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CATALYSIS AND SELECTIVITY IN PREBIOTIC SYNTHESIS: INITIATION OF THE FORMATION OF OLIGO(U)S ON MONTMORILLONITE CLAY BY ADENOSINE-5′-METHYLPHOSPHATE

KONG-JIANG WANG and JAMES P. FERRIS*
Department of Chemistry and the NY Center for Studies on the Origins of Life, Rensselaer Polytechnic Institute, Troy, NY 12180, U.S.A.
*(*author for correspondence, e-mail: ferrij@rpi.edu)*

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Abstract. Adenosine-5′-methylphosphate (MepA) initiates the oligomerization of the 5′-phosphorimidazolide of uridine (ImpU) in the presence of montmorillonite clay. Longer oligomers form because the 5′-phosphate is blocked with a methyl group that prevents the formation of cyclic- and pyrophosphate-containing compounds. The MepA initiates 69–84% of the 5–9 charge oligomers, respectively. The montmorillonite catalyst also provides selectivity in the oligomerization reactions so that the main reaction pathway is MepA → MepA$^{3'}$pU → MepA$^{3'}$pU$^{2'}$pU → MepA$^{3'}$pU$^{2'}$pU$^{3'}$pU. MepA did not enhance the oligomerization of ImpA. The relative rates of the reactions were determined from an investigation of the products in competitive reactions. Selectivity was observed in the reaction of ImpU with equimolar amounts of MepA$^{3'}$pU and MepA$^{2'}$pU where the relative reaction rates are 10.3:1, respectively. In the reaction of ImpA with MepA$^{3'}$pA and MepA$^{2'}$pA the ImpA reacts 5.2 times faster with MepA$^{3'}$pA. In the competitive reaction of ImpU and ImpA with MepA$^{3'}$pA and MepA$^{3'}$pU the elongation proceeds on MepA$^{3'}$pA 5.6 times more rapidly than with MepA$^{3'}$pU. There is no correlation between the extent of binding to the montmorillonite and reaction rates in the formation of longer oligomers. The formation of more than two sequential 2′,5′-linkages in the oligomer chain proceeds more slowly than the addition to a single 2′,5′-link or a 3′,5′-link and either chain termination or elongation by a 3′,5′-linage occurs. The central role that catalysis may have had in the prebiotic formation of biopolymers is discussed.

Keywords: adenosine-5′-methylphosphate initiation, catalysis, montmorillonite, oligo (U), RNA formation, selectivity

1. Introduction

The montmorillonite-catalyzed reaction of activated mononucleotides in aqueous solution yields polynucleotides containing oligomers longer than 40 mers (Ferris, 2002) (Huang and Ferris, 2003). It is a general reaction that occurs with the 5′-activated phosphates of adenosine, guanosine, uridine, cytidine and inosine (Ferris and Ertem, 1993; Ding *et al.*, 1996; Ertem and Ferris, 1997; Kawamura and Ferris,

Note added in proof: There are errors in the high resolution mass spectral data given in Section 4.2.1. The high resolution mass spectrum found for the cyclic dimer of UpUp (C-UpUp) was 657.02260. $C_{18}H_{21}N_4O_{16}P_2Na_2$ requires 657.02232. The high resolution mass spectrum found for the cyclic dimer of ApAp (C-ApAp) was 725.05850. $C_{20}H_{22}N_{10}O_{12}P_2Na_3$ requires 725.05839.

1999). Studies on the reaction pathway suggest that it proceeds at proximate sites in the interlayer between the clay platelets (Ertem and Ferris, 1998; Wang and Ferris, 2001) and binding studies demonstrate that purine nucleotides adhere more strongly to the montmorillonite than do pyrimidine nucleotides. Hydrophobic (van der Waals) interactions between the purine rings and the silicate layer are consistent with the observed binding data where the nucleotides form a monolayer on the silicate surfaces (Kawamura and Ferris, 1999) (Ertem and Ferris, 2000, Wang and Ferris, 2001). Selectivity was observed in the reaction of a mixture of the 5′-activated nucleotides of adenosine and cytidine and rules for the relative reactivity's of the activated nucleotides of purines and pyrimidines were derived from this study (Miyakawa and Ferris, 2003).

In the process of investigating the inhibition of the montmorillonite-catalyzed formation of RNA oligomers it was observed that MepA enhanced the formation of longer oligomers when reacted with ImpU. This present research reports the investigation of the pathway by which these longer oligomers formed.

2. Results and Discussion

2.1. Reaction of MepA with ImpU

It was observed in this study that a mixture of adenosine-5′-methylphosphate (MepA) (Figure 1a) (Wang and Ferris, 2001) with 15 mM ImpU (Figure 1b) gave longer oligomers than ImpU alone. (Table I) (Figure 2). The peak widths of the oligomers in the 5–11 mer fractions in the MepA-ImpU reaction are narrower than those in the reaction of ImpU alone indicative of diminished heterogeneity in the oligomers present in each fraction. This is consistent with the formation of fewer isomers due to the absence of $A^{5'}$ppA groups in the oligomers. A detailed investigation of the structure of the products formed was undertaken to determine other effect of MepA on the reactions of activated RNA monomers on montmorillonite clay.

Structure analysis revealed that the formations of the majority of oligomers in fractions 5–9 (Figure 2B) were initiated by MepA. Each peak was collected from the ion exchange HPLC column (Figure 2) and was rechromatographed on

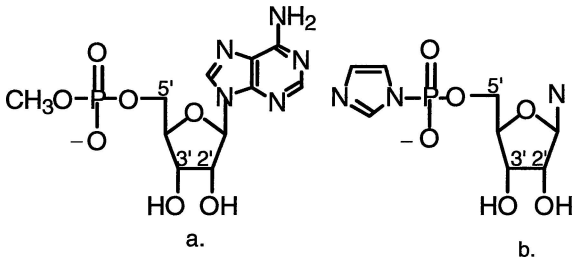

Figure 1. (a) MepA, (b) ImpA, N = A; ImpU, N = U.

TABLE I

Yields (%) of the oligomers in the reactions described[a,b,c]

	0	−1	−2	−3	−4	−5	−6	−7	−8	−9	−10	−11	12
15 ImpU	7.4	–	76.8	7.12	2.76	1.44	0.73	0.33	0.15	0.07	+	–	–
15 ImpU + 15 MepA	1.31	33.3	57.9	6.36	0.98	0.14	+	–	–	–	–	–	–
15 ImpU + 1.5 MepA	3.54	23.4	45.5	8.82	6.15	3.35	2.05	1.41	0.87	0.49	0.29	0.14	+
ImpA	8.55	–	37.8	27.5	13.1	7.02	2.67	1.35	0.60	0.24	0.07	+	
15 ImpA + 15 MepA	1.56	40.6	35.5	14.1	5.97	1.65	0.43	+	–	–	–	–	–
15 ImpA + 1.5 MepA	1.23	13.9	32.7	24.6	15.2	7.48	3.34	1.19	0.34	+	+	–	–
7.5 MepA$^{3'}$pA + 7.5 ImpA	0.37	35.1	59.9	4.60	0.03	–	–	–	–	–	–	–	–
7.5 MepA$^{2'}$pA + 7.5 ImpA	1.00	29.8	61.5	7.18	0.52	–	–	–	–	–	–	–	–
3.75MepA$^{3'}$pA + 3.75 MepA$^{2'}$pA + 7.5 ImpA	0.53	26.4	69.3	4.54	0.23	–	–	–	–	–	–	–	–
7.5 MepA$^{3'}$pU + 7.5 ImpU	1.66	25.8	68.2	4.01	0.23	–	–	–	–	–	–	–	–
7.5 MepA$^{2'}$pU + 7.5 ImpU	1.38	26.1	66.4	5.65	0.37	–	–	–	–	–	–	–	–
3.75 MepA$^{3'}$pU + 3.75 MepA$^{2'}$pU + 7.5 ImpU	1.46	23.9	69.7	4.92	0.24	–	–	–	–	–	–	–	–
3.75 ImpA + 3.75 ImpU	2.48	28.0	18.3	45.4	5.45	0.43	+	+	–	–	–	–	–
7.5 MepA + 3.75 ImpA + 3.75 ImpU	0.65	55.6	36.3	5.91	1.62	+	+	–	–	–	–	–	–
7.5 MepA$^{3'}$pA + 3.75 ImpA + 3.75 ImpU	2.18	27.8	65.1	4.89	+	–	–	–	–	–	–	–	–
7.5 MepA$^{2'}$pA + 3.75 ImpA + 3.75 ImpU	2.15	21.2	71.2	5.41	+	–	–	–	–	–	–	–	–
7.5MepA$^{3'}$pU + 3.75 ImpA + 3.75 ImpU	2.60	24.5	68.3	4.56	+	–	–	–	–	–	–	–	–
7.5MepA$^{2'}$pU + 3.75 ImpA + 3.75 ImpU	2.71	20.2	68.4	8.70	+	–	–	–	–	–	–	–	–
1.5 mM MepA$^{3'}$pU + 15 mM ImpU	–	6.89	65.9	13.7	5.60	4.08	1.99	0.87	0.34	0.16	0.06	–	–
1.5 mM MepA$^{2'}$pU + 15 mM ImpU	–	3.65	79.1	9.95	3.61	2.17	0.95	0.38	0.10	–	–	–	–

The number at the top of each column gives 'a' the number of negative charges in the oligomers eluted from the ion exchange HPLC. The number charges rather than the number of monomers units is used because the pA-initiated oligomers that have the same number of monomer units as the MepA-initiated oligomers have an additional negative charge. Reactant concentrations are millimolar.

[b]Percent of each fraction is the ratio of the UV absorbance of that fraction to the total UV absorbance of all components in the reaction mixture.

[c]"+" Designates a peak in the HPLC trace that was too small to be integrated.

a reverse phase column. The principal components of the four-charge and longer fractions were observed to be in a cluster of more intense peaks with the longer retention times on the reverse phase column. The extent of the MepA-initiation of these oligomers was determined by phosphodiesterase I (PD I) hydrolysis which cleaves 3′,5′-; 2′,5′- and 5′,5′-phosphodiester bonds (Richards *et al.*, 1967; Itkes

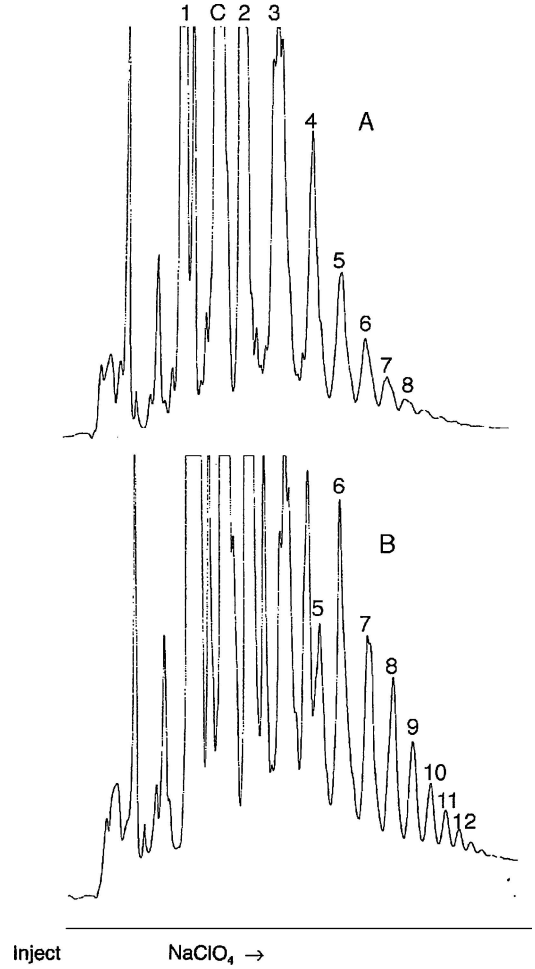

Figure 2. Ion exchange HPLC traces of reactions on montmorillonite where the number of monomers in each peak is designated. (A) 15 mM ImpU; (B) 1.5 mM MepA + 15 mM ImpU. (B) Mainly pA-initiated oligomers are present in fractions 2 and 3. The HPLC peaks for MepA$(pU)_4$ and the longer oligomers initiated by MepA are designated by numbers. The area under HPLC trace B is greater than that under A because of the greater UV absorption of MepA at 260 nm (15%) in the MepA-initiated reaction.

et al., 1988). PD I also cleaves the methyl grouping from MepA to give adenosine and 5′-AMP but at a slower rate than it cleaves the phosphodiester bond between nucleotides. Alkaline phosphatase (APH) hydrolysis of the cleavage products of the MepAp, Up and AMP generated by PD I cleavage yielded MepA, U and adenosine. The amount of MepA incorporation into the longer oligomers was calculated from the percentage of MepA, A and U formed as determined by reverse phase HPLC (Figure 3). The oligomers to which MepA was bound by a 3′,5′-link were determined by RNase T_2 and the subsequent APH hydrolyses to cleave the

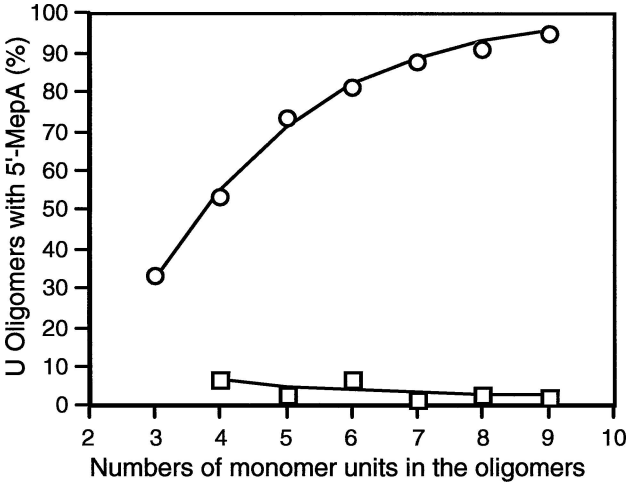

Figure 3. Percent of the oligo(U)s containing MepA groups in the reaction of 1.5 mM MepA with 15 mM ImpU. (○) Oligomers where the MepA is bound to the end of oligomers by a 3′,5′-phosphodiester bond and (□) where the MepA is linked by a 2′,5′-phosphodiester bond.

3′-phosphate groupings. The oligomers with unreacted 5′-MepA$^{2'}$pU groups were collected from an ion exchange HPLC and subjected to further hydrolysis with PD I followed by APH hydrolysis. The percent MepA linked by 2′,5′-bonds was determined from the sum of the peak areas of MepA and adenosine from the reverse phase chromatogram (Figure 3). Only about 5% of the longer oligomers have MepA linked by a 2′,5′-phosphodiester bond.

The formation of mainly oligomers with a terminal 5′-MepA$^{3'}$pU grouping was confirmed by detailed structural analysis of the longer oligomers present in the two-charge to four-charge (dimer to tetramer) fractions (Figure 4). The proportion of oligomers with a 5′-MepA$^{3'}$pU group increases in the order 15–24 to 38% going from the two charge to four-charge fractions. The reverse phase HPLC determination of the peak areas of the MepA-initiated oligomers of the five- through nine-charge fractions indicated they increased from 69 to 84% of the reaction products. This study and studies described below revealed that MepA$^{3'}$pU elongates much more rapidly than MepA$^{2'}$pU as shown by the low yields of MepA$^{2'}$pU-initiated oligomers as reaction products (Figure 4).

Shorter oligomer chains are formed in the reaction between 15 mM amounts of MepA and ImpU than with 1.5 mM MepA and 15 mM ImpU (Table I). The lower yields of longer oligomers demonstrates that the reaction between ImpU and MepA is faster than the reaction of ImpU with the U groups on the 3′-end of the oligomers. Structure analysis of the reaction products revealed that the relative amounts of the dimers, trimers and tetramers with a 5′-MepA group in the 1:1 MepA–ImpU mixture is comparable to that observed in the reaction of 1.5 mM MepA with ImpU (Figure 5).

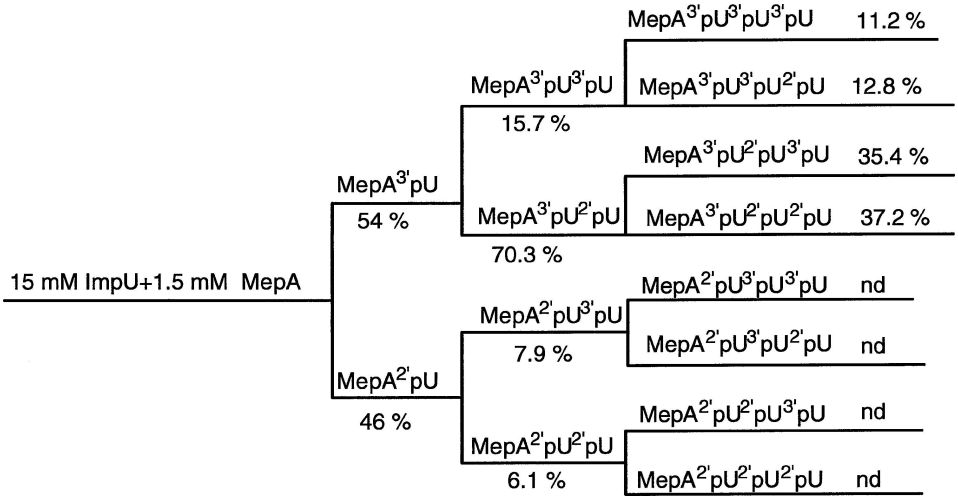

Figure 4. The proportion of MepA-initiated dimers, trimers and tetramers in the two-charge, three-charge and four-charge fractions formed in the reaction of 15 mM ImpU with 1.5 mM MepA. nd is not detected.

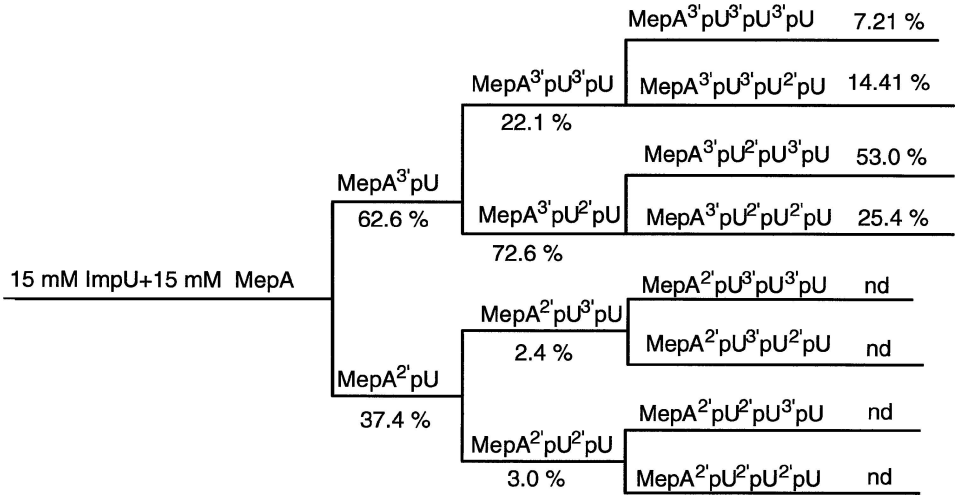

Figure 5. The proportion of MepA-initiated dimers, trimers and tetramers in the two-charge, three-charge and four-charge fractions in the reaction of 15 mM ImpU with 15 mM MepA. nd is not detected.

2.2. Reaction of MepA with ImpA

The reaction of a mixture of 1.5 mM MepA and 15 mM MepA with that of 15 mM ImpA demonstrated that MepA does not enhance the rate of oligomer formation from ImpA. The yields of oligomers with a 5′-MepA grouping decrease as the

TABLE II

Composition of the 5′-MepA oligomers formed in the reaction of a mixture of 15 mM ImpA + 1.5 mM MepA[a]

	5′-MepA products (%)
Two-charge fraction	
MepA$^{3'}$pA	72.1
MepA$^{2'}$pA	27.9
MepApA is 14.9% of the total chromophore	
Three-charge fraction	
MepA$^{3'}$pA$^{3'}$pA	40.0
MepA$^{3'}$pA$^{2'}$pA	60.0
MepA$^{2'}$pA$^{3'}$pA	nd[b]
MepA$^{2'}$pA$^{2'}$pA	nd
MepApApA is 8% of the total chromophore	

[a]Fractions collected from the ion exchange HPLC column, then purified by reverse phase HPLC and characterized by standard enzymatic hydrolysis. Peak areas are the ratio of the UV absorbance of compound to the total UV absorbance of the components listed.
[b]nd: Not detected.

chain length increases (Tables II and III). The percent of MepA-initiated oligo(A)s changes from 15 to 8% going from two-charged to three-charged fractions in the reaction of a mixture of 1.5 mM MepA and 15 mM ImpA. A corresponding drop in the MepA-initiated oligomers of 37–25% is observed in the two-charge to three-charge products of the reaction of a mixture of 15 mM MepA and 15 mM ImpA. These data show that the MepA-initiated reaction of ImpA proceeds at about the same rate as the reaction of ImpA alone since there is no increase in the extent of MepA incorporation with chain length. As was observed with the MepA-initiated reaction of ImpU, the 3′,5′-linked two-charged isomer (MepApA) is a much better initiator of the formation of three-charged products than the corresponding 2′,5′-linked isomer (MepA$^{2'}$pA).

2.3. Relative reaction rates

The relative rates of the elongation of MepA with ImpU and ImpA were studied in competitive reactions to gain insight into the polymerization pathways. Equimolar amounts of ImpU and ImpA were reacted together with MepA and the structures of the reaction products were determined (Equation (1)) (Table IV). The proportion of MepApA (41%) is less than that of MepApU (59%) in the two-charge fraction demonstrating that the addition of activated pyrimidine nucleotides is faster than that of purines to the 3′-end of the oligomers. The formation of the three-charge fraction is a competition between the four isomers of MepApN (N = A and U) with the binary mixture of ImpA and ImpU. The proportion of MepA$^{3'}$pA-initiated

TABLE III

Composition of the 5′-MepA oligomers formed in the reaction of a mixture of 15 mM ImpA + 15 mM MepA[a]

	5′-MepA products (%)
Two-charge fraction	
MepA$^{3'}$pA	71.6
MepA$^{2'}$pA	28.4
MepApA is 37.2% of the total absorbance of the two-charge fraction	
Three-charge fraction	
MepA$^{3'}$pA$^{3'}$pA	30.2
MepA$^{3'}$pA$^{2'}$pA	63.0
MepA$^{2'}$pA$^{3'}$pA	2.0
MepA$^{2'}$pA$^{2'}$pA	4.80
MepApApA is about 25% of the total absorbance of the four-charge fraction	
Four-charge fraction	
MepApApApA is about 12% of the total absorbance of the four-charge fraction	

[a]See footnote in Table 2.

products in the three-charge fraction was significantly greater than those initiated by MepA$^{3'}$pU (36.8–6.6% or 5.6:1). The proportion of the starting MepA$^{3'}$pA (31%) is less than the MepA$^{3'}$pU (46%) but more MepA$^{3'}$pA-initiated oligomers were formed. This shows that the elongation of MepA$^{3'}$pA is significantly faster than that of MepA$^{3'}$pU. Only the MepA$^{3'}$pN and not the MepA$^{2'}$pN addition products were detected.

$$\text{MepA} + \text{ImpA} + \text{ImpU} \rightarrow \underset{41\%}{\text{MepApA}} + \underset{51\%}{\text{MepApU}} \rightarrow \underset{37\%}{\text{MepApApN}} + \underset{7\%}{\text{MepApUpN}} \quad (1)$$

The relative rates of reaction of equal amounts of ImpA and ImpU with MepA$^{3'}$pA and MepA$^{3'}$pU were discerned from the composition of the three-charge fraction obtained in the reaction of equal amounts of ImpA and ImpU with MepA (Equations (2) and (3)) (Table V). Approximately equal amounts of MepA$^{3'}$pApA and MepA$^{3'}$pApU (31.0 and 31.4%, respectively) formed while the addition of ImpA and ImpU to MepA$^{3'}$pU gave a greater proportion (59:41%) of U-addition products.

$$\text{MepA}^{3'}\text{pA} + \text{ImpA} + \text{ImpU} \rightarrow \underset{49\%}{\text{MepA}^{3'}\text{pApA}} + \underset{51\%}{\text{MepA}^{3'}\text{pApU}} \quad (2)$$

$$\text{MepA}^{3'}\text{pU} + \text{ImpA} + \text{ImpU} \rightarrow \underset{41\%}{\text{MepA}^{3'}\text{pUpA}} + \underset{59\%}{\text{MepA}^{3'}\text{pUpU}} \quad (3)$$

TABLE IV

Yields of the products from the reaction of 7.5 mM MepA with a mixture of 3.75 mM ImpU + 3.75 mM ImpA[a]

	Yield (%)
Two-charge fraction	
MepA$^{3'}$pA	30.9
MepA$^{2'}$pA	10.5
MepA$^{3'}$pU	46.2
MepA$^{2'}$pU	12.4
Three-charge fraction	
pU$^{2'}$pU	0.32
pA$^{2'}$pU	2.38
pA$^{2'}$pA	1.83
C-A$^{3'}$pU$^{3'}$p[b] (cyclic)	29.8
pA$^{3'}$pU	6.51
pA$^{3'}$pA	0.82
C-A$^{3'}$pA$^{3'}$p[b] (cyclic)	5.72
MepA$^{3'}$pA$^{3'}$pA	4.04
MepA$^{3'}$pA$^{2'}$pA	8.07
MepA$^{3'}$pA$^{3'}$pU	7.51
MepA$^{3'}$pA$^{2'}$pU	17.2
MepA$^{3'}$pU$^{2'}$pA	2.50
MepA$^{3'}$pU$^{2'}$pU	4.06
Not identified	9.20

[a]See footnote in Table II.

[b]Cyclic dimers elute from the ion exchange column in the three-charge fraction.

Structure analysis of the oligomers formed from the MepA-initiated reactions of ImpU show the elongation of MepA$^{3'}$pU proceeds more rapidly than that of MepA$^{2'}$pU (Figures 3 and 4). This was tested by synthesizing 3′,5′- and 2′,5′-isomers of MepApU and determining the structures of the trimers formed in their reactions with ImpU. Structure analysis of the products formed from the 1.5 mM MepA$^{3'}$pU with ImpU (Table I) established that the oligomers with five or greater monomer units had 70–100% 5′-MepA groups (Figure 6). Reaction of 1.5 mM MepA$^{2'}$pU with 15 mM ImpU generated oligomers in which only 9.5–8.1% of the 5–9 mers had 5′-MepA groups (Figure 6) so the principal linear reaction products had 5′-pU groups. The HPLC trace of the oligomerization initiated by MepA$^{2'}$pU resembled that of the ImpU reaction (Figure 2A) while HPLC trace of the oligomers initiated by MepA$^{3'}$pU resembled that of the HPLC Figure 2B (HPLC traces not shown).

TABLE V

Yields of the 5′-MepA oligomers in the reaction of a mixture of 3.75 mM ImpU and 3.75 mM ImpA with the individual isomers of 7.5 mM MepApN (N = A or U)[a]

	Percent of 3-charge fraction
MepA$^{3'}$pA	
MepA$^{3'}$pA$^{3'}$pA	12.4
MepA$^{3'}$pA$^{2'}$pA	18.6
MepA$^{3'}$pA$^{3'}$pU	15.8
MepA$^{3'}$pA$^{2'}$pU	15.6
The three-charge fraction is 4.9% of the total absorbance	
MepA$^{2'}$pA[b]	
MepA$^{2'}$pA$^{3'}$pU	17.1
MepA$^{2'}$pA$^{3'}$pA	12.1
The three-charge fraction is 5.4% of the total absorbance	
MepA$^{3'}$pU	
MepA$^{3'}$pU$^{3'}$pA	8.15
MepA$^{3'}$pU$^{2'}$pA	15.5
MepA$^{3'}$pU$^{3'}$pU	9.0
MepA$^{3'}$pU$^{2'}$pU	23.7
The three-charge fraction is 4.6% of the total absorbance	
MepA$^{2'}$pU[c]	
The three-charge fraction is 8.7% of the reaction mixture	

[a]See footnote in Table II.

[b]The three-charge fraction contains pNpN, cyclic dimers and MepA$^{2'}$pNpN(N = A, U). The identified peaks were based on the RNase T_2 hydrolysis. These assignments were confirmed by their resistance to APH hydrolysis.

[c]Based on RNase T_2 and APH hydrolysis of the major purified peaks by RP HPLC, the major peaks were not addition products of MepA$^{2'}$pU.

These findings are consistent with those reported in Figures 3 and 4 where it was observed that the elongation of an oligomer with a 3′-pyrimidine linked by a 2′,5′-phosphodiester bond proceeds more slowly than one with a 3′,5′-phosphodiester bond in montmorillonite-catalyzed reactions.

The relative reaction rate studies were extended by investigation of the competitive reactions of equimolar amounts of MepA$^{3'}$pA and MepA$^{2'}$pA with ImpA (Table VI, #3) (Equation (4)) and the corresponding reaction of MepA$^{3'}$pU and MepA$^{2'}$pU with ImpU (Table VI, #6) (Equation (5)). The reaction products were identified from the products observed in the reactions of the individual MepA$^{2'}$pN isomers with ImpA or ImpU (Table VI, #1, 2, 4, 5). It was possible to identify

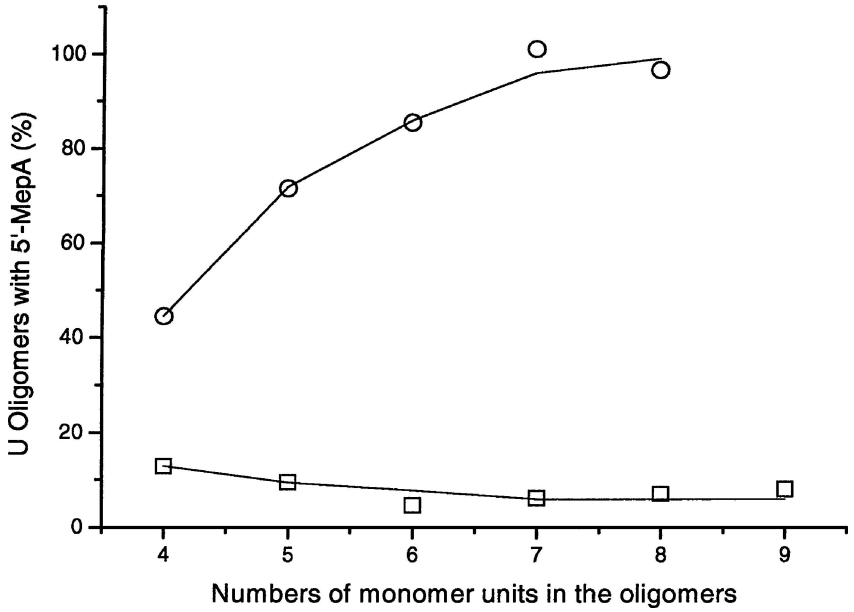

Figure 6. Percent of oligomers with 5′-MepA groups in the reaction of 1.5 mM MepA$^{3'}$pU with ImpU (○) and percent of oligomers in the reaction of 1.5 mM MepA$^{2'}$pA with 15 mM ImpU (□).

the addition products from the reverse phase HPLC trace since we knew the retention times of the pNpN and cyclic dimer (C-NpNp, N = A or U) products. The major peaks remaining were then assigned to the MepApNpN addition products. The presence of the 5′-MepA group in these oligomers was confirmed by the observation that the retention times of these peaks did not change on treatment of the reaction mixture with APH. A 5.2:1 ratio of MepA$^{3'}$pA to MepA$^{2'}$pA addition products was obtained (Table VII) indicative of a five-fold greater rate of reaction of the 3′,5′-isomer.

In the competitive reaction of ImpU with equimolar amounts of MepA$^{3'}$pU to MepA$^{2'}$pU a 10.3:1 ratio of MepA$^{3'}$pU to MepA$^{2'}$pU addition products observed (Table VI) indicative of a 10-fold greater rate of reaction of the 3′,5′-linked isomer. These data directly demonstrate that the MepA$^{3'}$pN derivatives (N = A or U) elongate 5–10 times more rapidly than the corresponding MepA$^{2'}$pN derivatives. The differences in rates are mirrored in the higher yields of the MepA$^{3'}$pN (N = A, U) addition products in the reactions with the corresponding ImpN derivatives (Table VI).

$$\begin{aligned} &\text{MepA}^{3'}\text{pA} + \text{MepA}^{2'}\text{pA} + \text{ImpA} \\ &\rightarrow \underset{23\%}{\text{MepA}^{3'}\text{pA}^{3'}\text{pA}} + \underset{31\%}{\text{MepA}^{3'}\text{pA}^{2'}\text{pA}} + \underset{10\%}{\text{MepA}^{2'}\text{pApA}} \end{aligned} \tag{4}$$

TABLE VI

Yields in the reactions of individual compounds and binary mixtures of MepApN isomers with ImpA and/or ImpU[a,b]

Reaction	Products	Percentage of three-charge fraction
(1) 7.5 $MepA^{3'}pA + 7.5$ ImpA	$MepA^{3'}pA^{3'}pA$	28.3
	$MepA^{3'}pA^{2'}pA$	43.6
(2) 7.5 $MepA^{2'}pA + 7.5$ ImpA	$MepA^{2'}pA^{3'}pA$	14.2
	$MepA^{2'}pA^{2'}pA$	12.3
(3) 3.75 $MepA^{3'}pA + 3.75$ $MepA^{2'}pA$	$MepA^{3'}pA^{3'}pA$	22.5
+ 7.5 ImpA	$MepA^{3'}pA^{2'}pA$	31.0
	$MepA^{2'}pA^{3'}pA$	4.05
	$MepA^{2'}pA^{2'}pA$	6.23
	$MepA^{3'}pApA/MepA^{2'}$	pApA = 5.2/1
(4) 7.5 $MepA^{3'}pU + 7.5$ ImpU	$MepA^{3'}pU^{3'}U$	22.1
	$MepA^{3'}pU^{2'}U$	48.6
(5) 7.5 $MepA^{2'}pU + 7.5$ ImpU	$MepA^{2'}pU^{3'}U$	10.1
	$MepA^{2'}pU^{2'}U$	6.50
(6) 3.75 $MepA^{3'}pU + 3.75$ $MepA^{2'}pU$	$MepA^{3'}pU^{3'}pU$	13.9
+ 7.5 ImpU	$MepA^{3'}pU^{2'}pU$	34.8
	$MepA^{2'}pU^{3'}pU$	3.25
	$MepA^{2'}pU^{2'}pU$	1.48
	$MepA^{3'}pUpU/MepA^{2'}$	pUpU = 10.3/1

[a]The HPLC peaks due to the addition products $MepA^{2'}pA$ and $MepA^{2'}pU$ were assigned on the basis of the retention times of peaks observed in the reaction of ImpA with $MepA^{2'}pA$.

$$\begin{aligned} &MepA^{3'}pU + MepA^{2'}pU + ImpU \\ &\rightarrow \underset{}{\overset{3'}{M}epA\,pU^{3'}pU_{14\%}} + \underset{35\%}{MepA^{3'}pU^{2'}pU} + \underset{5\%}{MepA^{2'}pUpU} \end{aligned} \tag{5}$$

2.4. Selectivity in phosphodiester bond formation

The selectivity observed in the reaction of MepA with ImpU can be explained from the data obtained in the reaction of mixtures of activated monomers (Ertem and Ferris, 2000) and the reactivity rules derived from the reaction of a mixture of ImpA and ImpC (Miyakawa *et al.*, 2002). The high proportion longer oligomers with the 5′-MepA grouping in the reaction of MepA with ImpU is

TABLE VII

The 2′,5′-linked groups released on enzymatic hydrolysis of the oligomers formed in the reaction of 1.5 mM MepA with 15 mM ImpU[a,b,c]

Oligomers	$\sim^{3'}pU$	$\sim^{3'}pU^{2'}pU$	$\sim U^{2'}pU^{3'}p\sim$	$\sim^{3'}pU^{2'}pU^{2'}pU$	$\sim U^{2'}pU^{2'}pU^{3'}p\sim$
3-Mers	22.6	77.4	0	0	0
4-Mers	49.3	12.6	35.6	36.7	0
5-Mers	22.5	47.9	59.8	29.6	10.2
6-Mers	31.4	29.3	76.4	39.4	18.7
7-Mers	21.8	30.9	100	47.4	26.6
8-Mers	24.0	30.2	119	45.8	41.7
9-Mers	20.4	30.7	150	48.9	54.6

[a]The percentage of terminal $\sim^{3'}pU^{2'}pU$ is based on $U^{2'}pU$ formed after RNase T_2 hydrolysis, terminal $\sim^{3'}pU^{2'}pU^{2'}pU$ is based the $U^{2'}pU^{2'}pU$ formation after RNase T_2 hydrolysis. $\sim^{3'}pU^{2'}pU^{3'}\sim$ is based on the peak area increase of $U^{2'}pU$ after the follow-up APH hydrolysis. And $\sim U^{2'}pU^{2'}pU^{3'}\sim$ is based on the peak area increase of $\sim^{3'}pU^{2'}pU^{2'}pU$ after the follow-up APH hydrolysis.

[b]Uridine released by RNase T_2 hydrolysis could not be determined because it had the same retention time as another compound as shown its decrease in intensity on treatment with APH. It was determined by subtraction the sum of $U^{2'}pU$ and $U^{2'}pU^{2'}pU$ percentage from 100%.

[c]The peak clusters purified were actually all with $MepA^{3'}pU$ initiation based on the MepA released after RNase T_2 and the follow-up APH hydrolysis.

consistent with the more rapid reactions of activated pyrimidine nucleotides at the 3′-positions of purine nucleotides than at the 3′-position of pyrimidine nucleotides.

The favored elongation of $MepA^{3'}pU$ over $MepA^{2'}pU$ is a consequence of the faster rate of elongation of a nucleotide bound to the 3′-end of the oligomer by a 3′,5′-phosphodiester bond versus that of one bound by a 2′,5′-phosphodiester bond. The formation of a trimer linked by a 2′,5′-phosphodiester bond is consistent with the observed favored elongation of a 3′-pyrimidine nucleotide via a 2′,5′-phosphodiester bond (Miyakawa and Ferris, 2003).

The formation of longer oligomers in the reaction of MepA with ImpU versus that of ImpU alone is explained by the initial formation of higher yields of $MepA^{3'}pU$ that in turn undergoes a more rapid reaction to form $MepA^{3'}pUpU$ than ImpU reacts with $pU^{2'}pU$ to form the corresponding $pU^{2'}pU^{3'}pU$.

A 10:1 selective advantage of the formation of $MepA^{3'}pUpU$ over $MepA^{2'}pUpU$ in the competitive reaction of $MepA^{3'}pU$ and $MepA^{2'}pU$ with ImpU was observed (Table VI). The absence of $MepA^{2'}pNpN$ products in the competitive reactions of MepA with a mixture of ImpA and ImpU (Table IV) demonstrates the importance of this reaction rate difference on the products formed. This reactivity is consistent with the sequence of steps in the reactions in Equation (6).

$$\text{MepA} \rightarrow \text{MepA}^{3'}pU \rightarrow \text{MepA}^{3'}pU^{2'}pU \rightarrow \text{MepA}^{3'}pU^{2'}pU^{3'}pU \quad (6)$$

The absence of the formation of oligomers longer than seven mers with mainly 5′-MepA groups in the reaction of 1.5 mM MepA with 15 mM ImpA is due to an absence of a significant difference in the rates of elongation of ImpA and MepA. The 10-fold higher amount of ImpA over MepA results in only small amounts of MepA-initiated oligomers because their rates of elongation are the same.

2.5. Consecutive and 3′-terminal 2′, 5′-phosphodiester bonds

The products of the RNase T_2 and the subsequent APH hydrolysis of the oligomers formed by montmorillonite catalysis were used to determine the number of consecutive and terminal 2′,5′-links in the oligomers. The products of the reaction of 1.5 mM MepA with 15 mM ImpU were separated by reverse phase HPLC after RNase T_2 hydrolysis and again after the subsequent APH hydrolysis.

The cluster of peaks with retention times on a reverse phase column the same as $U^{2'}pU$ and longer were collected and separated on an ion exchange column. Two main peaks were assigned to $U^{2'}pU$ and $U^{2'}pU^{2'}pU$. In addition a third peak, which is due to two compounds present in comparable amounts with close to the same retentions times, was observed. If one of these two peaks is assumed to be due to $U^{2'}pU^{2'}pU^{2'}pU$, it occurs rarely in the oligomers since the absorbance ratio of $U^{2'}pU^{2'}pU$:$U^{2'}pU^{2'}pU^{2'}pU$ is about 8:1. The ratio of the peak area ratio for $U^{2'}pU$ to the terminal $U^{2'}pU^{2'}pU$, as determined by reverse phase HPLC, is about 3.5:1. This means that the rate of elongation of $U^{2'}pU$ by a 3′,5′-phosphodiester bond is 3.5 times faster than elongation with a 2′,5′-linkage. The amounts of the putative $U^{2'}pU^{2'}pU^{2'}pU$ are very small so the rate of elongation of $U^{2'}pU^{2'}pU$ by a 2′,5′-link is very slow. Similar 2′,5′-linked products were obtained in the reaction of 1.5 mM MepA with 15 mM ImpA.

It was possible to determine the groups on the 3′-ends of the longer RNAs formed in the MepA-initiated oligomerization of ImpU after first separating the oligomers on an anion exchange column by charge and then on a reverse phase HPLC column (Table VII). The RNase T_2 hydrolysis products are U, MepAp, Up, $U^{2'}pU$, $U^{2'}pUp$, $U^{2'}pU^{2'}pU$ and $U^{2'}pU^{2'}pUp$ and the subsequent hydrolysis by APH gave MepA, U, $U^{2}pU$ and $U^{2'}pU^{2'}pU$. The percentages of $U^{2'}pU$ and $U^{2'}pU^{2'}pU$ in the RNase T_2 cleavage products were used to calculate the percentage of 3,5′-phosphodiester bonds at the 3′-end of the growing chain. This value levels off at about 20% at chain lengths of 7–9. The percentages of $U^{2'}pU$ and $U^{2'}pU^{2'}pU$ units at the 3′-end of the chains increases to about 30 and 45%, respectively, in the 7–9 mers. No $U^{2'}pU^{2'}pU^{2'}pU$ was observed in these studies.

2.6. Binding and selectivity

The selectivity in the reactions of activated purine and pyrimidine nucleotides in reactions catalyzed by montmorillonite clay differ. It was observed in previous

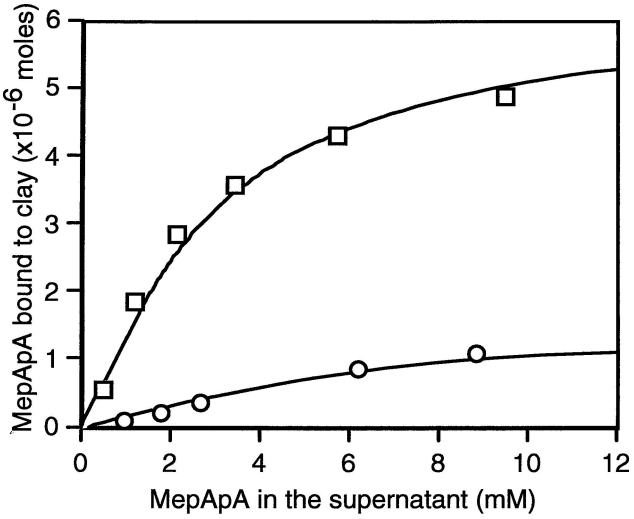

Figure 7. Adsorption isotherms of the MepApA isomers on montmorillonite. Mep$A^{3'}$pA (□), Mep$A^{2'}$pA (○).

studies that the binding of activated purine and pyrimidine nucleotides to montmorillonite are markedly different (Ferris *et al.*, 1989; Kawamura and Ferris, 1999). The extent of binding of some of the reactants to montmorillonite was measured to determine if there was a correlation between binding and reactivity. Three reactions are described below where there is no agreement between the extent of binding and the reaction rate of the activated nucleotide on montmorillonite.

The extent of binding was measured by the Langmuir absorption coefficients ($K_{L.}$) calculated from the binding isotherms for the isomers of MepApA (Figure 7) and MepApU (Figure 8) (Table VIII). These K_L values, together with those measured previously (Wang and Ferris, 2001), were used to determine the role of binding in the reactivity and selectivity observed in the following studies.

(1) The Mep$^{3'}$pN isomers bind more strongly (K_L) to montmorillonite than the corresponding Mep$^{2'}$pN derivatives (Figures 7 and 8). Since the Mep$^{3'}$pN isomers elongate faster than the corresponding Mep$^{2'}$pN isomers with ImpU and ImpA these data suggest a direct correlation between reactivity and binding.
(2) ImpA binds more strongly to montmorillonite than ImpU ($K_L = 45$ and 15, respectively; Wang and Ferris, 2001) but the reaction of an equimolar mixture of these activated nucleotides with Mep$A^{3'}$pU gives approximately equal yields of addition products (Table V) indicative of a inverse relationship between binding and reactivity. These findings differ from those obtained in example (1) above.
(3) The reaction of the ImpA–ImpU mixture with MepApU gives a higher yield of the addition of the U products than A (59%:41%, respectively). This finding

TABLE VIII

Langmuir adsorption coefficient (K_L 1/mol) and saturation binding (a_s) of 3′,5′-linked dimers and 2′,5′ -linked dimers

	MepA$^{3'}$pA	MepA$^{2'}$pA	MepA$^{3'}$pU	MepA$^{2'}$pU
K_L	397	39.2	47.7	31.8
a_s	0.148	0.094	0.230	0.086

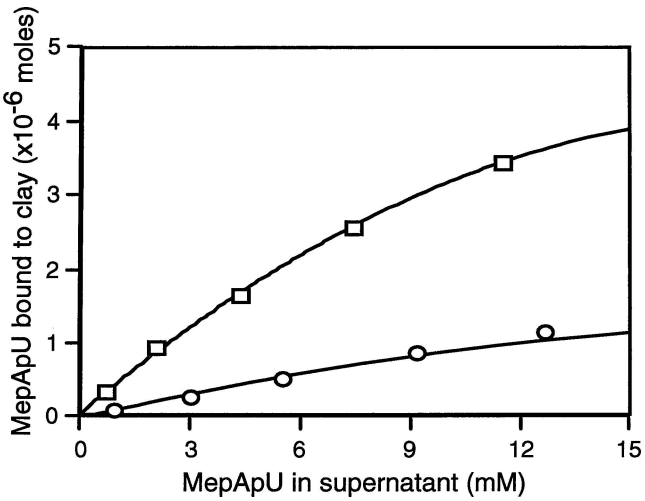

Figure 8. Absorption isotherms of the MepApU isomers on montmorillonite. MepA$^{3'}$pU (□), MepA$^{2'}$pU (○).

is also differs from the result in example (1) in that the results are consistent with an inverse relationship between binding and elongation.

The differences in these three examples clearly demonstrate that there is no correlation between the extent of binding of the activated nucleotide to montmorillonite and the rate of oligomer elongation.

2.7. Structural analysis of cyclic nucleotides

In the course of this research we reinvestigated the structure of the cyclic nucleotides of adenylic and uridylic acid the structures of which were reported to be cyclic trimers (Prabahar *et al.*, 1994; Ferris and Ertem, 1993; Ding *et al.*, 1996). These compounds have now been assigned the cyclic dimer structure on the basis of their molecular weights and empirical formulas determined from high-resolution mass spectra. Previously these compounds were assumed to be trimers because they elute in or near the three-charge fraction of the ion exchange HPLC chromatogram. In addition they appeared to be resistant to hydrolysis because their RNase T_2

hydrolysis rates are much slower than those of the linear dimers. Long-term hydrolysis with RNase T_2 resulted in their degradation to Ap and Up, respectively, indicative that both phosphodiester bonds are 3′,5′-linked (the RNase T_2 hydrolysis of the cyclic nucleotides is unpublished work from this laboratory by P. C. Joshi).

3. Conclusions

The first step in the reaction of ImpU with MepA is faster than the self-condensation of ImpU. This is because the reaction of a pyrimidine nucleotide with a purine is faster than its reaction with another pyrimidine nucleotide (Miyakawa and Ferris, 2003).

The second step in the elongation is the addition of ImpU to the linear dimers. The rate of elongation of a dimer in which there is a 3′,5′-phosphodiester bond linking the 3′-nucleotide is faster than the reaction with a 2′,5′-link (Miyakawa and Ferris, 2003). The first two steps in the MepA-initiated reaction of ImpU are faster than the corresponding steps in the steps in the elongation of ImpU alone. This results in a jump-start in the MepA-initiated reaction of ImpU. The absence of this jump-start in the MepA-initiated reaction of ImpA is due to the absence of the difference in rates of condensation of ImpA with MepA. Consequently, there is no increase in the rate of reaction ImpA in the presence of MepA.

It is possible to propose, from the results obtained in this study and previous studies (Miyakawa and Ferris, 2003), the following reaction pathway between a purine and a pyrimidine nucleotide on montmorillonite:

$$\mathrm{ImpA} + \mathrm{ImpU} \rightarrow \mathrm{pA^{2'}pN} + \mathrm{pA^{3'}pN} \xrightarrow{\mathrm{ImpN}} \underset{\text{major}}{\mathrm{pA^{3'}pN^{2'}pN}} + \underset{\text{minor}}{\mathrm{pA^{3'}pN^{3'}pN}}$$

$$\xrightarrow{\mathrm{ImpN}} \underset{\text{major}}{\mathrm{PA^{3'}pN^{2'}pN^{3'}pN}} + \underset{\text{minor}}{\mathrm{pA^{3'}pN^{2'}pNp^{2'}pN}}$$

The observed selectivity in product formation is impressive when compared with the same reactions performed in the absence of catalysis. Comprehensive studies by Kanavarioti and coworkers have shown that the reaction of the phosphorimidazolides of nucleosides using concentrations in the 1 M range yield dimers as the major reaction products. Only "... trace amounts of longer oligomers are formed ..." (Kanavarioti, 1998). In addition, approximately equal amounts of all possible dimers are obtained in a reaction of a ternary mixture of the phosphorimidazolides of the nucleosides of G, C, and U (Kanavarioti, 1997, 1998). The absence of the formation of longer oligomers and the absence of selectivity demonstrates that it was not possible to have formed RNAs of sufficient length on the primitive Earth to initiate the RNA world in the absence of catalysis. Scenarios that propose mechanisms for the concentration of activated monomers on the primitive Earth in the absence of catalysis will not result in the generation of polymers. Instead the

monomers will react to form low molecular weight oligomers that are a mixture of all possible isomers (Kanavarioti, 1998).

Catalysis was essential to generate the long biopolymers essential to initiate the first life on Earth. Catalysts have the capability of restricting the available reaction pathways so that the monomers are converted to polymers that have limited structural heterogeneity. It is possible that RNAs formed in this way would have been long enough to have served as catalysts and to have undergone replication with limited mutation, requisites for the RNAs needed to initiate the RNA world (Joyce and Orgel, 1999; Szostak and Ellington, 1993).

4. Experimental Section

4.1. General

Most of the materials and experimental methods used in this study were described previously (Wang and Ferris, 2001). Homoionic Na^+-montmorillonite was prepared from Volclay (American Colloid Co., SPV-200) by titration method (Banin *et al.*, 1985). All the binding and oligomerization reaction were carried out in the standard buffer solution (0.1 M HEPES, 0.2 M NaCl and 0.075 M $MgCl_2$, pH 8.0). The phosphorimidazolides of adenosine and uridine were prepared by the literature procedure (Joyce *et al.*, 1984). Mass spectra were obtained at the School of Chemical Sciences, University of Illinois at Urbana.

The anion exchange HPLC column was a HEMA IEC BIO Q and the elution conditions described previously was used (Ferris and Ertem, 1993). Reverse phase chromatography was performed on a Waters C18 μBondapak column (3.9 mm $\times$ 300 mm) and an Alltima C18 column (Alltech, 5 μm, 4.6 mm $\times$ 250 mm). In the reverse phase HPLC analysis, solvent A is 0.02 M KH_2PO_4 buffer (pH 5.8), solvent B is 30 % of acetonitrile in solvent A, and the elution gradient was from 4% B to 35% B in 27 min.

Ribonuclease T_2 (RNase T_2) and bacterial alkaline phosphatase (APH) were obtained from Sigma. Phosphodiesterase I (PD I) was from Sigma (*Crotalus Adamanteus* venom) and USB. The Sigma preparation had nucleotidase activity while the nucleotidase activity was inhibited in the USB enzyme. MepA was synthesized by the reported method (Wang and Ferris, 2001), and its purity was checked by both reverse phase and ion exchange HPLC. The authentic sample of $U^{2'}pU$ was from Sigma.

The molar extinction coefficients of ImpA and MepA were assumed to be the same as that of 5′-AMP. The molar extinction coefficient of ImpU was assumed to be the same as that of 5′-UMP. There is no molar extinction coefficient data available for $MepA^{2'}pA$, $MepA^{3'}pA$, $MepA^{2'}pU$, $MepA^{3'}pU$, $U^{2'}pU$ and $U^{2'}pU^{2'}pU$. For MepApA and MepApU, the hypochromicity for $A^{3'}pA$ and $A^{3'}pU$ has been adopted (9.4, 5.0%) and the hypochromicity of $U^{2'}pU$ was assumed to be the

same as that of $U^{3'}pU$ (1.7%) (Warshaw and Tinoco, 1966). The hypochromicity of $U^{2'}pU^{2'}pU$ was also assumed to be 1.7% because the oligomers of U are not stacked (Warshaw and Tinoco, 1966). The molar extinction coefficients of 2′,5′- and 3′,5′-linked dimers were assumed to be the same (Kierzek *et al.*, 1992). Based the number of the monomers, the composition and the hypochromicities of the oligomers at 260 nm, the molar extinction coefficients of MepApA, MepApU, $U^{2'}pU$ and $U^{2'}pU^{2'}pU$ were calculated to be $2.72 \times 10^{+4}$, $2.38 \times 10^{+4}$, $1.97 \times 10^{+4}$ and $2.95 \times 10^{+4}$, respectively.

The procedures for RNase T_2 and APH hydrolysis have been reported (Ding *et al.*, 1996). It was found that in addition to 3′,5′-linkages, 2′,5′-linkages and 5′,5′-linkages, PD I cleaved the methyl phosphate group of MepA to 5′-AMP (Richards *et al.*, 1967; Itkes *et al.*, 1988). The substrate was dissolved in 0.11 M Tris, 0.11 M NaCl and 0.015 M $MgCl_2$ (pH 9.0) as described in the 1988 Worthington manual, page 269. To 400 μl of substrates 50 μl PD I (1 unit/ml) was added. The reaction mixture was kept at RT for about 15 h. The Alltima reverse phase column was used for the analysis of enzymatic hydrolysis products.

The components analyzed by reverse phase chromatography were identified by their retention times. Their structures were confirmed, when possible by co-injecting authentic samples, with the collected fractions on the reverse phase HPLC. In several cases, fractions collected from reverse phase analytical HPLC profiles were checked using ion exchange HPLC to determine the charges of the components. The structure assignment of the oligomers was based on both the numbers of charges in the sample as determined from the retention time on ion exchange HPLC as well as from the enzymatic hydrolysis data.

4.2. Preparation of reactants and products

4.2.1. *Cyclic Dimers (C-UpUp, C-ApAp and C-ApUp)*

Oligomerization reactions of 15 mM ImpU, 15 mM ImpA and a 7.5 mM mixture of equal amounts of ImpU and ImpA were carried out on montmorillonite according to the reported procedures (Ding *et al.*, 1996). The mixtures of the oligomers were separated using an ion exchange HPLC column. The fraction between two-charge fraction (mainly 5′-UMP) and three-charge fraction was the cyclic dimers of 5′-UMP, which were about 30% of the whole chromatogram. The product was found to be 98% pure by ion exchange HPLC. The C-UpUp fraction was characterized by RNase T_2 and the subsequent APH hydrolysis (unpublished work of P. C. Joshi). FAB^+ MS showed no major peak near 918, the molecular weight of the cyclic trimer of 5′-UMP. The three major peaks detected were 657.1 ($M+2Na^+ + H^+$, calculated 657), 679.1 ($M+3Na^+$, calculated 679) and 701.6 ($M+4Na^+ - H^+$, calculated 701). The observed high-resolution peak was 657.0220 ($C_{18}H_{21}N_4O_{16}P_2Na_2$ requires 657.0223).

Oligomerization of 15 mM ImpA on montmorillonite was carried out according to the reported procedures (Ferris and Ertem, 1993). The three-charge fraction was collected from the anion exchange HPLC column and concentrated using a rotary evaporator. The yield of the three-charge fraction was about 35% of the total chromophore. The product was found to be 97% pure by both ion exchange and reverse phase HPLC. The C-ApAp was characterized by RNase T_2 and subsequent APH hydrolysis (unpublished work by P. C. Joshi). FAB^+ MS showed no major peak near 987, the molecular weight of the cyclic trimer of 5′-AMP. In the FAB^+ spectrum of the cyclic dimers of 5′-AMP, 703 ($M + 2Na^+ + H^+$, calculated 703) and 725.1 ($M + 3Na^+$, calculated 725) were the major peaks. In ES^{-1} spectrum, one peak 657.4 ($M\text{-}H^+$, calculated 657) was detected. The high-resolution peak was 725.0589 ($C_{20}H_{22}N_{10}N_{12}P_2Na_3$ requires 725.0587).

The three-charge fraction of 3.75 mM ImpU with 3.75 mM ImpA oligomerization reaction on clay was collected from the anion exchange HPLC column. Reverse phase HPLC analysis demonstrated that there was a major fraction with retention time much shorter than C-ApAp that was shown to be C-ApUp. The fraction was purified using a reverse phase HPLC. C-ApUp resisted APH hydrolysis. RNase T_2 hydrolysis (10 unit/mL) of the C-ApUp gave equal molar amounts of 3′-AMP and 3′-UMP. The subsequent APH hydrolysis gave equal molar amounts of adenosine and uridine.

4.2.2. *Me$A^{2'}$pA and Mep$A^{3'}$pA*

The oligomerization reaction of 15 mM ImpA with 10 mM MepA on montmorillonite was carried out following the reported procedure (Ferris and Ertem, 1993). The reactions were allowed to proceed at room temperature for 24 h. The combined supernatant was separated by an ion exchange HPLC. The two-charge fractions were collected (about 25% of the total absorbance) and treated with APH to convert 5′-AMP to adenosine. Mep$A^{2'}$pA and Mep$A^{3'}$pA were separated and collected by reverse phase HPLC (μBondapak). It was found that the purity of Mep$A^{2'}$pA was only about 80% after reverse phase HPLC purification. It was further purified on a μBondapak HPLC column. The yields of Mep$A^{2'}$pA and Mep$A^{3'}$pA were about 10% of the total absorbance of whole chromatogram.

The purified Mep$A^{2'}$pA and Mep$A^{3'}$pA were desalted using a preparative column (C18 resin, 37–55 μm Waters, 3.4 cm × 29.5 cm). The sample was added to the top of the column and then the column eluted with 500 ml double-distilled water and then with 500 ml 5% acetonitrile water. The separation was monitored by reverse phase HPLC. The fractions containing the dimers were combined, concentrated and lyophilized to dryness. The samples were dissolved in 1 mL of distilled water and the insoluble impurities were removed by centrifugation for 10 min at 14,000 rpm.

The purity of Mep$A^{2'}$pA and Mep$A^{3'}$pA was determined by both ion exchange and C18 reverse phase HPLC (97 and 98% pure, respectively). Both Mep$A^{2'}$pA and

MepA$^{3'}$pA resisted APH hydrolysis. MepA$^{2'}$pA also resisted RNase T_2 hydrolysis. After RNase T_2 hydrolysis, the products from MepA$^{3'}$pA were MepA$^{3'}$p and adenosine. The subsequent APH hydrolysis converted MepA$^{3'}$p to MepA. PD I hydrolysis yielded MepA and AMP. The subsequent APH hydrolysis converted AMP into adenosine. About 20% of the MepA was demethylated by PD I to 5′-AMP.

4.2.3. *MepA$^{2'}$pU and MepA$^{3'}$pU*

The same procedure used for the preparation of the isomers of MepApA was used for the formation of MepApU isomers. The two-charge fraction was separated by anion exchange HPLC was collected (about 56% of the total UV absorption). Reverse phase HPLC analysis of the two-charge fraction from ion exchange purification showed that MepA$^{2'}$pU (18%) and MepA$^{3'}$pAU (46%) accounted for about 64% of this fraction. MepA$^{2'}$pU and MepA$^{3'}$pU were purified from the concentrated two-charge fractions by reverse phase HPLC. The purified MepA$^{2'}$pU and MepA$^{3'}$pU were desalted by passing through a preparative C18 column as described for the isomers of MepApA. The MepA$^{2'}$pU and MepA$^{3'}$pU were found to be pure by both ion exchange and C18 reverse phase HPLC (96 and 98%, respectively). Both MepA$^{2'}$pU and MepA$^{3'}$pU resisted APH hydrolysis. MepA$^{2'}$pU also resisted RNase T_2 hydrolysis. After RNase T_2 hydrolysis, the products from MepA$^{3'}$pU were MepA$^{3'}$p and uridine. The subsequent APH hydrolysis converted MepA$^{3'}$p to MepA. PD I hydrolysis yielded MepA and UMP. The subsequent APH hydrolysis converted UMP into uridine. About 20% MepA was hydrolyzed to 5′-AMP by PD I.

The preparation of U$^{2'}$pU$^{2'}$pU and A$^{2'}$pA$^{2'}$pA is part of a separate study and will be described in a separate paper.

4.3. Adsorption isotherms

The binding of MepA$^{3'}$pA and MepA$^{2'}$pA to montmorillonite was determined by the reported procedures (Wang and Ferris, 2001) with the exception that the studies were carried out on a 200 μL scale. A solution of the purified dimers was prepared in 0.2 M NaCl, 0.075 M $MgCl_2$ and 0.1 M HEPES (pH 8) and the concentration of the dimers was determined by a UV spectroscopy. The dimers were added to 10 mg of clay in a 1.7 mL centrifuge tube and the volume was adjusted to 200 μL by adding the standard buffer. The samples were vortexed and kept at room temperature for 1 h and then centrifuged at 14,000 rpm for 10 min. The concentration of the dimers in the supernatant was determined by reverse phase HPLC. By comparing the peak area decrease of the samples with a control solution, which had no clay, the binding on clay was determined. The same procedure was used to measure the binding of MepA$^{3'}$pU and MepA$^{2'}$pU.

4.4. Elongation of the purified dimers and the characterization of the products

The purity of the starting compounds (MepA$^{2'}$pA, MepA$^{3'}$pA, MepA$^{2'}$pU, MepA$^{3'}$pU, ImpU and ImpA) was monitored by both reverse phase and ion exchange HPLC (more than 96% pure). The dimers were dissolved in the standard buffer and then their concentration was determined by UV spectroscopy. The dimers and activated monomers were added to 5 mg of montmorillonite and the total volume was quickly adjusted to 100 μL by the addition of the standard buffer. The samples were vortexed and kept at room temperature for 18 h. The supernatant was removed after centrifuging the samples at 14,000 rpm for 10 min and the residual montmorillonite was washed twice with 300μL 0.1 M ammonium acetate. Analysis of the supernatant by ion exchange HPLC showed that the three-charge compounds was the major products (4–8.7% of the total UV absorption) and that four-charge and the longer products were only 0.2% of the total UV absorption. The one-to-three charge fractions were each collected using an ion exchange HPLC, and lyophilized. Each fraction was dissolved in 1 mL double distilled water, and analyzed by reverse phase HPLC. The components in the three-charge fraction were further separated by reverse phase HPLC. The purified products were dialyzed for 5 h at 4 °C and lyophilized. The products were dissolved in 700 μL of double-distilled water. The purities of MepA$^{2'}$pA, MepA$^{3'}$pA, MepA$^{2'}$pU, MepA$^{3'}$pU were 97, 98, 96 and 98%, respectively. Structure analysis was performed by mixing 100 μL solution of oligomers with 100 μL 0.1 M Tris buffer (pH 9) and 25 μL APH (1 unit/ml in 0.1 M Tris buffer), and then was incubated at 37 °C overnight. To 400 μl samples was added 200 μL RNase T_2 (2 unit/ml in 0.01 M ammonium acetate, pH 4.2) for overnight at 37 °C. After reverse phase HPLC analysis of the samples, 150 μL APH Tris buffer (pH 9) and 40 μL APH solution (1 unit/ml in 0.1 M Tris, pH 9) was added into 300 μL solutions after RNase T_2 hydrolysis. Then the mixture was vortexed and kept at 37 °C overnight.

The competitive addition reactions of the 2′,5′-linked dimers and 3′,5′-linked dimers and the characterization of the products were identical to the above-mentioned procedures.

The oligomerization of a mixture of 3.75 mM ImpA, 3.75 mM ImpU and 7.5 mM MepA and of a mixture of 3.75 mM ImpU + 3.75 mM ImpA was done following the standard procedure for the monomer oligomerization. The combined supernatants were separated using ion exchange HPLC, then the three-charge fraction of 3.75 mM ImpA + 3.75 mM ImpU reaction and the two-charge and three-charge fractions of 7.5 mM MepA + 3.75 mM ImpU + 3.75 mM ImpA were further purified using reverse phase HPLC. After the dialysis and lyophilization, the collected fractions were characterized by RNase T_2 and APH hydrolysis.

For the competitive reactions of the MepApN dimers (N = A, U), the purified compounds were dissolved in the standard buffer, their concentration was determined UV spectroscopy, and then they were added to a 5 mg of clay. Then

12.5 μL 30 mM ImpU and 12.5 μL 30 mM ImpA were added into the same 1.7 mL centrifuge tube. The total volume was adjusted to 100 μL by adding the standard buffer. The samples were vortexed and kept at room temperature for 18 h. The products were separated and characterized by the same procedures mentioned above.

4.5. Oligomerization and characterization of the MepA-initiated oligomers

The oligomerization of 15 mM ImpU with 15 mM MepA was carried out following standard procedures (Ferris and Ertem, 1993). Anion exchange HPLC analysis demonstrated that in the 15 mM ImpU + 15 mM MepA reaction, the longest oligomers had 5 charges (0.14% of the total UV absorption). The oligomer mixtures in the combined supernatant of 15 mM ImpU with 15 mM MepA reaction were separated according to the numbers of charges in the oligomers by ion exchange HPLC. The one-charge, two-charge, three-charge and four-charge fractions were collected. Each fraction was then analyzed by reverse phase HPLC. The oligomers from 15 mM ImpU oligomerization were 34% in the three-charge fraction and 26% in the four-charge fraction. The percentage of MepA-initiated oligomers was increased as the chain got longer (from 65.7% in three-charge fraction to 73.9% in four-charge fraction). Because MepApU-initiated oligomers are more hydrophobic than the oligomers of 15 mM ImpU oligomerization, MepA-initiated oligomers in the two-charge fraction, three-charge fraction and four-charge fraction had longer retention times on reverse phase HPLC than the oligomers from 15 mM ImpU alone. Materials in the two-charge fraction, the three-charge and four-charge fractions were purified and collected by reverse phase HPLC. After concentrating using a rotary evaporator, the fractions were dialyzed for 5 h at 4 °C and lyophilized. The purified fractions were dissolved in the double distilled water, and then analyzed by C18 reverse phase HPLC to determine their homogeneity. In this experiment, the MepA-initiated oligomers were the target peaks. One general characteristic of MepA-initiated oligomers is their resistance to APH hydrolysis because of their lack of a terminal phosphate. Therefore, an aliquot of the fractions (300 μL) was mixed with 200 μL Tris buffer (pH 9) and 40 μL APH solution (1 unit/mL in 0.1 M Tris), and was incubated for 15 h at 37 °C. Reverse phase HPLC analysis could distinguish the molecules with terminal phosphate (non-MepA-initiated products). Then RNase T_2 and the subsequent APH hydrolysis (Ding *et al.*, 1996) were used to characterize the structure of the oligomers. The analysis of the hydrolyzed samples was performed on an Alltima C18 reverse phase HPLC column.

The oligomerization reaction of 15 mM ImpU with 1.5 mM MepA on montmorillonite was carried out following the same procedures for 15 mM ImpU with 15 mM MepA oligomerization. In 15 mM ImpU with 1.5 mM MepA reaction, the longest oligomers contained eleven charges (Table I). After ion exchange HPLC separation

of the oligomers, each fraction was analyzed by reverse phase HPLC. It was found that MepA-initiated oligomers in two-charge fraction, three-charge fraction were separated effectively from the oligomers of 15 mM ImpU oligomerization using a reverse phase HPLC. However, for the longer oligomers, there was not a clear single peak but rather a peak cluster in the profiles of the fractions. The peak clusters in MepA-initiated tetramers and the longer oligomers were purified by reverse phase HPLC. It is impossible to figure out the structures of all the compounds in the peak clusters because there was no method available to separate the substances in the peak clusters to single compounds. However, the structural units present were determined by RNase T_2 and APH hydrolysis. All the peaks in the peak clusters were found to resist APH hydrolysis. After RNase T_2 hydrolysis, uridine, $U^{2'}pU$ and $U^{2'}pU^{2'}pU$ were cleaved from the 3′-terminal of the oligomers. After the subsequent APH hydrolysis, the peak area increase of $U^{2'}pU$ and $U^{2'}pU^{2'}pU$ meant that these additional molecules were from the internal sequences of the oligomers. The MepA produced was used to calculate the percentage of molecules initiated by $MepA^3pU$.

The oligomerization of 15 mM ImpA with 15 mM MepA on montmorillonite was carried out following the same procedures used in the 15 mM ImpU with 15 mM MepA reaction. Ion exchange HPLC showed that the longest oligomers had six charges, and the last two fractions accounted for 2.1% of the total UV absorption (Table I). After ion exchange HPLC separation of the combined supernatant of 15 mM ImpA with 15 mM MepA, the fractions with one to four charges were collected. It was found that the target peaks in two-charge fraction (MepA-initiated dimers, two peaks) could be separated from the oligomers of 15 mM ImpA oligomerization by reverse phase HPLC. In three-charge fraction (MepA-initiated trimers), reverse phase analysis demonstrated that MepA-initiated trimers were mixed with the oligomers of 15 mM ImpA oligomerization. The three-charge fraction was treated by APH first. Ion exchange analysis demonstrated that after APH hydrolysis the three-charge fraction was divided into two portions: one-charge portion and three-charge portion. Then ion exchange HPLC was used to separate the products and the remaining three-charge fraction was collected. Reverse phase HPLC analysis of the remaining three-charge fraction demonstrated that there were about six major peaks. The six major peaks were collected and separated by reverse phase HPLC. Reverse phase analysis of the four-charge fraction (MepA-initiated tetramers) demonstrated that MepA-initiated tetramers oligomers were mixed with the oligomers of 15 mM ImpA oligomerization. The four-charge fraction was hydrolyzed by APH and ion exchange HPLC analysis demonstrated that after APH hydrolysis the four-charge fraction was divided into two portions: two-charge portion and four-charge portion. The remaining four-charge fraction was collected from the ion exchange column. Reverse phase analysis of the remaining four-charge fraction demonstrated that there were five major peaks. All the five major peaks were purified using reverse phase HPLC. After concentration, dialysis and lyophilization of the purified fractions, they were analyzed for purity and characterized by RNase

T_2 and APH hydrolysis. In this fraction the identified MepA-initiated tetramers accounted for about 5% of the UV absorption.

The oligomerization of 15 mM ImpA with 1.5 mM MepA on montmorillonite was carried out following the same procedures for 15 mM ImpU with 15 mM MepA reaction. Ion exchange HPLC analysis showed that the longest oligomers contained ten charges (Table I). The MepA-initiated dimers and trimers were purified and characterized. MepA-initiated dimers in two-charge fraction were separated from the oligomers of 15 mM ImpA oligomerization by reverse phase HPLC. In three-charge fraction (MepA-initiated trimers), reverse phase analysis demonstrated that MepA-initiated trimers oligomers were mixed with the oligomers of 15 mM ImpA oligomerization. Therefore three-charge fraction was hydrolyzed by APH and ion exchange HPLC was used to separate and the collect the three-charge fraction. Reverse phase analysis of the three-charge fraction demonstrated that there were six major peaks. All the six major peaks were purified using a reverse phase HPLC. After concentration, dialysis and lyophilization, the structure analysis of the oligomers in each fraction was performed using RNase T_2 and APH hydrolysis.

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