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REGIO- AND DIASTEREO-SELECTIVITY OF MONTMORILLONITE-CATALYZED OLIGOMERIZATION OF RACEMIC ADENOSINE 5′-PHOSPHORIMIDAZOLIDE

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□ *Clay is a possible candidate for an effective catalyst in prebiotic chemical evolution of biomolecules. Montmorillonite was reported to effectively catalyze oligomerization of racemic adenosine 5′-phosphorimidazolide (DL-ImpA). In the oligomerization reaction, considerable amounts of cyclic dimers as well as linear dimers were produced in the oligomerization reactions. To assess the regio- and diastereo-selectivities of the oligomerization reaction, the dimer products including cyclic dimers were completely identified by means of enzymatic degradation reactions of the products.*

Keywords RNA; mononucleotide; racemate; clay-catalyzed oligomerization; cyclic nucleotide

INTRODUCTION

Biomolecules such as amino acids, sugars, and nucleic acids are chiral, and the chirality of biomolecules is thought to have been essential for the origins of life.[1,2] Indeed, the homochirality of biomolecules plays an important role for the specificity in the recognition between them.[3] Since the discovery of ribozymes,[4,5] RNA is thought to have been one of the important precursors of life.[6] Under prebiotic conditions, biomolecules such as nucleotides are thought to have been formed as a racemate. Considering the chemical evolution of RNA, there are serious problems how racemic nucleotides have been oligomerized, and how the homochirality of RNA has been established.[7]

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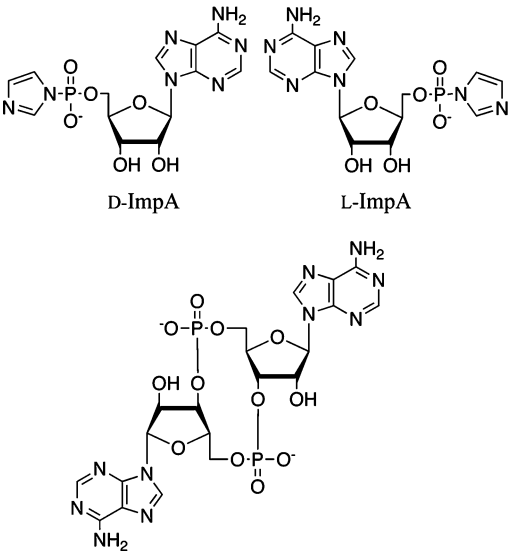

FIGURE 1 Structures of D-, L-ImpA (upper) and D-homochiral cyclic $pA^{3'}pA^{3'}$ (bottom).

Template-directed oligomerization of activated mononucleotide D-ImpA (Figure 1) effectively yielded polynucleotides,[8] but was unsuccessful for oligomerization of L-ImpA and racemic ImpA.[9] Since Ferris and coworkers have reported effective oligomerization of D-ImpA on montmorillonite clay,[10,11] we noticed the achirality of montmorillonite and its potential to catalyze oligomerization for L-ImpA or racemic ImpA as effective as for D-ImpA. Then, we found that montmorillonite serves an effective catalyst for the oligomerization of L-ImpA and racemic ImpA.[12] Independently, Ferris and coworkers have also reported the montmorillonite-catalyzed reaction of racemic ImpA.[13] Although the formation of considerable amounts of cyclic dimers (Figure 1) were observed in the reaction, the identification of cyclic dimers formed by the oligomerization of *racemic* ImpA was not reported because of the possibility of formation of various kinds of regio-, enantio-, and diastereo-isomers. Very recently, Ferris and coworkers have reported the identification of homochiral D,D-cyclic $pA^{3'}pA^{3'}$ and heterochiral cyclic $pA^{3'}pA^{3'}$ as well as linear dimers produced by the montmorillonite-catalyzed oligomerization of racemic ImpA.[14] However, under their experimental conditions to suppress the formation of longer oligomers, they have found neither the regio-isomers of the phosphodiester bond of cyclic $pA^{3'}pA^{3'}$ nor HEPES-pApA, which is an adduct of the dimer with HEPES used as a buffer. We found that the HEPES-pApA adduct shows the same chromatographic behavior with one of the cyclic dimers in reversed phase HPLC. The chromatographic overlap of HEPES-pApA and cyclic dimers makes the identification of them difficult, leading to decrease of the accuracy of quantitative product analysis. To solve

such difficulty, we employed imidazole as a buffer in place of HEPES to avoid formation of HEPES-pApA. In this article, we report on the complete identification of the dimer products, including the first identification of homochiral cyclic $pA^{2'}pA^{3'}$ produced by the montmorillonite-catalyzed oligomerization of racemic ImpA.

EXPERIMENTAL SECTION

General

D- and L-ImpAs were synthesized by a literature procedure from adenosine 5′-phosphoric acid,[8] of which L-isomer was synthesized by phosphorylation[15] of L-adenosine.[6] Calf intestine alkaline phosphatase and RNase T_2 was purchased from Takara Bio Inc. (Shiga, Japan) and Sigma (St. Louis, MO, USA), respectively. Reversed phase HPLC was performed on a column of μBondasphere 5C18 100Å with a linear gradient of acetonitrile (0–10%) in 50 mM potassium phosphate (pH 4.0) by a Shimadzu LC-10A system. Anion exchange HPLC was performed on a column of TSK GEL DNA-NPR (TOSOH) with a linear gradient of $NaClO_4$ (0–0.15 M/30 minutes) in 20 mM Tris-HCl (pH 9.0).

Oligomerization of ImpA

To 50 mg of Na^+-montmorillonite, 14 mM ImpA solution containing 0.2 M NaCl, 75 mM $MgCl_2$, 0.1 M HEPES or imidazole (pH 8.0) was added. After the pH was adjusted to 8.0 by adding diluted NaOH or HCl, respectively, reactions were stand for 7 days at 25°C.

Alkaline Phosphatase Treatment

A solution of dimers (0.2 OD units/200 μL) containing 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) was incubated with 0.4 μL of calf intestine alkaline phosphatase (0.5 units/μL) at 37°C for 3 hours. The mixture was filtered through Ultrafree-MC 5000 NMWL filter unit (Millipore) and was analyzed by a reversed phase HPLC.

RNase T_2 Treatment

A solution of dimers (0.015 OD units/12 μL) was added 0.2 M ammonium acetate (pH 5.0) 1.5 μL and RNase T_2 (0.1 units/μL) 1.5 μL, and was incubated at 37°C for 3 hours. After addition of 10 mM $CuSO_4$ (1.65 μL), the mixture was filtered through Ultrafree-MC 5000 NMWL filter unit and was analyzed by a reversed phase HPLC.

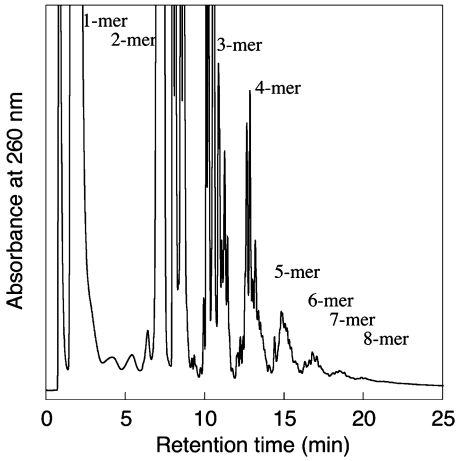

FIGURE 2 An anion exchange HPLC chromatogram of montmorillonite-catalyzed oligomerization products of racemic ImpA.

RESULTS AND DISCUSSION

Oligomerization reactions were carried out by adding 50 mg of Na^+-montmorillonite to a solution (1 mL) containing 14 mM racemic ImpA, 0.2 M NaCl, 75 mM $MgCl_2$, 0.1 M HEPES buffer (pH 8.0) at 25°C for 7 days. The reaction efficiently proceeded to give oligomers up to 8-mers (Figure 2). To identify the dimer products (Figure 3), the dimer fraction was collected by using anion exchange HPLC and desalted. Figure 4 shows the HPLC chromatograms of the dimer fraction; (a) anion exchange, (b) reversed phase HPLC chromatograms, and (c) a HPLC chromatogram of the dephosphorylated product of the dimer fraction with alkaline phosphatase. This enzyme shows an enough dephosphorylation activity for L-nucleotides as well as for D-nucleotides,[16,17] and the only isomers of pApA among possible dimer products (Figure 3) are converted to 5′-dephosphorylated dimers. The peaks for the four linear dimers shifted to more lipophilic region (**C–F**) by this enzyme, however dimers **A** and **B** were not affected. This result indicates that dimers **C–F** are the regio- and diastereo-isomers of ApA produced from the corresponding pApA isomers. These dimers **C–F** were easily identified as homochiral (D,D- and L,L-) $A^{2'}pA$, heterochiral (D,L- and L,D-) $A^{2'}pA$, homochiral (D,D- and L,L-) $A^{3'}pA$ and heterochiral (D,L- and L,D-) $A^{3'}pA$, respectively by comparison with authentic samples.[16,17] On the other hand, dimers **A** and **B** should be cyclic dimers or 5′-capped dimers such as AppApA or HEPES-pApA (Figure 3). In the preliminary degradation experiments of dimer **A** with alkaline hydrolysis followed by alkaline phosphatase treatment, dimer **A** was found to contain HEPES-pApA as well as cyclic dimers by detecting HEPES-pA.[18]

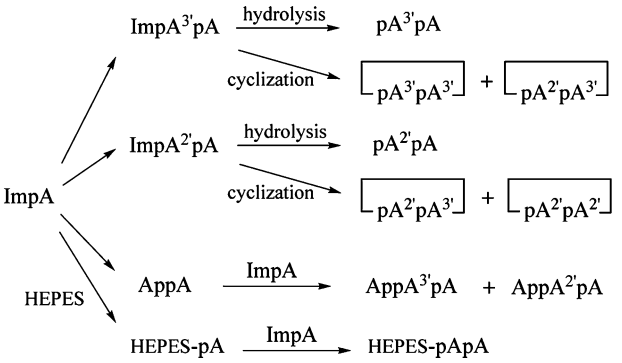

FIGURE 3 Possible dimer products formed by the condensation reaction of D-ImpA catalyzed by Na^+-montmorillonite. In the reaction of *racemic* ImpA, additional enantio- and diastereo-isomers are formed.

Alternatively, we carried out the oligomerization reaction of racemic ImpA under the similar condition by using imidazole buffer in place of HEPES buffer to avoid forming HEPES-pApA. Both the reactions proceed similarly, and the yields of oligomers produced by each reaction are compared in Table 1. After isolation of the dimer fraction from the reaction in imidazole buffer, it was analyzed by an anion exchange HPLC

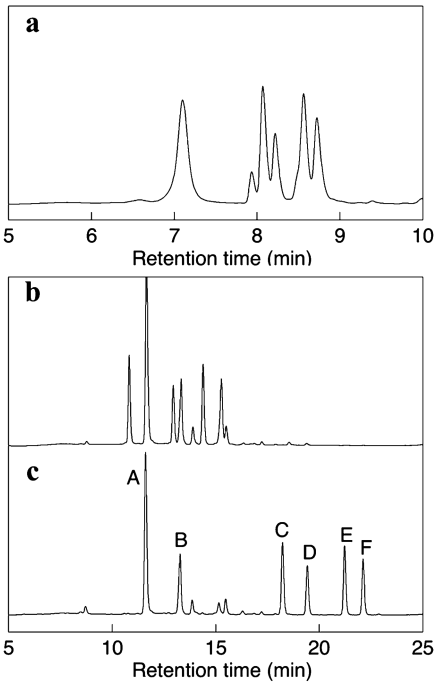

FIGURE 4 HPLC analyses of the dimer fraction produced by clay-catalyzed oligomerization of racemic ImpA in HEPES buffer. (a) Anion exchange, and reversed phase HPLC chromatograms (b) before and (c) after alkaline phosphatase treatment of the dimer fraction.

TABLE 1 Yields of oligomers produced by montmorillonite-catalyzed oligomerization of D,L-ImpA[a]

	Oligomers (%)							
Buffer	1 mer	2 mer	3 mer	4 mer	5 mer	6 mer	7 mer	8 mer
HEPES	47.8	29.4	14.2	5.53	2.01	0.75	0.28	0.06
Imidazole	47.0	30.1	13.3	5.73	2.28	1.02	0.42	0.11

[a]Reactions were carried out at 25°C for 7 days. D,L-ImpA concentration was 14 mM (Each enantiomer concentration is 7 mM) in 0.2 M NaCl, 75 mM $MgCl_2$, and 0.1 M buffer, pH 8.0. Percentages listed are the uncorrected HPLC absorbance readings.

(Figure 5a). The chromatogram lacks a peak at 7.9 minutes, which was observed in the dimer fraction from the reaction in HEPES buffer (Figure 4a). It was proved that this peak corresponds to HEPES-pApA by degradation experiments (data not shown). Figure 5b and 5c shows reversed phase HPLC chromatograms of the dimer fraction and its dephosphorylated product after alkaline phosphatase treatment, respectively. Dimers **C–F** were readily assigned to the isomers of ApA as described above.

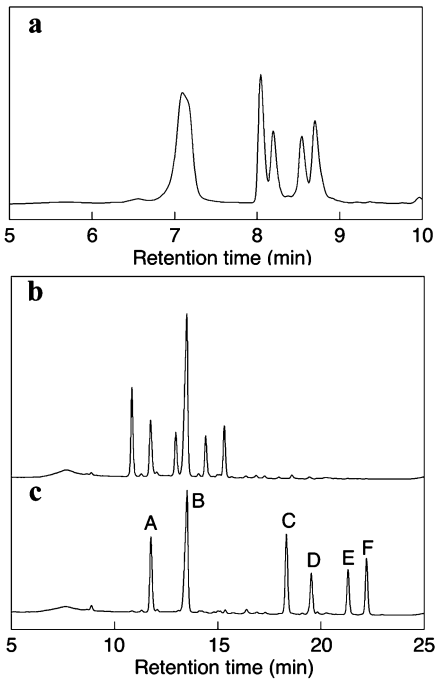

FIGURE 5 HPLC analyses of the dimer fraction produced by clay-catalyzed oligomerization of racemic ImpA in imidazole buffer. (a) Anion exchange, and reversed phase HPLC chromatograms (b) before and (c) after alkaline phosphatase treatment of the dimer fraction.

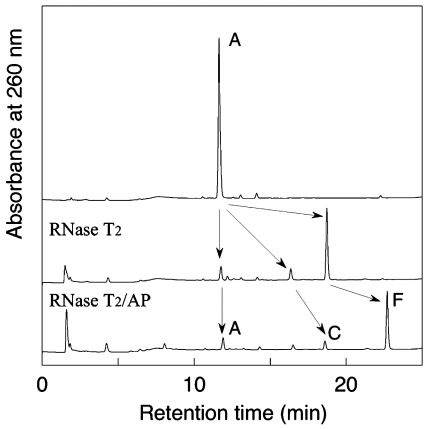

FIGURE 6 Reversed phase HPLC analyses of RNase T_2 followed by alkaline phosphatase (AP) treatment of dimer A.

In this case, degradation experiments with alkaline hydrolysis followed by alkaline phosphatase treatment demonstrated that dimers **A** and **B** contained neither AppApA nor HEPES-pApA. Therefore, these two peaks were thought to contain only cyclic dimers. After isolation of dimers **A** and **B**, we carried out RNase T_2 treatment followed by alkaline phosphatase treatment to characterize these cyclic dimers. Figure 6 shows the result for the enzymatic digestion of dimer **A**. As a result, dimer **A** afforded three peaks corresponding to dimers **A**, **C**, and **F**. RNaseT_2 can cleave the 3′-5′ phosphodiester linkage, but can cleave neither the 2′-5′ phosphodiester linkage,[19] nor the 3′-5′ phosphodiester linkage of which 5′-end side nucleoside has an L-stereochemistry.[17] The cyclic dimer has ten kinds of regio-, enantio- and diastereo-isomers (Figure 7). Among them, the isomers that afford homochiral $A^{2'}pA$ (dimer **C**) and heterochiral $A^{3'}pA$ (dimer **F**) after these treatments are homochiral D,D-cyclic $pA^{2'}pA^{3'}$ and heterochiral cyclic $pA^{3'}pA^{3'}$, respectively. Therefore, it is very likely that dimer **A** consists of heterochiral cyclic $pA^{3'}pA^{3'}$ as a major constituent and homochiral cyclic $pA^{2'}pA^{3'}$ as a minor one. It is also plausible that homochiral L,L-cyclic $pA^{2'}pA^{3'}$ was not hydrolyzed and is still observed as dimer **A**.

Figure 8 shows the results of the enzymatic digestion with RNase T_2 and alkaline phosphatase for dimer **B**. Dimer **B** afforded nearly equal amounts of two products corresponding to dimer **B** and 3′-AMP with RNase T_2 treatment. 3′-AMP was converted to adenosine with alkaline phosphatase treatment. Among the cyclic dimers shown in Figure 7, only homochiral D,D-cyclic $pA^{3'}pA^{3'}$ affords 3′-AMP and adenosine with these treatments. Thus, the unreacted constituent is considered to be homochiral L,L-cyclic $pA^{3'}pA^{3'}$.

The percentage of each isomer contained in the dimer fraction is summarized in Table 2. The homochiral dimers are preferentially

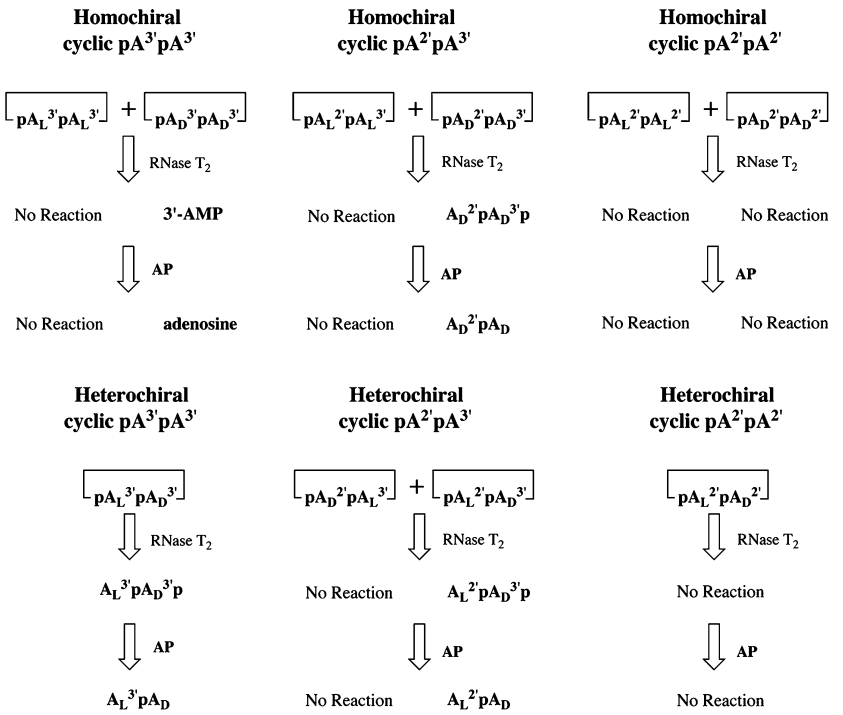

FIGURE 7 Regio-, enantio-, and diastereo-isomers of cyclic adenylyladenosine, and their expected degradation products with RNase T_2 and alkaline phosphatase (**AP**) treatments. Since heterochiral cyclic $pA_L{}^{3'}pA_D{}^{3'}$ and cyclic $pA_L{}^{2'}pA_D{}^{2'}$ are mesomeric, their enantiomers do not exist.

produced (66.0%) relative to the heterochiral ones (33.9%), and the 3′-5′ phosphodiester linkages are more efficiently formed (78.3%) than the 2′-5′ phosphodiester linkages (21.7%). These proportions are basically similar to those reported by Ferris and coworkers,[14] although their experimental

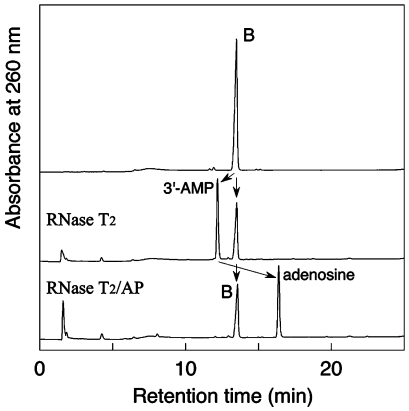

FIGURE 8 Reversed phase HPLC analyses of RNase T_2 followed by alkaline phosphatase (AP) treatment of dimer B.

TABLE 2 Relative yields of dimers formed by montmorillonite-catalyzed oligomerization reaction of racemic ImpA[a]

	Products	Yield (%)
D-D & L-L dimer (homochiral)	$A^{2'}pA$	18.5
	$A^{3'}pA$	9.8
	cyclic $pA^{2'}pA^{3'}$	4.0
	cyclic $pA^{3'}pA^{3'}$	33.7
D-L & L-D dimer (heterochiral)	$A^{2'}pA$	9.9
	$A^{3'}pA$	12.4
	cyclic $pA^{3'}pA^{3'}$	11.6

[a]Yields were estimated based on HPLC peak area of the dimer fraction after alkaline phosphatase treatment (Figure 5c).

conditions are considerably low ImpA concentration (0.15 or 0.6 mM) to suppress the formation of longer oligomers relative to our experimental conditions (14 mM). It should be noted that the regioselectivity for the 3′-5′ phosphodiester linkages is relatively low in our conditions although the homochiral selectivity is very similar in both experimental conditions. The buffer condition and/or ImpA concentration may affect on the regioselectivity of the reaction. The content of the cyclic dimers reaches 49.3% of the total dimer products. This feature of the reaction may lead to the some decrease of the oligomerization efficiency for racemic ImpA on montmorillonite compared with that for D- or L-ImpA. It can be considered that the formation of the cyclic dimers may serve as the inactivating pathway of reactive dimers, or that when large amounts of dimers are accumulated, the formation of cyclic dimers may serve as the storage of the quasi-reactive form of dimers.

CONCLUSION

In conclusion, we have successfully characterized the cyclic dimers as well as linear dimers in the montmorillonite-catalyzed oligomerization products of racemic ImpA. We have reported that the diastereo-isomers of ApA show the different hydrolytic reactivity.[20] The elucidation of the chemical properties of the cyclic dimers and its difference between the isomers would be important to understand the characteristics of clay-catalyzed[12,13] and metal ion-catalyzed[21] oligomerization reactions of racemic mononucleotides.

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