-Ge-132}]_0 = [Nd^{3+}\text{-Ge-132}]_0 = [\text{Ge-132}]_0 = 9.6$ mmol/l, $[Pr^{3+}]_0 = [Nd^{3+}]_0 = 9.6$ mmol/l, $[5'\text{-Amp}]_0 = [5'\text{-Gmp}]_0 = 0.2$mmol/l: $[\ ]_0$-initial concentration.

Sample	% of 5′-AMP cleaved[a]	% of 5′-GMP cleaved[a]
Pr^{3+}-Ge-132	16.4 ± 0.2	12.4 ± 0.2
Nd^{3+}-Ge-132	15.5 ± 0.2	10.8 ± 0.2
Ge-132	6.6 ± 0.1	5.5 ± 0.2
Pr^{3+}	0	0
Nd^{3+}	0	0

[a] Values are mean ± SD.

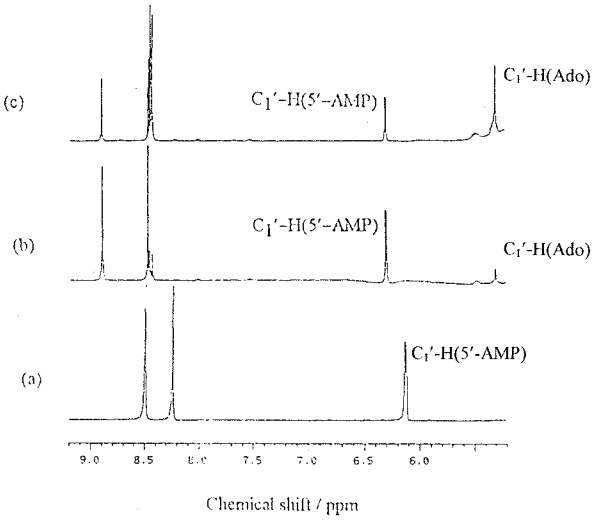


Figure 6. ^{1}H NMR spectra for the cleavage of 5′-AMP(12mM) by Pr^{3+}-Ge-132 (30.8mM) at pD 6 and 50°C, DSS as internal reference. (a) 0 h, (b) 25 h, (c) 65 h.

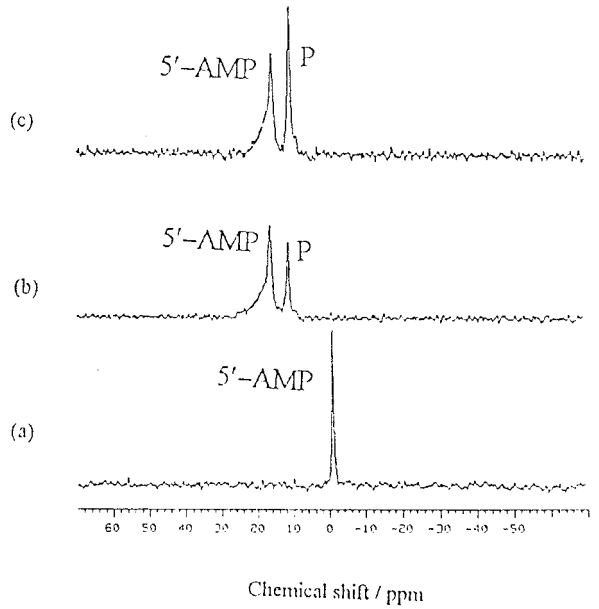


Figure 7. ^{31}P NMR spectra for the cleavage of 5′-AMP (12mM) by Pr^{3+}-Ge-132 (30.8mM) at pH 6 and 50°C, 85% phosphoric acid as external reference. (a) 0 h, (b) 45 h, (c) 65 h

that the complex hydrolyze 5′-GMP to Guanosine (Guo) and free phosphates. Hydrolytic scission is the mechanisms of cleavage of both 5′-AMP and 5′-GMP. The same results were also observed by using Yb^{3+} and Lu^{3+} complexes of Ge-132.

In order to verify our results, cleavage of 5′-AMP by Pr^{3+}-Ge-132 was further investigated by high-performance (HPLC); by comparing the retention time of the components of the hydrolysis mixture with those of standard compounds; the formation of adenosine (Ado) was confirmed (Figure 8). Adenine formation, which should take place if the reaction involved cleavage of the C-N bond of 5′-AMP, was not observed (no peak at 260.5nm) thus confirming the results of NMR and the method of measuring the released inorganic phosphate. The mechanism of cleavage of 5′-AMP is hydrolytic scission.

It was proposed by Komiyama *et al.* that Thymidyl (3′-5′) thymidine (TpT) hydrolysis involved intramolecular attack by the Ce(IV)-bond hydroxide ion towards the phosphate of TpT (Komiyama *et al.* 1994). Lanthanide ions cannot form metal hydroxide clusters at pH 6.0, thus they are inactive in the hydrolysis of 5′-AMP and 5′-GMP. Ge-132 is soluble in water to about 0.98% and in solution this compound is hydrolyzed and dissociated to the monomer, trihydroxygermylpropanoic acid. This fact was clarified from ^{17}O-NMR spectrum of [^{17}O]-labeled Ge-132 and ^{13}C-NMR spectrum of Ge-132 in the solid state and in solution (Akiba & Kakimoto 1994; Tsutsui *et al.* 1976). The proposed mechanism for the cleavage involves coordination of the phosphate of 5′-AMP to the Ln^{3+} complexes, as depicted in Scheme 1, thus leading to the weakening of the P-O bond. Then the hydroxide ions on the complexes function as a nucleophile and intramolecular nucleophilic attack to the phosphorus atom of 5′-AMP. This results in the cleavage of phosphate monoester linkage of mononucleosides.

In conclusion, these results confirm the hydrolytic cleavage of 5′-AMP and 5′-GMP by Ce^{4+} and the lanthanide complexes of Ge-132. These are two kinds of homogeneous hydrolytic systems. These findings are crucially important for the molecular design of artificial hydrolytic nucleases and are advantageous to medication, therapy and other fields.

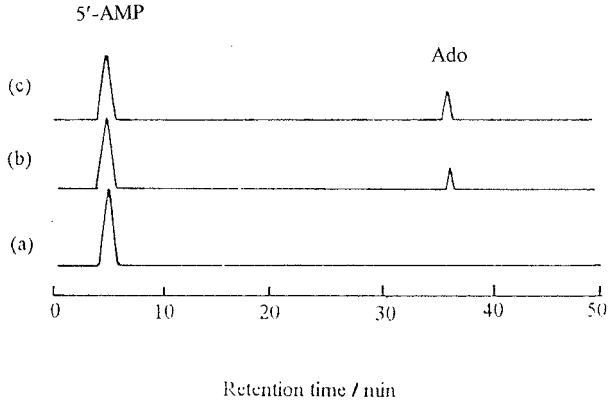
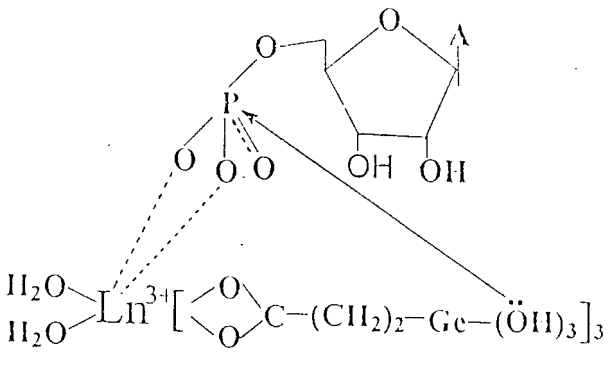


Figure 8. HPLC patterns for the cleavage of 5′-AMP(2.5mM) by Pr^{3+}-Ge-132(10mM) at pH 6 and 50°C. (a) 0 h, (b) 15 h, (c) 30 h



Scheme 1. Proposed mechanism for the Ln^{3+}-Ge-132 cleavage of 5′-AMP.

Acknowledgements

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