One-Step, Regioselective Synthesis of up to 50-mers of RNA Oligomers by Montmorillonite Catalysis

Wenhua Huang[†] and James P. Ferris*

Contribution from the Department of Chemistry and Chemical Biology and New York Center for Studies on the Origins of Life and Rensselaer Polytechnic Institute, Troy, New York 12180

Received March 15, 2006; E-mail: ferrij@rpi.edu

Abstract: 5′-Nucleotides of A and U with the phosphate activated with 1-methyladenine generate RNA oligomers containing 40−50 monomers in 1 day in reactions catalyzed by montmorillonite. The corresponding monomers of C give oligomers that are 20−25-mers in length after a 9-day reaction. It was not possible to determine the chain lengths of the oligomers of G since they did not give well-defined bands on gel electrophoresis. Co-oligomers of A and U as well as A, U, G, and C were also prepared. The oligo(A)s formed were separated by gel electrophoresis, and the bands of the 7−39-mers were isolated, the 3′,5′-phosphodiester bonds were cleaved by RNase T_2, and the terminal phosphate groups were cleaved with alkaline phosphatase. HPLC analysis revealed that the proportions of A5′pp5′A, A, A2′pA, and A2′pA2′pA formed were almost the same for the long and shorter oligomers. A similar structure analysis performed on the oligo(U)s established that the proportions of U5′pp5′U, U, U2′pU, U2′pU2′pU, U2′pU2′pU2′pU, and U2′pU2′pU2′pU2′pU did not vary with chain length. The structural analysis of the oligomers of A revealed that 74% of the phosphodiester bonds were 3′,5′-linked a value slightly greater than 67% observed when imidazole was the activating group. 61% of the bonds in the U oligomers were 3′,5′-linked, which is almost 3 times greater than the 20% measured when imidazole was the activating group. The potential significance of these data to the origin and early evolution of life is discussed.

Introduction

Studies on the role of catalysis in prebiotic synthesis have focused on the montmorillonite-catalyzed formation of RNA oligomers. Oligomers that were 10-mers in length were observed in our initial studies with imidazole as the phosphate-activating group (Figure 1a).[1−3] Elongation of a decameric primer, bound to montmorillonite, by the daily addition of the 5′-phosphorimidazolide of adenosine (ImpA)(Figure 1a) for 12−14 days resulted in the formation of up to 50-mers.[4,5]

The search for more effective activating groups for nucleotides resulted in the discovery that 4-(dimethylamino)pyridine and 1-methyladenine accelerated the condensation of monomers of RNA to oligomers.[6,7] The reactivity of adenine-5′-phosphoro-1-methyladeninium (MeadpA) (Figure 1c) was investigated in greater detail because methylated adenines may have been present on the primitive Earth.[8] These studies demonstrated that it was not necessary to elongate a 10-mer primer for 12−14 days to obtain oligomers containing 30−50 monomer units since
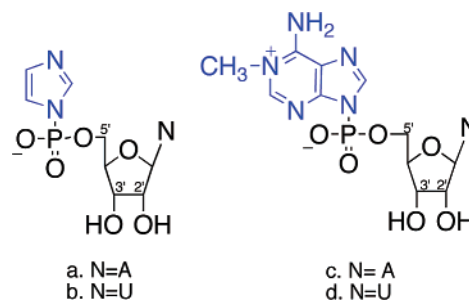

Figure 1. Activated monomers of nucleotides: (a) 5′-phosphorimidazolide of adenosine (ImpA), (b) 5′-phosphorimidazolide of uridine (ImpU), (c) adenosine 5′-phosphoro-1-methyladenine (MeadpA), (d) uridine 5′-phosphoro-1-methyladenine (MeadpU).

these formed in 1 day.[9] An objective of the present research was the determination of whether the 3′,5′-regioselectivity of phosphodiester bond formation changed in the longer oligomers as suggested by the regioselectivity observed in the dimers, trimers, and tetramers formed.[3,10] The proportion of 3′,5′-selectivity was found to be about 67% in the dimers, trimers, and tetramers formed from ImpA and 20% in those formed from ImpU.[3,11] The 3′,5′-regioselectivity exceeded 80% in the dimers, trimers, and tetramers formed from MeadpA (Figure 1c).[7] Dimers were the only oligomers formed in the absence of montmorillonite.[7] This regioselectivity study will be extended

† Current address: Department of Chemistry, Tianjiin University, 92 Weijin Road, Nankai District, Tianjin 300072, P. R. China.
(1) Weimann, B. J.; Lohrmann, R.; Orgel, L. E.; Schneider-Bernloehr, H.; Sulston, J. E. *Science* **1968**, *161*, 387−388.
(2) Ferris, J. P.; Ertem, G. *Science* **1992**, *257*, 1387−1389.
(3) Ferris, J. P.; Ertem, G. *J. Am. Chem. Soc.* **1993**, *115*, 12270−12275.
(4) Ferris, J. P.; Hill, A. R., Jr.; Liu, R.; Orgel, L. E. *Nature* **1996**, *381*, 59−61.
(5) Ferris, J. P. *Origins Life Evol. Biosphere* **2002**, *32*, 311−332.
(6) Prabahar, K. J.; Cole, T. D.; Ferris, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 10914−10920.
(7) Prabahar, K. J.; Ferris, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 4330−4337.
(8) Miller, S. L. *Nat. Struct. Biol.* **1997**, *4* (3), 167−169.
(9) Huang, W.; Ferris, J. P. *Chem. Commun.* **2003**, 1458−1459.
(10) Miyakawa, S.; Ferris, J. P. *J. Am. Chem. Soc.* **2003**, *125*, 8202−8208.
(11) Ding, Z. P.; Kawamura, K.; Ferris, J. P. *Origins Life Evol. Biosphere* **1996**, *26*, 151−171.
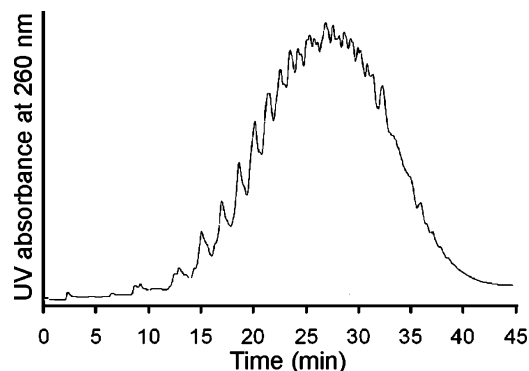
Figure 2. Anion exchange HPLC analysis of the 7−50-mers of the oligomers formed by the 3 day reaction of MeadpA on montmorillonite. The supernatant was removed by centrifugation and extraction of the oligomers from the montmorillonite with pH 9, 0.1 M $Na_4P_2O_7$, dialysis, and two concentrations using Microcon spin tubes.
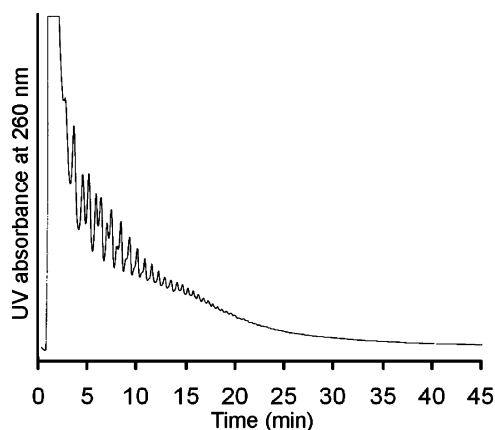

Figure 3. Anion exchange analysis of the 9 day reaction of MeadpC on montmorillonite. The reaction mixture was centrifuged to remove the supernatant, the montmorillonite was extracted with pH 9, 0.1 M $Na_4P_2O_7$, and the extract was analyzed by anion-exchange HPLC.

to oligomers formed from MeadpU (Figure 1d). Finally, the formation of oligomers from MeadpC and MeadpG and mixtures of activated nucleotides are discussed. The objective here was to determine if the montmorillonite-catalyzed reaction of methyladenine activated monomers also gives good yields of long oligomers composed of 2, 3, and 4 different monomers. Such oligomers will be useful in studies on the origins of, and processes in, the RNA world.

Results and Discussion

In the present study 40−50-mers of A and U formed in 1 day, in the absence of a primer, when 1-methyladenine was used as the activating group (Figure 2). In addition, the ratio of montmorillonite to monomer was reduced by a factor of 5 over that used in our previous studies,[5] while the concentrations of activated monomers remained the same at 15 mM. The oligomers of G had longer HPLC retention times but no bands for individual oligomers when we attempted to determine their chain lengths by gel electrophoresis. This may be due to the association of the oligo(G)s.[12] The oligomers formed from MeadpC were shorter than those from A and U (Figure 3). The highest yields of C oligomers were attained after a 7 to 9 day reaction.
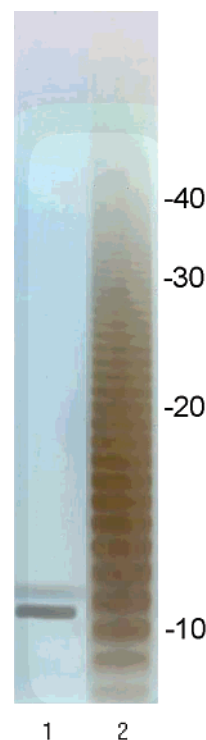
(12) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984; pp 315−320.

Figure 4. Gel electrophoresis of the oligomers formed from the reaction of a 1:1 mixture of MeadpA and MeadpU on montmorillonite (lane 2) together with the electrophoresis of A(pA)$_9$ (lane 1) which migrates one step slower than (pA)$_{10}$ due to the absence of the 5′-terminal phosphate. Reaction conditions and workup were the same as those described in Figure 2. The oligomers were visualized with silver stain.[13]

Oligomers formed in the reaction of mixtures of the activated monomers of A and U; A and G; A and C; and A, U, G, and C. Gel electrophoresis indicated that maximum chain lengths of the AU oligomers were about the same as those formed from the activated monomers of A and U (Figure 4). Hydrolytic studies indicate that approximately equal amounts of A and U were incorporated into the AU copolymer[9] alone, while the oligomers prepared from a mixture of A, U, G, and C were shorter. These conclusions were reached by comparison of the anion exchange HPLC retention times of the longest oligomers in the reactions. The longest oligomers from the reaction of A, U, G, and C had retention times of 17.9 min which is 2.7−5.5 min less than that of the longest oligomers of A, U, G, and AU. The formation of shorter oligomers may be the result of the slower reaction of the activated monomers of C noted above.

Since the maximum chain lengths of the oligomers of A and U never exceeded 40−50-mers, experiments were performed to see if this was due to the degradation of the oligomers during the course of their synthesis. The possibility of the presence of microorganisms in the reaction mixture that degraded the oligomers was considered. Measures to exclude microorganisms included heating montmorillonite in the dry state at 150 °C for 60 h and heating for 30 min in an autoclave at 120 °C in the presence of steam at 2 atm of pressure. The sterile reaction tubes were washed with 70% ethanol before use, and extra care was taken to avoid contamination by ribonuclease (RNase). However no longer chain lengths of oligo(U)s and oligo(A)s were observed by HPLC analysis of the oligomers indicating that neither microorganisms nor RNase were responsible for the termination of RNA oligomer formation at 40−50-mers.
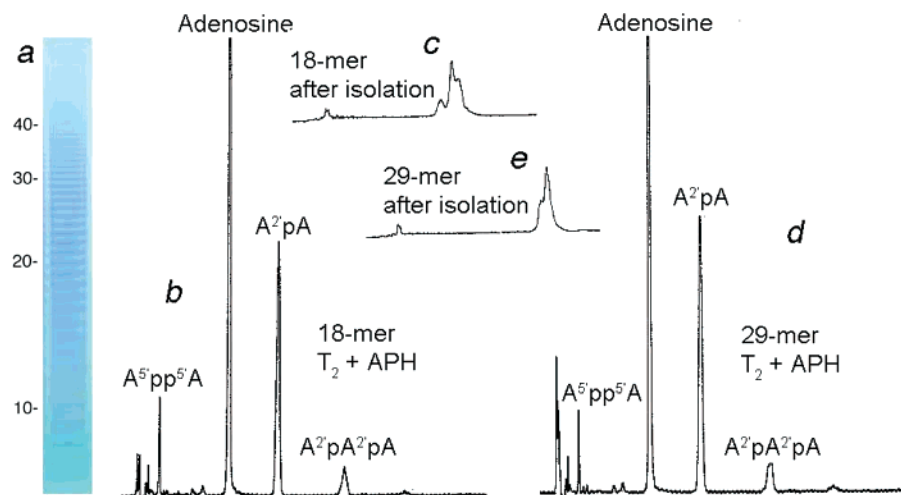
Figure 5. Determination of the structural units present in the oligo(A)s formed by the reaction of MeadpA on montmorillonite: (a) gel electrophoresis analysis of the reaction products, with detection using methylene blue, (c and e) anion-exchange HPLC analysis of the 18- and 29-mers, respectively, extracted from the gel, (b and d) reversed-phase HPLC analysis of the products formed by hydrolysis of the 18- and 29-mers, respectively, with RNase T_2 followed by APH hydrolyses.

The regioselectivity in the phosphodiester bonds in the 7–39-mers formed by the montmorillonite-catalyzed reaction of 1-MeadpA was determined by selective enzymatic hydrolysis. The reaction products were first separated by gel electrophoresis and visualized with methylene blue[13] (Figure 5a). The 5′-phosphorylated 10-mer of A in the reaction mixture was identified by the identity of its migration distance on the gel with that of a synthetic 5′-phosphorylated 10-mer of A (Figure 5a).[5] Each of the bands from 7- to 39-mers were sliced from the gel, and the oligomers were isolated by the crush and extract procedure.[14] The additional peaks in the HPLC traces may be due to oligomers containing pyrophosphate groups that have migration times similar to those of the 5′-phosphorylated oligomer.[5] Hydrolysis of the eluted bands with ribonuclease T_2 (RNase T_2) cleaved the 3′,5′-links and the subsequent treatment with alkaline phosphatase (APH) generated adenosine, $A^{5'}pp^{5'}A$, and dimers and trimers with 2′,5′-linkages (Figure 5b and d).

The structures of the hydrolysis products were established by their coelution with authentic samples on a reversed-phase HPLC column. The structure assigned to $A^{2'}pA^{2'}pA$ was consistent with the molecular weight determined by electrospray mass spectrometry: calculated for the free acid of $C_{30}H_{37}N_{15}O_{16}P_2$ 925.2; found M $-$ H$^+$ 924.3, M $-$ 2H$^+$ 461.6, and M $-$ 2H$^+$ + Na$^+$ 946.0. The relative HPLC peak areas of the hydrolysis products from the 18- and 29-mers were virtually identical (Figure 5b and 5d) indicative of comparable proportions of the same structural units in both oligomers. Hydrolytic data for all the 7–39 oligomers also showed there was little variation in the regioselectivity (Figure 6). This finding demonstrates that clay mineral catalysis generates a structurally similar group of oligomers that have different chain lengths but comparable proportions of 2′,5′- and 3′,5′-linkages.

A comparable analysis was performed on the oligo(U)s formed in the reaction of the MeadpU (Figure 1d.). Since silver stain, methylene blue, and other dyes investigated do not stain oligo(U)s, it was not possible to detect these oligomers on an
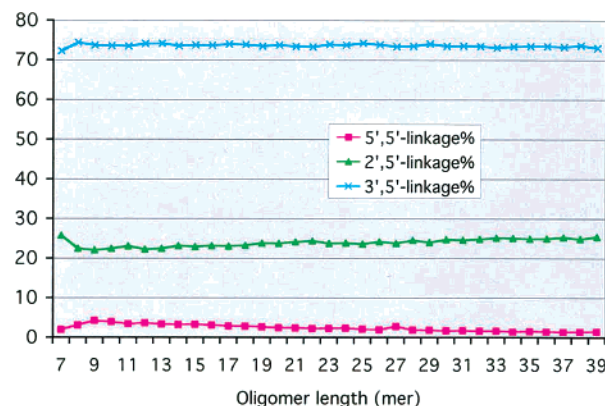
(13) Sambrook, J.; Russell, D. W. *Molecular Cloning A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Press: 2001; Vol. 3.
(14) Sambrook, J.; Russell, D. W. *Molecular Cloning A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Press: 2001; Vol. 1.

Figure 6. Regioselectivity of the bonds formed in the 7- to 39-mers of oligo(A)s as determined by their treatment with RNase T_2 followed by APH.

electrophoresis gel. They were separated on an anion exchange HPLC column and isolated by collecting fractions every 1.8 min (Figure 7a). There was no attempt to collect individual oligomers because they are not completely resolved. HPLC analysis of the collected fractions on an anion exchange column indicated the presence of several oligomers. For example the fourth and eighth fractions gave peaks with retention times between 10.6 and 12.4 min and 17.8–19.6 min, respectively (Figure 7c and 7d). RNase T_2 hydrolysis followed by APH hydrolysis of the fourth and eighth fractions resulted in the formation of $U^{5'}pp^{5'}U$, uridine, and four 2′,5′-linked oligomers (Figures 7b and 7e), the identities of which were established by co-injection with authentic samples, and their structures were consistent with the molecular weights determined by mass spectrometry. The structures of the 2′,5′-linked 2-, 3-, 4-, and 5-mers were also consistent with the Up/U ratios of observed after their phosphodiesterase I hydrolysis.[15] The relative yields of the $U^{5'}pp^{5'}U$, uridine, and four 2′,5′-linked oligomers obtained in fractions 4 and 8 were quite similar. This was demonstrated for the oligomers in all the fractions collected (Figure 8).

Catalysis not only provided long oligomers but also higher proportions of 3′,5′-phosphodiester bonds (61%) using MeadpU

(15) Richards, G. M.; Tutas, D. J.; Wechter, W. J.; Laskowski, M. *Biochemistry* **1967**, *6* (9), 2908–2914.
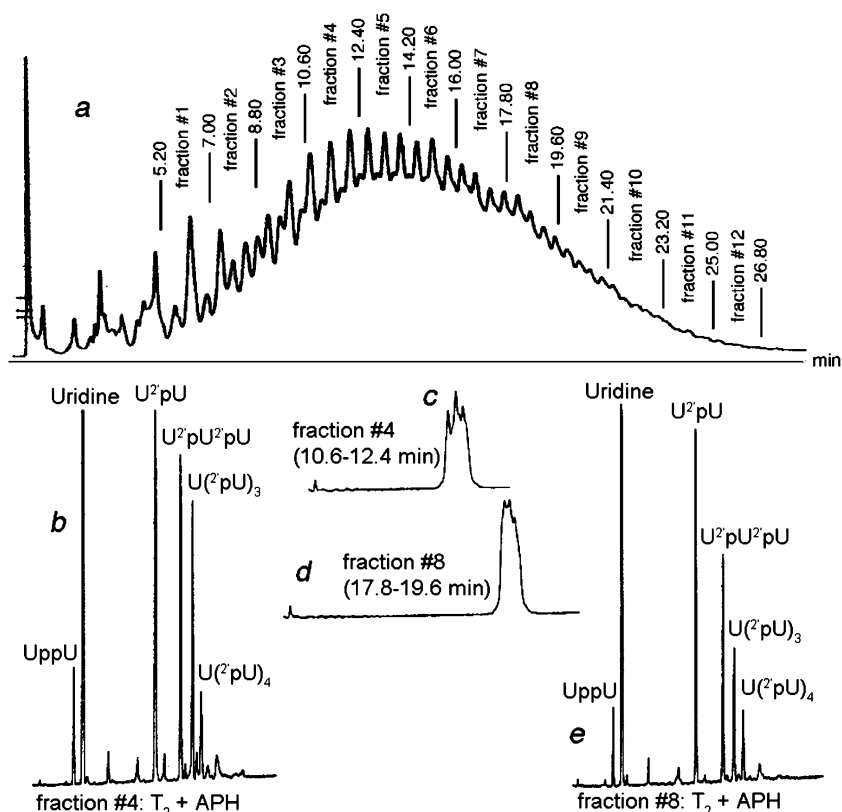
Figure 7. Analysis of the structural units present in the oligo(U)s formed by the reaction of MeadpU on montmorillonite: (a) HPLC analysis of the reaction products showing the fractions collected for analysis, (c and d) anion-exchange HPLC analysis of the fractions 4 and 8, (b and e) reversed-phase analysis of the products formed by hydrolysis of the fractions 4 and 8 with RNase T$_2$ followed by APH.
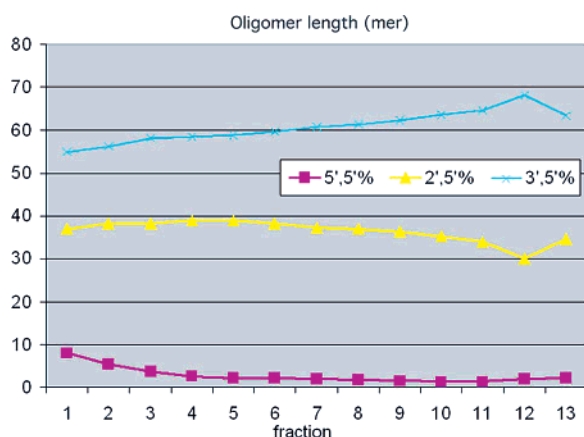

Figure 8. Regioselectivity of the bonds formed in oligo(U)s by montmorillonite catalysis followed by RNaseT$_2$ and APH hydrolyses.

(Figure 1d) than the 20% observed using ImpU (Figure 1b).[11] The corresponding proportion for the oligo(A)s was 74%, a value slightly higher than the 67% observed in the dimers, trimers, and tetramers formed from ImpA.[16] This regioselectivity is lower than the average value of 84% for the dimers, trimers, and tetramers formed from MeadpA.[7] While the actual source of the differences in regioselectivity has not been determined, it could be due to the transition from trimers and tetramers to 7-mers and/or the change of the activating group from imidazole to methyladenine. The dramatic increase in the 3′, 5′-regioselectivity in the oligomers formed from MeadpU is more likely due to the use of methyladenine instead of imidazole as the

(16) Ferris, J. P.; Ertem, G. *Origins Life Evol. Biosphere* **1993**, *23*, 229−241.

activating group. A higher proportion of 3′,5′-links could have resulted in the more rapid evolution to all 3′,5′-phosphodiester bonds during the replication of the RNA oligomers.

The uniform regioselectivity with chain length was not expected. Since oligomers with a 3′-terminal of 3′,5′-phosphodiester bond elongate faster than those with the 2′,5′-bond, we predicted the longer oligomers would have a great proportion of 3′,5′-links.[3,10] Our data show that the proportion of 2′,5′- and 3′,5′-links is almost identical in the oligomers longer than 6-mers. This is consistent with the catalytic sites on montmorillonite interacting only at the 3′-end of the elongating chain where the activated monomer adds to it. This postulate is also consistent with studies on the sequence selectivity of the reaction, which depends only on the nucleotide bound to the 3′-end of the elongating chain, the phosphodiester bond (2′,5′- or 3′,5′-link of the 3′-nucleotide), and the structure of the activated nucleotide reacting with it.[10] The rest of the oligomer chain has little effect on the observed regioselectivity.

The compositions of the 32-mers of the oligomers of A and U formed by montmorillonite catalysis have been derived from the analytical data to be approximately $(A^{5'}pp^{5'}A)_{0.59}A_{16.3}$-$(Ap^{2'}A)_{6.2}(A^{2'}pA^{2'}pA)_{0.76}$ with 74% of the monomers linked by 3′,5′-phosphodiester bonds. The average composition of the mixture containing 32-mers (fraction 10) is estimated to be $(U^{5'}pp^{5'}U)_{0.38}(U)_{13.8}(U^{2'}pU)_{3.7}(U^{2'}pU^{2'}pU)_{1.6}(U^{2'}pU^{2'}pU^{2'}pU)_{0.9}$-$(U^{2'}pU^{2'}pU^{2'}pU^{2'}pU)_{0.32}$ with 61% of the monomers linked by 3′,5′-phosphodiester bonds. The other oligo(A)s formed will have the same proportions of each of the structural units as the 32-mer and 75% of the phosphodiester bonds will be 3′,5′-

linked. The formula for the 32-mers of (oligo)Us reflects the 61% of 3′,5′-links as compared with the 75% in oligo(A)s.

Montmorillonite is a "selective" catalyst in that not all the reaction products have the same structures. It differs from "specific" catalysts that have a high probability for producing the same product each time as is observed in most of the enzymatic catalyses of the reactions of biomolecules. The selectivity of the montmorillonite catalyst has been demonstrated here by the limited variation in the regioselectivity with the length of the oligomers formed from MeadpA (1c) and MeadpU (1d). Sequence selectivity was observed in the reaction of an equimolar mixture of ImpA and ImpC,[10] and the chiral selectivity was observed in the individual reactions of D, L-ImpA and D, L-ImpU.[17,18]

Selective catalysts are more likely than specific catalysts for the formation of the biomolecules that instituted the origins of life. This is because specificity requires a specific catalyst for the formation of a specific product. It is unlikely that specific catalysts were prevalent on the primitive Earth since the available catalytic substances were likely to have been minerals and metal ions. Neither of these is likely to catalyze the reaction of only one substrate, nor will they have the complex structures, like enzymes, required to control a reaction pathway. Selective catalysts generate the formation of a family of structurally related compounds. In addition, in some instances a selective catalyst will catalyze more than one reaction as shown by the montmorillonite-catalyzed formation of vesicles as well as the formation of RNA oligomers.[19] The array of structurally related oligomers formed is equivalent to the quasi-species of Eigen and co-workers that were replicated as a group until a more efficient replicase evolved that took over the replication process.[20]

These studies illustrate how naturally occurring minerals could have catalyzed the formation of the biopolymers that initiated and maintained the first life on Earth. The selectivity of the montmorillonite made it possible to generate an excess of 3′,5′-linked oligomers with similar sequences and regioselectivity. The catalyst would have continued to make these oligomers if the activated monomers were available on the primitive Earth. A problem with the RNA world scenario is the absence of a plausible prebiotic synthesis of the requisite activated mononucleotides

Experimental Section

Materials and General Procedures. MeadpA, MeadpU, MeadpG, and MeadpC were prepared as described in the literature.[7] 4-[*N*-Morpholino]butanesulfonic acid (MOBS) and methylene blue were purchased from Sigma. Acrylamide-bisacrylamide (29:1, 40%) was purchased from Krackeler Scientific, Inc. Dialysis was carried out using membranes from Spectrum Laboratories, while desalting and concentration of the longer oligomers were performed using Millipore Microcon YM-3 spin filters. HPLC was performed using two Waters 501 pumps, a Waters automated gradient controller (model 680), and a Hitachi AS-2000 autosampler. The wavelength of the Hitachi L-4200H detector was set at 260 nm, and the flow rate was 1.0 mL/min. RNase T_2 (500 units/mL) was purchased from Sigma and diluted to 100 units/mL with

(17) Joshi, P. C.; Pitsch, S.; Ferris, J. P. *Chem. Commun.* **2000**, 2497−2498.
(18) Joshi, P. C.; Pitsch, S.; Ferris, J. P. *Origins Life Evol. Biosphere* **2006**, in press.
(19) Hanczyc, M. M.; Fujikawa, S. M.; Szostak, J. W. *Science* **2003**, *302* (5645), 618−622.
(20) Eigen, M.; McCaskill, J.; Schuster, P. *J. Phys. Chem.* **1988**, *92* (24), 6881−6891.

0.015 M NH_4Ac to pH 4.2 before use. Shrimp APH, 1 unit/μL with 10× buffer, was purchased from USB. Phosphodiesterase I (PDI) (129 units/vial) was purchased from Worthington and dissolved in 2.0 mL of buffer (0.11 M Tris, 0.11 NaCl, 15 mM $MgCl_2$, pH 8.9) before use. RNase free water (ultrapure, 0.2 μm filtered) was purchased from USB. Water for dialysis and the mobile phase of HPLC was obtained using a Barnstead Diamond water purification system. Centrifugation was performed on an Eppendorf 5415D instrument. Electrospray ionization (in negatively charged ion mode) mass spectra were obtained on an Agilent 1100 series LC/MSD system operated by Agilent Chem Station (Version A.09.01) and LC/MSD Trap software (Version 4.1). The ion trap was optimized for detection of singly charged ions at *m/z* 900.

General Procedure for the Reaction of 1-MeadpN. To a Pall Nanosep MF 0.45 μm centrifuge device was added sequentially to the filter tube 2.0 mg of Na^+-montmorillonite, 100 μL of 2 × MOBS buffer electrolyte (0.2 M MOBS, 0.4 M NaCl, 0.15 $MgCl_2$, pH 8.0), 50 μL of RNase free water, and 50 μL of 0.06 M 1-MeadpN. The reaction mixture was stirred with a sterile inoculation loop and allowed to stand in a closed container containing water, to limit evaporation of the water in the reaction tube, for 1−3 days at room temperature. The tube was then centrifuged to remove the supernatant from the montmorillonite. Fresh 1-MeadpN and buffer (total volume 200 μL) were added to the clay−oligomer complex in the filtration tube, and the reaction was allowed to proceed for 1 day. The oligomers were eluted from the montmorillonite with 2 × 200 μL washes with 0.1 M pyrophosphate (pH 9.0). The combined washes were desalted using a Microcon tube and then subjected to APH hydrolysis. In some preparations the products were desalted by dialysis against water with an MWCO 2000 membrane, lyophilized, and dissolved in RNase free water (1.8 mL). The resulting solution was filtered through a Nanosep centrifuge tube and further concentrated using a Microcon centrifugal filter device. The same procedure was used for the reactions of mixtures of activated monomers. Equal amounts of each monomer were used, and the combined concentration of all the monomers was 15 mM.

HPLC Separation of MeadpN Oligomers. The separation of oligomers was performed using a Hydrocell NS 1000, 4.6 mm × 150 mm ion-exchange column from BioChrom Labs. The mobile phase was as follows: A, 2 mM Tris, pH 8; B, 2 mM Tris, 0.4 M $NaClO_4$, pH 8. Gradient: linear, 96−46% A and 4−54% B in 50 min. For most samples (60 μL), the fractions were collected every 1.8 min starting from 5.20 min with a total of 12 fractions. For other samples (30 μL), one fraction at 10−30 min was collected. These fractions were either desalted using a Microcon centrifugal filter or dialyzed using a membrane (MWCO 500 or 2000) followed by concentration with a Microcon centrifugal filter.

HPLC Analysis of Oligomer Hydrolysis Products. HPLC analyses were performed using an HEMA-IEC BIO 1000Q 10U, 4.6 mm × 150 mm ion-exchange column and an Alltima C18 5μ, 4.6 mm × 250 mm Alltech reversed-phase column. The eluants from the HEMA column were as follows: mobile phase A, 2 mM Tris, mobile phase B pH 8.0; B, 2 mM Tris, 0.4 M $NaClO_4$, pH 8.0; gradient, linear, 98−48% A and 2−52% B in 50 min. For the Alltima column, the eluants were mobile phase A, 50 mM NaH_2PO_4; B, 50 mM NaH_2PO_4:CH_3CN (70:30); linear gradient for analysis of U oligomers, 100−75% A and 0−25% B in 50 min; isocratic for analysis of A oligomers, 85% A and 15% B.

Enzymatic Hydrolyses. The isolated oligomers (5−60 μL) were digested with RNase T_2 (0.063−0.095 unit/ μL) for 24 h at 37 °C and further hydrolyzed using shrimp APH (0.04−0.12 unit/μL) for 1 h at 37 °C followed by denaturing the enzyme by heating at 65 °C for 15 min. The product was desalted using a Microcon filter. Phosphodiesterase I (PDI) hydrolyses were carried out using 0.6 units/mL for 24 h at 37 °C. The resulting products were analyzed directly by HPLC.

Identification and Isolation of the Enzymatic Degradation Products. Adenosine, 3′-AMP, A²′pA, App⁵′A, uridine, 3′-UMP, U²′pU, and Upp⁵U were identified by co-injection with standard

samples. $A^{2'}pA^{2'}pA$, $U^{2'}pU^{2'}pU$, $U^{2'}pU^{2'}pU^{2'}pU$, and $U^{2'}pU^{2'}pU^{2'}pU^{2'}pU$ were isolated by reversed-phase HPLC, lyophilized, redissolved in 5.0 mL of water, dialyzed against water, lyophilized again, and then dissolved in 200 μL of water. The isolated products were identified by enzymatic degradation using phosphodiesterase I. Each product was further characterized by electrospray mass spectroscopy of each isolated product m/z: $A^{2'}pA^{2'}pA$, calculated for $C_{30}H_{37}N_{15}O_{16}P_2$ 925.0, found 924.0 $[M - H]^-$, 946.0 $[M - 2H + Na]^-$, $U^{2'}pU^{2'}pU$ ($C_{27}H_{34}N_6O_{22}P_2$) 856.1; found ($M - H^+$, 855.2, $M - 2H^+ + Na^+$, 877.1), calculated for the free acid of $U^{2'}pU^{2'}pU^{2'}pU$ ($C_{36}H_{45}N_8O_{30}P_3$) 1162.1; found ($M - H^+$ 1161.1, $M - 2H^+$ 580.3, $M - 2H^+ + Na^+$ 1183.1, $M - 3H^+ + 2Na^+$ 1205.1) and calculated for the free acid of $U^{2'}pU^{2'}pU^{2'}pU^{2'}pU$ ($C_{45}H_{56}N_{10}O_{38}P_4$) 1468.2 ($M - H^+$ 1467.0, $M - 2H^+$ 733.2, $M - 2H^+ + Na^+$ 1489.0).

5′-Phosphorylation of RNA Oligomers. To a mixture of 20 μL of the desalted APH hydrolysis product were added 2.0 μL of 10 × buffer, 2.0 μL of T$_4$-polynucleotide kinase (New England Biolabs), and [γ-^{32}P]-ATP (Amersham), and the mixture was incubated at 37 °C for 1 h and then denatured at 70 °C for 10 min. The resulting mixture was desalted using a Microcon filter and then subjected to gel electrophoresis.

Gel Electrophoresis. Gel electrophoresis (Owl Scientific) using an EC-500 power supply (Apparatus Corporation) was performed on a 20% acrylamide−bisacrylamide denaturing gel on 16 × 28 cm^2 plates. Equal aliquots of the oligomer solution were loaded into the wells of the electrophoresis gel with a decamer (A$_{10}$, Dharmacon Co.) as a marker, and the gel was run for 2.5 h at 1000 V. Then the gels were visualized by an autoradiogram, methylene blue, or silver stain.[13] In the case of the oligomers of A visualized with methylene blue, the bands corresponding to oligomers with the same length were cut and combined, from which the oligomers were recovered by the crush and soak method.[13] The eluates were dialyzed against water (MWCO 2000), lyophilized, and dissolved in RNase free water. The resulting solution was filtered through a Nanosep MF centrifugal device and further concentrated using a Microcon centrifugal filter.

The proportions of $A^{5'}pp^{5'}A$, $A^{2'}p^{5'}A$, and $A^{2'}p^{5'}A^{2'}pA$ in each oligomer was estimated from the expressions below and their HPLC peak areas that were calibrated versus authentic standards. The same procedure was used for the calculation of the number of these functional groups in the oligo(U)s.

$$(A^{5'}pp^{5'}A)_x(A)_y(A^{2'}p^{5'}A)_z(A^{2'}p^{5'}A^{2'}A)_w =$$

formula for the oligomer

$$N = 2X + Y + 2Z + 3W$$

where N is the total number of nucleotides