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Abiotic formation of RNA-like oligomers by montmorillonite catalysis: part II

Gözen Ertem[1,4,5], Ann M. Snellinger-O'Brien[2], M. C. Ertem[3], D. A. Rogoff[4], Jason P. Dworkin[5], Murray V. Johnston[2] and Robert M. Hazen[1]

[1]*Geophysical Laboratory and NASA Astrobiology Institute, 5251 Broad Branch Road NW, Washington, DC 20015, USA*
[2]*Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716, USA*
[3]*University Research Foundation, Maryland Advanced Development Laboratory, Greenbelt, MD 20770*
[4]*Space Science Division, NASA Ames Research Center, Moffett Field, CA 94035, USA*
[5]*Astrochemistry Branch, NASA Goddard Space Flight Center, Greenbelt, Maryland 20771, USA*

Abstract: This work is an extension of our previous studies carried out to investigate the possible catalytic role of minerals in the abiotic synthesis of biologically important molecules. In the presence of montmorillonite, a member of the phyllosilicate group minerals that are abundant on Earth and identified on Mars, activated RNA monomers, namely 5′-phosphorimidazolides of nucleosides (ImpNs), undergo condensation reactions in aqueous electrolyte solution producing oligomers with similar structures to short RNA fragments. Analysis of the linear trimer isomers formed in the reaction of a mixture of activated adenosine and cytidine monomers (ImpA and ImpC, respectively) employing high-performance liquid chromatography, selective enzymatic hydrolysis and matrix-assisted laser desorption/ionization mass spectroscopy molecular weight measurements demonstrate that montmorillonite catalysis facilitates the formation of hetero-isomers containing 56% A- and 44% C-monomer incorporated in their structure. The results also show that 56% of the monomer units are linked together by RNA-like 3′,5′-phosphodiester bonds. These results follow the same trend observed in our most recent work studying the reaction of activated adenosine and uridine monomers, and support Bernal's hypothesis proposing the possible catalytic role of minerals in the abiotic processes in the course of chemical evolution.

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Key words: chemical evolution, HPLC, MALDI-MS, mineral catalysis, montmorillonite, oligonucleotides, RNA.

Introduction

In his well-known lecture delivered in 1947, John Bernal proposed that minerals may have served as catalysts for the formation of bio-oligomers at the early stages of chemical evolution by adsorbing the monomers on their surfaces, thereby bringing them into favourable orientation to react with each other (Bernal 1949). He also suggested that adsorption of these molecules on mineral surfaces would protect them from the harmful effects of UV radiation. Our model studies designed to investigate the possible role of mineral catalysis in the abiotic synthesis of bio-oligomers show that the clay mineral montmorillonite serves as an effective catalyst in aqueous electrolyte solution to link activated monomer units together producing RNA-like oligomers[1] (see reviews by Ertem (2004), Ferris (2006) and Shapiro (2006)). Our most recent studies demonstrate that preferential formation of hetero-oligomers observed in the dimer isomers (Ertem & Ferris 2000) is preserved in their elongation products of trimer fractions formed in the montmorillonite catalysed reaction of ImpA with ImpU (Ertem *et al*. 2007). This finding is a significant step in the right direction, because our eventual objective is to form RNA-like oligomers. Here in Part II, we extended these studies to investigate the regio- and sequence selectivity of trimer isomers formed in the montmorillonite-catalysed reaction of ImpA with ImpC.

Montmorillonite, which is used as a catalyst in our research, belongs to the phyllosilicate group minerals, which form large deposits on Earth. Bibring *et al*. (2005) have identified phyllosilicates on Mars by OMEGA, a visible–near infrared hyperspectral imager. The structural formula of the particular montmorillonite used in our studies is derived from its elemental analysis results (Brindley & Ertem 1971) following Ross & Hendricks model (1945), and is depicted as follows:

$$\underbrace{[Si_{3.89}\ Al_{0.11}]^{IV}}_{\text{Tetrahedral sheet}}\ \underbrace{[Al_{1.57}\ Fe_{0.17}{}^{3+}\ Fe_{0.02}{}^{2+}\ Mg_{0.27}]^{VI}}_{\text{Octahedral sheet}}\ O_{10}\ (OH)_2\ Na_{0.40}$$

In this formula, $[\ldots]^{IV}$ denotes tetrahedrally coordinated and $[\ldots]^{VI}$ denotes octahedrally coordinated cations.

[1] We use the term oligomer to refer to any product that is produced in the condensation of mononucleotides by the formation of a phosphodiester bond, and can be as short as two monomer units long.

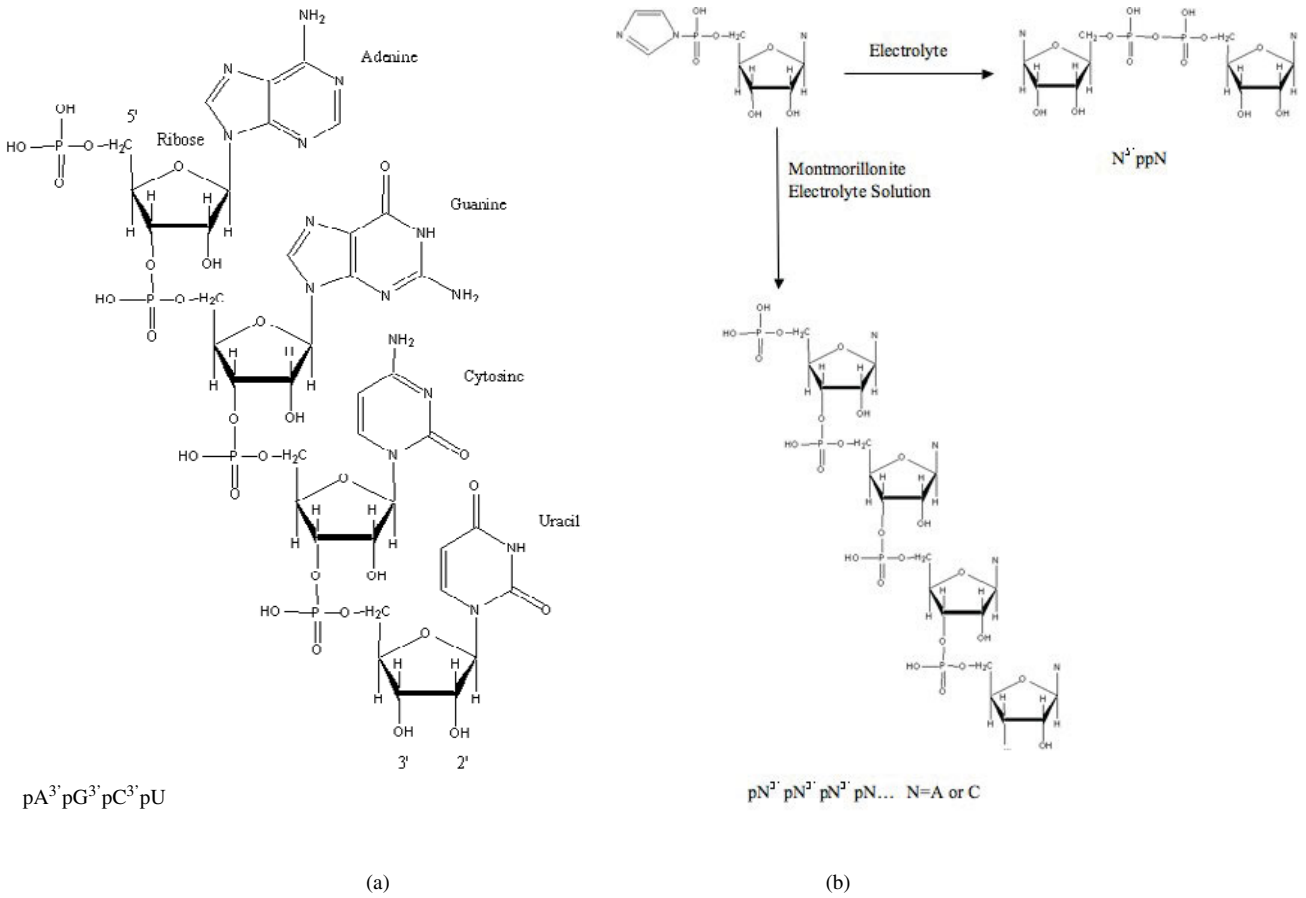

Fig. 1. (a) A short segment of an RNA strand and conventional short-hand notation showing its sequence. (b) A short segment of a synthetic RNA-like oligonucleotide formed in the reaction of activated monomers by montmorillonite catalysis. N represents any nucleobase, in this case adenine (A) or cytidine (C).

Montmorillonite owes its catalytic activity to the isomorphic substitutions mainly occurring in the octahedral sheet, as explained in detail in a previous work (Ertem 2004, pp. 551–552). Pyrophillite, which is another clay mineral with a similar structure to that of montmorillonite but has no substitutions, offers no catalytic activity:

$$\underset{\text{Tetrahedral sheet}}{[Si_{4.0}]^{IV}} \quad \underset{\text{Octahedral sheet}}{[Al_{2.0}]^{VI}} \quad O_{10}(OH)_2$$

Studies designed to establish the site of catalysis, i.e. whether the reaction of activated monomers take place at the edge sites or on the basal surfaces of montmorillonite by selectively blocking these sites prior to running the reactions, showed that the main oligomerization occurs on the basal surfaces of montmorillonite (Ertem & Ferris 1998).

Figures 1(a) and (b) show the structures of short segments of RNA and the synthetic oligomer chain formed in the montmorillonite catalysed reaction of ImpA with ImpC, respectively. In present-day RNA, all of the internucleotide linkages are of the 3′,5′-type, whereas the synthetic oligomer chains formed in the ImpA–ImpC reaction contain both 3′,5′- and 2′,5′-phosphodiester bonds.

Materials and methods

Volclay SPV-200 was a gift from American Colloid Company, Arlington Heights, IL. Polyadenylic-cytidylic acid (Poly[A, C]) was purchased from SIGMA. High-performance liquid chromatography (HPLC) grade solvents, enzymes and chemicals were purchased from the same sources listed previously (Ferris and Ertem 1993; Ertem *et al.* 2007).

The Na-form of the montmorillonite from Volclay was prepared according to Banin *et al.* (1985), and removal of the organics was achieved by hydrogen peroxide treatment (Ferris *et al.* 1989). The activated mononucleotides ImpA and ImpC were prepared as described by Ertem *et al.* (2007) and references therein.

HPLC analysis was performed on an Agilent 1050 instrument. A C-18 reverse-phase column was purchased from Alltech. A Biosphere GMB 1000 Q anion exchange column was obtained from Puresyn, Inc., Malvern, PA. The mobile phase composition and the gradient program were as described by Ertem and Ferris (2000). The C-18 ZipTip was from Millipore. Matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS) data were obtained on a BRUKER OmniFlex-NT at the University of Delaware.

Table 1. *Products obtained by RNase T_2 hydrolysis followed by APH hydrolysis of each trimer fraction formed in the partial hydrolysis of Poly[A,C]*

Starting isomer	RNase T_2 hydrolysis product	APH hydrolysis product
$A^{3'}pA^{3'}pA$	$A^{3'}p + A^{3'}p + A$	A + A + A
$A^{3'}pA^{3'}pC$	$A^{3'}p + A^{3'}p + C$	A + A + C
$A^{3'}pC^{3'}pC$	$A^{3'}p + C^{3'}p + C$	A + C + C
$C^{3'}pC^{3'}pA$	$C^{3'}p + C^{3'}p + A$	C + C + A
$C^{3'}pA^{3'}pA$	$C^{3'}p + A^{3'}p + A$	C + A + A
$A^{3'}pC^{3'}pA$	$A^{3'}p + C^{3'}p + A$	A + C + A
$C^{3'}pA^{3'}pC$	$C^{3'}p + A^{3'}p + C$	C + A + C
$C^{3'}pC^{3'}pC$	$C^{3'}p + C^{3'}p + C$	C + C + C

A detailed account of the experimental procedure was given by Ertem *et al.* (2007).

Preparation of standards

The homo-trimers $N^{2'}pN^{2'}pN$, $N^{3'}pN^{2'}pN$, $N^{3'}pN^{3'}pN$ were prepared from the momontmorillonite catalysed self-condensation reactions of ImpA and ImpC.

Hetero-trimers $A^{3'}pA^{3'}pC$, $A^{3'}pC^{3'}pC$, $C^{3'}pC^{3'}pA$, $C^{3'}pA^{3'}pA$, $A^{3'}pC^{3'}pA$ and $C^{3'}pA^{3'}pC$ were isolated from the partial base hydrolysis products of Poly[A,C]: 1 mg of Poly[A,C] was dissolved in 1 mL of 0.1 M NaOH and hydrolysed for 40–50 min at 70 °C to produce $(N^{3'}p)_n$ type oligomers (Lohrmann *et al.* 1980). As the distribution of A- and C-monomers in Poly[A,C] strands is random, the trimer fraction isolated from its partial hydrolysis products using anion exchange column contains all six of the hetero-isomers along with $A^{3'}pA^{3'}pA^{3'}p$ and $C^{3'}pC^{3'}pC^{3'}p$. The isolation of the $N^{3'}pN^{3'}pN$ mixture from the trimer fraction and the identification of each isomer, where N = A or C, were carried out according to Ertem *et al.* (2007). The trimer fraction was first digested with alkaline phosphatase (APH) to hydrolyse the 3′-phosphate groupings. The $N^{3'}pN^{3'}pN$ mixture thus formed was first hydrolysed with RNase T_2, followed by APH enzymes. Hydrolysis products were identified by coinjecting them with authentic standards on a reverse-phase column. Table 1 lists the hydrolysis products of each isomer.

By coinjecting these hydrolysis products with authentic standards, the retention time of each isomer was established. Elution of the 2′-linked analogues of these isomers, which are not commercially available, has been assumed to follow the same order but with shorter retention times (Ertem & Ferris 2000).

Reactions

Montmorillonite catalysed ImpA–ImpC reaction was prepared in 0.1 M HEPES, 0.2 M NaCl and 0.075 M $MgCl_2$, at pH 8 in solution to contain 50 mg of Na-montmorillonite/mL, and each activated monomer at a final concentration of 0.014 M (Ertem & Ferris 2000), allowed to stand at 25 °C for 7 days, centrifuged and the supernatant was removed. The oligonucleotides adsorbed on the montmorillonite were washed with 0.5 mL of 0.5 M ammonium acetate solution, the mixture was centrifuged and the wash was combined with the supernatant. The pH of the combined supernatant and wash was adjusted to 4–5 with the addition of 10% $HClO_4$ to hydrolyse the 5′-imidazolide groupings (Schwartz & Orgel 1985). Then 40 μL of the reaction mixture was injected onto an anion exchange column, trimer fractions were collected and stored at −20 °C until use. A number of such injections were made to obtain sufficient amounts of the trimer fraction. Linear isomers with the general formula of NpNpN, where N = A or C, were isolated from the cyclic isomers and pyrophosphate derivatives with the same procedure as described by Ertem *et al.* (2007). The reverse-phase HPLC elution profile of linear trimer isomers is shown in Fig. 2.

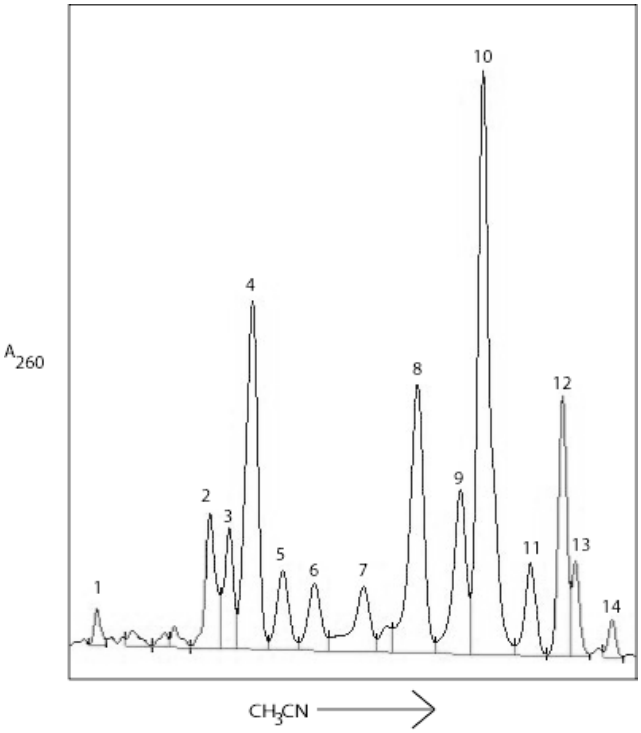

Fig. 2. Reverse-phase HPLC elution profile of linear trimer isomers formed in the montmorillonite catalysed reaction of ImpA with ImpC. Alltima C-18, 5 μ, 4 × 250 mm reverse-phase column. Mobile phase: Buffer A, 0.02 M NaH_2PO_4 in 0.2% w/v trifluoroacetic acid solution at pH 2.5; Buffer B, 0.2% trifluoroacetic acid, pH 2.5 in 30% acetonitrile v/v. Flow rate: 1.0 mL min^{-1}. Gradient: initial time to 10 min, Buffer B 0–15%; 10–14 min, Buffer B 15%; 14–29 min, Buffer B 15–30%; 29–33 min, Buffer B 30–40% (HPLC conditions: Kanavarioti (1997)). Due to their close retention times, fractions (2 and 3), (4, 5 and 6), (7 and 8), (9 and 10) and (11, 12 and 13) are collected together. They produce different hydrolysis products.

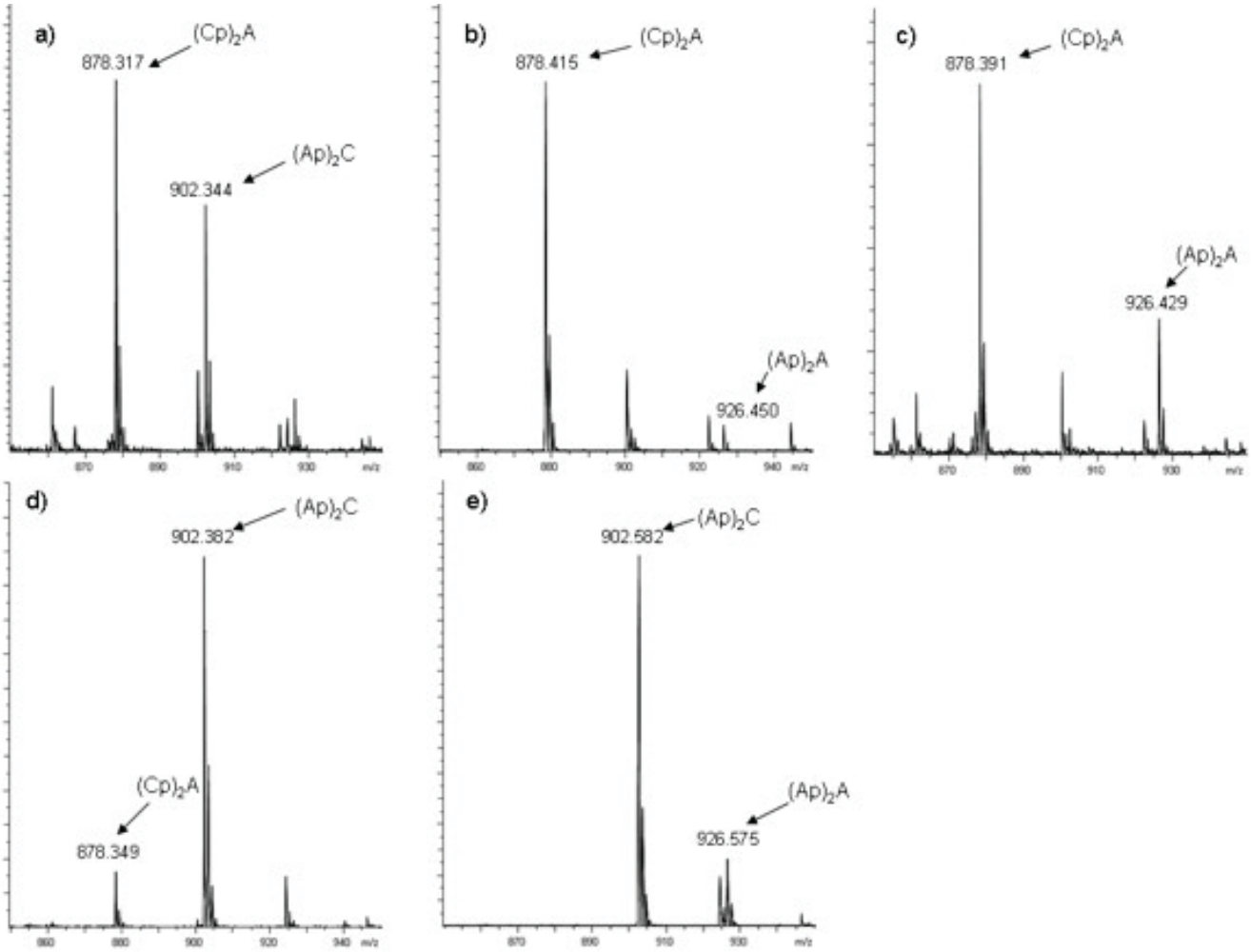

Fig. 3. MALDI spectra of trimer fractions formed in the montmorillonite catalysed reaction of ImpA with ImpC: (a) fractions 2 and 3; (b) fractions 4, 5 and 6; (c) fractions 7 and 8; (d) fractions 9 and 10; and (e) fractions 11, 12 and 13.

Identification of trimer isomers

Enzymatic hydrolysis

Fractions were collected from the reverse-phase column, either separately or two at a time depending on the difference between their retention times, as shown in Fig. 2, digested first with RNase T_2 followed by APH as described in detail previously (Ertem *et al.* 2007, Figure 3). Identification of enzymatic hydrolysis products was achieved by coinjecting them with authentic standards.

Molecular weight identification by MALDI-MS

Sequence information thus obtained was further confirmed by MALDI-MS molecular weight measurements. First, isomers were collected from the reverse-phase column. Following dialysis, freeze-drying and dissolution in water, each mixture was passed through a C-18 ZipTip and spotted on a MALDI target with α-cyano-4-hydroxycinnamic acid. The matrix concentration was 10 mg mL^{-1} in a mixture containing 70% acetonitrile and 0.1% triflouroacetic acid in water. MALDI spectra were collected in positive ion mode using reflectron detection. The resulting spectra had baseline isotopic resolution. Figure 3 shows the MALDI spectra of trimer fractions formed in the montmorillonite catalysed ImpA–ImpC reaction.

Since the spectra were obtained in positive ion mode, the peaks detected represent the $(M+H)^+$ of the analyte, i.e. the molecular mass of the analyte plus a hydrogen ion. Represented in Fig. 3 are partial spectra in the region of 850–950 m/z, which is the region where ImpA and ImpC trimers would be found. For example, the spectra in Fig. 3(a) displays the presence of $(Cp)_2A$ at 878.317 and $(Ap)_2C$ at 902.344. There is no peak at 854.2, which would be indicative of $(Cp)_2C$. The other peaks in the spectra are salt adducts and other contaminants that are not representative of the presence of additional oligomers. The spectra in Fig. 3(b)–(e) were interpreted in the same fashion. It is important to note that although MALDI-MS data provides compositional identification of the trimers, it does not allow for sequential identification. The $(Ap)_2C$ isomers (ApApC, ApCpA, CpApA) and the $(Cp)_2A$ isomers (CpCpA, CpApC and ApCpC) are isobaric and are not distinguishable in

Table 2. *Sequences and enzymatic hydrolysis products of linear trimer isomers formed in the montmorillonite catalysed ImpA–ImpC reaction. Yields are calculated as the area ratio of each peak to the total area on the chromatogram from the average of two measurements. No correction was made for hyperchromicity or molar extinction coefficients*

Peak #	% Yield	Isomer	RNase T_2 products	APH products
1	1.2	$C^{2'}pC^{2'}pC$	$C^{2'}pC^{2'}pC$	$C^{2'}pC^{2'}pC$
2	1.1 and/or	$C^{2'}pA^{2'}pC$ $C^{2'}pC^{2'}pA$	$C^{2'}pA^{2'}pC$ $C^{2'}pC^{2'}pA$	$C^{2'}pA^{2'}pC$ $C^{2'}pC^{2'}pA$
	3.1	$A^{2'}pC^{2'}pC$	$A^{2'}pC^{2'}pC$	$A^{2'}pC^{2'}pC$
3	3.4	$A^{2'}pA^{2'}pC$	$A^{2'}pA^{2'}pC$	$A^{2'}pA^{2'}pC$
4	6.7 5.6	$C^{3'}pA^{2'}pC$ $A^{2'}pC^{3'}pC$	$C^{3'}p + A^{2'}pC$ $A^{2'}pC^{3'}p + C$	$C + A^{2'}pC$ $A^{2'}pC + C$
5	2.7	$A^{2'}pA^{2'}pA$	$A^{2'}pA^{2'}pA$	$A^{2'}pA^{2'}pA$
6	2.5	$C^{3'}pA^{3'}pC$	$C^{3'}p+A^{3'}p + C$	$C + A + C$
7	2.8	$A^{2'}pA^{3'}pA$	$A^{2'}pA^{3'}p + A$	$A^{2'}pA + A$
8	10	$A^{3'}pC^{2'}pC$	$A^{3'}p + C^{2'}pC$	$A + C^{2'}pC$
9	4.4	$A^{3'}pC^{3'}pC$	$A^{3'}p + C^{3'}p + C$	$A + C + C$
10	26	$A^{3'}pA^{2'}pC$	$A^{3'}p + A^{2'}pC$	$A + A^{2'}pC$
11	3.9	$A^{3'}pC^{3'}pA$	$A^{3'}p + C^{3'}p + A$	$A + C + A$
12	9.5	$A^{3'}pA^{3'}pC$	$A^{3'}p + A^{3'}p + C$	$A + A + C$
13	1.5	$A^{3'}pA^{2'}pA$	$A^{3'}p + A^{2'}pA$	$A + A^{2'}pA$
14	1.3	$A^{3'}pA^{3'}pA$	$A^{3'}p + A^{3'}p + A$	$A + A + A$
	14	Other isomers		

MALDI-MS. Information obtained by MALDI-MS analysis was combined with selective enzymatic hydrolysis data to validate the sequences of the trimer isomers.

Results are listed in Table 2.

Results and discussion

The highest number of isomers that can be formed in the ImpA–ImpC reaction, i.e., a binary monomer mixture, is $2^4 = 32$: two types of monomer and four possibilities for bond formation, namely, $pN^{2'}pN^{2'}pN$, $pN^{2'}pN^{3'}pN$, $pN^{3'}pN^{2'}pN$ and $pN^{3'}pN^{3'}pN$, where N = A or C. We have been able to identify the sequences of 16 isomers, which constitute 86 % of the linear isomers (Table 3). 89 % of these linear trimers are hetero-isomers and only 11 % are in the form of the eight possible homo-trimers. As the random formation of isomers, which would be the case in the absence of a catalyst, would result in 25 % homo-isomers and 75 % hetero-isomers, these results clearly demonstrate the effect of mineral catalysis. Overall incorporation of A- and C-monomers into the linear trimer isomers are 56 % and 44 % for A and C, respectively, based on their yields calculated from the normalized area ratios on the chromatogram. We found that 56 % of the monomer units are joined together via RNA-like 3′,5′-phosphodiester bonds (Table 3).

Table 3. *Analysis results of linear trimer isomers formed in the montmorillonite catalysed reaction of ImpC with ImpA. Yields are calculated from the average of two measurements*

Peak number	Isomer	Area (%)	Normalized area (%)	3′,5′-linkages (%)	Monomer incorporation %A	%C
1	$C^{2'}pC^{2'}pC$	1.2	1.4	0	0	1.4
2	$C^{2'}pA^{2'}pC$ and/or $C^{2'}pC^{2'}pA$ $A^{2'}pC^{2'}pC$	4.2	4.9	0	1.6	3.3
3	$A^{2'}pA^{2'}pC$	3.4	3.9	0	2.6	1.3
4	$C^{3'}pA^{2'}pC$ and	6.7	7.8	3.9	2.6	5.2
	$A^{2'}pC^{3'}pC$	5.6	6.5	3.3	2.2	4.3
5	$A^{2'}pA^{2'}pA$	2.7	3.1	0	3.1	0
6	$C^{3'}pA^{3'}pC$	2.5	2.9	2.9	1.0	2.0
7	$A^{2'}pA^{3'}pA$	2.8	3.3	1.7	3.3	0
8	$A^{3'}pC^{2'}pC$	10	12	6.0	4.0	8.0
9	$A^{3'}pC^{3'}pC$	4.4	5.1	5.1	1.7	3.4
10	$A^{3'}pA^{2'}pC$	26	30	15	20	10
11	$A^{3'}pC^{3'}pA$	3.9	4.5	4.5	3.0	1.5
12	$A^{3'}pA^{3'}pC$	9.5	11	11	7.3	3.7
13	$A^{3'}pA^{2'}pA$	1.5	1.7	0.9	1.7	0
14	$A^{3'}pA^{3'}pA$	1.3	1.5	1.5	1.5	0
Total % of identified isomers		86				
Total % of unidentified isomers		14				
Normalized values for identified isomers			100	56	56	44

Sequences of trimer, tetramer and pentamer fractions formed in the reaction of ImpA and ImpC by montmorillonite catalysis have been reported by Miyakawa & Ferris (2003). We failed to obtain reliable results for the sequences of oligomers longer than trimers due to the low yields of tetramer and pentamer fractions with large numbers of isomers, a suitable column to resolve these isomers and, more importantly, the absence of authentic standards. The yields of the dimer, trimer, tetramer and pentamer fractions in our reaction are 21 %, 9.5 %, 3.0 % and 1.1 %, respectively, based on the area ratios of peak of interest to the total area on the chromatogram. Linear isomers constitute 89 % of the dimer and 67 % of the trimer fractions.

One of the main challenges in studies of abiotic synthesis of bio-oligomers is the formation of phosphodiester bond in oligonucleotides, and peptide bond in proteins, under plausible prebiotic conditions. In one primitive Earth scenario, polymerization reactions would have had to take place in dilute aqueous solutions (Ferris & Usher 1988). The chemical synthesis of oligonucleotides in the laboratory has to be carried out under strictly anhydrous conditions (Sonveaux 1986). Our studies demonstrate that montmorillonite facilitates the formation of phosphodiester bond in dilute aqueous solutions by adsorbing the monomers on its basal surfaces,

therefore bringing them into close proximity of each other. Furthermore, it may orient them into a favourable position for intermolecular reactions to occur.

Reaction of ImpN to form oligonucleotides is initiated by protonation of imidazole grouping, which has a pK_a of 6.0 (Kanavarioti *et al.* 1989). Interlayer region of montmorillonite is acidic due to the polarization of water molecules in the hydration shell of interlayer cations (Ertem 2000, pp. 561–562). For highly electropositive cations such as Li^+ and Ca^{2+}, this polarization becomes more prominent and the local environment around the cation becomes more acidic. As a result, the extent of catalytic activity of montmorillonite increases with the electropositivity of the interlayer cation (Ferris & Ertem 1993).

Conclusions

The main objective of our research is to synthesize hetero-oligomers with sequence and regio-specificity similar in structure to RNA strands using minerals as catalyst. Analysis of trimer fractions formed in the reaction of ImpA and ImpC mixture demonstrates that sequence and regio-specificity observed in the dimer fractions are conserved in their elongation products and montmorillonite catalysis facilitates the formation of hetero-oligomers with sequence selectivity. We have been able to determine the sequences of 86% of the linear trimers: 89% of them are hetero-isomers containing 56% A-monomer and 44% C-monomer units. The ratio of 3′,5′-linkages, 49% observed for the dimer isomers, increases to 56% in trimer isomers, and the ratio of hetero-isomers, which was 73% for dimer isomers, increases to 89% in trimer isomers (see table I in Ertem & Ferris 2000). The same trend was also observed in the analysis of trimers formed in the ImpA–ImpU reaction (Ertem *et al.* 2007). Although, there seems to be a tendency to favour the formation of limited number of isomers (Ertem & Ferris 2000; Miyakawa & Ferris 2003), the ideal case would be to form as many isomers as possible, in this case 32 isomers, that would offer a richer pool for the selection of 'useful, functional' isomers in the course of evolution (Ertem *et al.* 2007). Therefore, our model studies demonstrating the 89% hetero-isomer formation is very significant for the abiotic synthesis of bio-molecules in the processes leading to the origin of life.

Abbreviations

RNA, ribonucleic acid; HPLC, high-performance liquid chromatography; MALDI-MS: matrix-assisted laser desorption/ionization-mass spectrometry; ImpN, 5′-phosphorimidazolide of nucleoside; ImpA, 5′-phosphorimidazolide of adenosine; ImpC, 5′-phosphorimidazolide of cytidine; ImpU, 5′-phosphorimidazolide of uridine; $pA^{2'}pC$, adenylyl(2′,5′) cytidine: an A–C dimer, where two monomer units are joined together by a phosphodiester bond formed between the 2′-OH group of 5′-adenosine monophosphate and the 5′-phosphate group of cytidine monophosphate; $pC^{3'}pA$, cytidyl(3′,5′)adenosine: an C–A dimer, where two monomer units are joined together by a phosphodiester bond formed between the 3′-OH group of cytidine monophosphate and the 5′-phosphate group of adenosine monophosphate; Poly[A,C], Poly[adenylic,cytidylic] acid; APH, alkaline phosphatase enzyme; RNase T_2, ribonuclease T_2 enzyme; HEPES, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid).

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