Adenine Derivatives as Phosphate-Activating Groups for the Regioselective Formation of 3′,5′-Linked Oligoadenylates on Montmorillonite: Possible Phosphate-Activating Groups for the Prebiotic Synthesis of RNA

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Received January 9, 1997[⊗]

Abstract: Methyladenine and adenine *N*-phosphoryl derivatives of adenosine 5′-monophosphate (5′-AMP) and uridine 5′-monophosphate (5′-UMP) are synthesized, and their structures are elucidated. The oligomerization reactions of the adenine derivatives of 5′-phosphoramidates of adenosine on montmorillonite are investigated. 1-Methyladenine and 3-methyladenine derivatives on montmorillonite yielded oligoadenylates as long as undecamer, and the 2-methyladenine and adenine derivatives on montmorillonite yielded oligomers up to hexamers and pentamers, respectively. The 1-methyladenine derivative yielded linear, cyclic, and $A^{5'}$ppA-derived oligonucleotides with a regioselectivity for the 3′,5′-phosphodiester linkages averaging 84%. The effect of pK_a and amine structure of phosphate-activating groups on the montmorillonite-catalyzed oligomerization of the 5′-phosphoramidate of adenosine are discussed. The binding and reaction of methyladenine and adenine *N*-phosphoryl derivatives of adenosine are described.

Introduction

The observation that RNA can function as an enzyme and store genetic information revealed that RNA or RNA-like molecules may have played a central role in the first primitive life on earth.[1,2] This scenario implies that organic precursors for the RNA-like monomers formed spontaneously and then underwent self-condensation reaction on the minerals catalysts to yield short oligonucleotides. The short oligomers could have been elongated on the mineral surface by reaction with RNA monomers.[3]

Studies from our laboratory demonstrated that the condensation of 5′-phosphoroimidazolides of nucleosides on montmorillonite in aqueous solution yields oligonucleotides as long as undecamer.[4−6] The heterogeneous RNA oligomers formed by mineral catalysis could have served as templates for the formation of their complementary oligomers.[7]

The condensation of mononucleotides on montmorillonite and the template-directed synthesis of complementary oligonucleotides are successful when the phosphate group of the mononucleotide is activated by imidazole or substituted imidazoles; nucleoside di- and triphosphates react slowly and undergo hydrolysis.[8] The effect of structure of the amine phosphate-activating group on the oligomerization of adenosine 5′-monophosphate on montmorillonite showed that 4-aminopyridine derivatives are the most effective phosphate-activating groups for oligoadenylate formation on montmorillonite. Oligomers containing up to 12-mers were produced in which the regioselectivity for 3′,5′-phosphodiester bond formation averaged 88%. It was concluded from the study that heterocyclic bases which have 4-aminopyridine type structural units may have been utilized as phosphate-activating groups for the condensation of mononucleotides on the primitive earth.[9] Since purines and pyrimidine derivatives have structural units similar to 4-aminopyridine and they were essential for the prebiotic synthesis of RNA monomers, purines and pyrimidines were investigated as phosphate-activating groups for the condensation of adenosine 5′-monophosphate on montmorillonite.

Experimental Section

General. ^{1}H NMR spectra were recorded on Varian XL-200 and Varian Unity 500 instruments, and ^{13}C NMR spectra were recorded on Varian Unity 500 instrument operating at 125 MHz. NMR spectra were obtained as DMSO and D_2O solutions using the following references: tetramethylsilane (TMS) and 3-(trimethylsilyl)-1-propanesulfonic acid (Tsp) for ^{1}H NMR and DMSO-d_6 solvent peaks for ^{13}C NMR. The chemical shifts are reported in ppm. UV spectra are recorded on Varian Cary 219 spectrophotometer. High-resolution mass spectra (FAB; matrix of dithiothreitol and dithioerythritol) were obtained at the School of Chemical Sciences, University of Illinois, Urbana, IL. The C_{18} Bondapak reverse phase gel 100 Å (mesh 15−20 μm) was purchased from Waters for the use in the preparative reverse phase column. The Dowex 50 W-X8 cation exchange resin (mesh 15−20) was purchased from Bio-rad laboratories and activated by the Cooper procedure.[10] Adenosine 5′-monophosphate (5′-AMP), uridine 5′-monophosphate (5′-UMP), 1-methyladenine, 2-methyladenine hemisulfate, adenine hemisulfate, 3-methyladenine, 1-ethyl-3-((dimethylamino)-propyl)carbodiimide (EDAC), ribonuclease T_2 (RNase T_2), and bacterial alkaline phosphatase (APH) were obtained from Sigma. 2,2′-Dipyridyl disulfide, triphenylphosphine, and 4-(dimethylamino)pyridine were obtained from Aldrich. DMF and DMSO were purchased from Fisher, and ether, acetone, and triethylamine were obtained from Mallinckrodt.

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

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S0002-7863(97)00076-0 CCC: $14.00

Volclay SPV-200 was a gift from the American Colloid Co., Arlington Heights, IL. The homoionic volclay montmorillonite was prepared by titration method.[11]

HPLC analyses were performed on a Waters HPLC system equipped with Lamda-Max model 481 UV detector, on a μ-Bondapak C_{18} reverse phase column using a gradient of 0.005 M NaH_2PO_4 in 5% methanol at pH 3.5 mixed with 0.01 M NaH_2PO_4 (40% methanol) at pH 4.0 and on a HEMA-IEC BIO Q anion exchange column from Alltech using a gradient of 0−0.4 M $NaClO_4$ at pH 8 with 2 mM Tris buffer. No Tris was used when samples were collected for further analysis, and the column was eluted in an isocratic mode using a buffer A (98%) and buffer B (2%) mixture for 6 min followed by a regular gradient elution mode.

General Procedure for the Preparation of Activated Nucleotides (4a, 5a).[12] A mixture of 5′-NMP·H_2O (free acid) (0.33 mmol) and 1-methyladenine (0.049, 0.33 mmol) was dissolved in water (2 mL), and the pH of the solution was adjusted to 5. 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride [EDAC] (0.191 g, 1 mmol) was added to this reaction mixture with stirring. Two additional portions of EDAC (2 × 0.0636 g, 2 × 0.33 mmol) were added at 1 h intervals, and the reaction was allowed to proceed for 4 h. During the reaction, the EDAC was hydrolyzed to the corresponding urea which was separated from the activated nucleotide by passing the reaction mixture through a DOWEX 50 W-X8 cation exchange column and elution with water (150 mL). The collected eluate was lyophilized to yield a colorless solid.

General Procedure for the Preparation of Activated Nucleotides (4b−d, 5b−d).[13] A mixture of 5′-NMP·H_2O (free acid, 0.5 mmol) and heterocyclic base **3** (0.5 mmol) was dissolved in DMF (10 mL) and DMSO (5 mL) in a 50 mL flask, and the solvents were evaporated to 2 mL at a reduced pressure to remove H_2O. The evaporation was repeated twice with DMF (2 × 10 mL). The residue was dissolved in DMF (10 mL) and cooled to −15 °C in an ice−salt mixture. Triethylamine (2 mL) was added to the reaction mixture with stirring followed by a solution of 2,2′-dipyridyl disulfide (0.333 g, 1.5 mmol) and triphenylphosphine (0.393 g, 1.5 mmol) in DMF (5 mL). The stirring was continued for 4 h, and the resulting clear yellow reaction mixture was added dropwise to a 1 L flask containing a cold solution of anhydrous sodium perchlorate (1 g) in a mixture of ether (100 mL), acetone (90 mL), and triethylamine (8 mL) with stirring under argon atmosphere. The stirring was continued for 1 h, and a colorless flocculant solid separated. The stirring was stopped, the solid was allowed to settle for 15 min, the supernatant was drained, and the remaining portion was centrifuged. The resulting colorless pellet was washed twice with 50% acetone−ether mixture, centrifuged, and dried under vacuum.

General Procedure for the Purification of Activated Nucleotides (4,5). The activated nucleotides **4** and **5** were purified using a preparative reverse phase column (30 × 300 mm). The column was eluted with water and then a water−acetonitrile mixture. The pH of the eluents was adjusted to 8−9 with a trace amount of triethylamine; 10 μL of triethylamine is sufficient for 1000 mL of water (excess triethylamine leads to the formation of triethylammonium salts of the activated nucleotides). The chromatography was performed at 4 °C, and the column was eluted under 5−10 psi of argon pressure with the flow rate of 7−10 mL/min. Fractions (100 mL) were collected and analyzed by reverse phase HPLC. The fractions which contained the activated nucleotides were pooled and lyophilized to yield the sodium salts of the activated nucleotides as colorless solids.

General Procedure for the Analysis of Activated Nucleotides (4−7) by Thin-Layer Chromatography (TLC).[14] Thin-layer chromatography was carried out on the precoated 0.25 mm silica gel plates by ascending technique. The solvent system was prepared from *n*-propanol, concentrated ammonia, and water (22:4:14 mL), and the products were identified by cochromatography with the authentic samples.

Adenosine 5′-Phosphoro-1-methyladeninium (1-CH_3adenpA) (4a). Compound **4a** was obtained as a colorless solid (0.130 g, 81.6%) and was shown to be 96% pure by HPLC on a reverse phase column. It showed poor solubility in water and gave a clear solution only if the concentration was <0.005 M. ^{1}H NMR (DMSO-d_6): δ 3.75 (s, 3H), 3.82 (m, 2H), 3.99 (m, 1H), 4.07 (m, 1H), 4.59 (m, 1H), 5.24 (bs, -OH), 5.47 (bs, -OH), 5.87 (d, 1H), 7.29 (s, -NH_2), 8.12 (s, 1H), 8.32 (s, 1H), 8.40 (s, 1H), 8.54 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 37.6, 65.9, 70.8, 73.7, 83.7, 86.9, 118.9, 120.1, 139.6, 146.9, 147.0, 148.6, 149.7, 150.7, 152.8, 156.1. High-resolution MS (positive ion FAB) calcd for $C_{16}H_{20}N_{10}O_6P$ [MH] 479.3711, found 479.1300.

Adenosine 5′-Phosphoro-2-methyladenine (2-CH_3adenpA) (4b). Compound **4b** was obtained as a colorless solid (0.200 g, 79.8%) and was shown to be 94% pure by HPLC on a reverse phase column. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H), 3.78 (m, 2H), 3.89 (m, 1H), 3.96 (m, 1H), 4.52 (m, 1H), 5.26 (bs, -OH), 5.45 (bs, -OH), 5.84 (d, 1H), 7.28 (s, -NH_2), 8.09 (s, 1H), 8.14 (s, 1H), 8.32 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 25.4, 65.0, 70.8, 73.5, 83.5, 87.0, 109.3, 119.4, 139.4, 147.5, 149.7, 152.6, 152.8, 156.2, 160.9, 161.6. High-resolution MS (positive ion FAB) calcd for $C_{16}H_{19}N_{10}O_6PNa$ [MH] 501.3529, found 501.1122.

Adenosine 5′-Phosphoroadenilide (AdenpA) (4c). Compound **4c** was purified by a fractional precipitation method. The colorless solid obtained from the synthesis was dissolved in cold DMF (5 mL), and the solution was centrifuged. The DMF soluble portion was reprecipitated as the sodium salt at 0−5 °C using cold acetone solution of sodium perchlorate. The solid was allowed to settle for 15 min, the supernatant was decanted, the remaining portion was centrifuged, and the supernatant was removed. The resulting colorless pellet was washed twice with acetone, centrifuged, and dried under vacuum. The dried sample was passed through a preparative reverse phase column and eluted with water (100 mL) followed by 15% acetonitrile−water (3 × 150 mL). The fractions which contained **4c** were lyophilized to yield the colorless solid (0.175 g, 72.0%). The compound was shown to be 94% pure by HPLC on an anion exchange column. ^{1}H NMR (DMSO-d_6): δ 3.32 (m, 2H), 3.65 (m, 1H), 4.00 (m, 1H), 4.54 (m, 1H), 5.33 (bs, -OH), 5.50 (bs, -OH), 5.86 (d, 1H), 7.28 (s, -NH_2), 8.09 (s, 1H), 8.11 (s, 1H), 8.19 (s, 1H), 8.31 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 65.0, 70.8, 73.5, 83.5, 87.0, 111.1, 119.1, 139.4, 147.8, 149.7, 152.9, 153.0, 156.2, 160.7, 162.5. High-resolution MS (positive ion FAB) calcd for $C_{15}H_{17}N_{10}O_6PNa$ [MH] 487.3261, found 487.1321.

Adenosine 5′-Phosphoro-3-methyladeninium (3-CH_3adenpA) (4d). Compound **4d** was obtained as a colorless solid (0.189 g, 79.0%) and was shown to be 75% pure by HPLC on a reverse phase column. Compound **4d** hydrolyzed during the purification process using the preparative reverse phase chromatography so was not purified further. ^{1}H NMR (DMSO-d_6): δ 3.80 (s, 3H), 3.85 (m, 2H), 4.00 (m, 1H), 4.25 (m, 1H), 4.50 (m, 1H), 5.80 (d, 1H), 7.32 (s, -NH_2), 8.08 (s, 1H), 8.17 (s, 1H), 8.45 (s, 1H), 8.58 (s, 1H).

Uridine 5′-Phosphoro-1-methyladeninium (1-CH_3adenpU) (5a). Compound **5a** was obtained as a colorless solid (0.120 g, 79.1%) and was shown to be 97% pure by HPLC on a reverse phase column. ^{1}H NMR (DMSO-d_6): δ 3.66 (s, 3H), 3.81 (m, 2H), 3.87 (m, 1H), 3.93 (m, 1H), 4.04 (m, 1H), 5.25 (bs, -OH), 5.47 (bs, -OH), 5.69 (d, 1H), 5.75 (d, 1H), 7.78 (d, 1H), 8.14 (s, 1H), 8.33 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 37.5, 65.7, 70.4, 73.2, 83.3, 87.5, 102.2, 120.4, 140.9, 146.7, 147.1, 148.4, 150.8, 151.0, 163.2. UV λ_{max} (0.01 M HEPES, pH 7): 257 nm. High-resolution MS (positive ion FAB) calcd for $C_{15}H_{19}N_7O_8P$ [MH] 456.3309, found 456.1039.

Uridine 5′-Phosphoro-2-methyladenine (2-CH_3adenpU) (5b). Compound **5b** was obtained as a colorless solid (0.185 g, 77.4%) and was shown to be 95% pure by HPLC on a reverse phase column. ^{1}H NMR (DMSO-d_6): δ 2.34 (s, 3H), 3.58 (m, 2H), 3.78 (m, 1H), 3.84 (m, 1H), 3.96 (m, 1H), 5.19 (bs, -OH), 5.39 (bs, -OH), 5.61 (d, 1H), 5.75 (d, 1H), 7.61 (d, 1H), 8.13 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 25.4, 65.0, 70.4, 73.3, 83.3, 87.2, 102.2, 109.2, 140.2, 147.5, 150.9, 152.6, 160.9, 161.6, 163.2. High-resolution MS (positive ion FAB) calcd for $C_{15}H_{18}N_7O_8PNa$ 478.3127, found 478.0855.

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Uridine 5′-Phosphoroadenine (AdenpU) (5c). Compound **5c** was obtained as a colorless solid (0.165 g, 71.1%) and was shown to be 94% pure by HPLC on a reverse phase column. ^{1}H NMR (DMSO-d_6): δ 3.63 (m, 1H), 3.80 (m, 1H), 3.85 (m, 1H), 3.96 (m, 1H), 5.27 (bs, -OH), 5.48 (bs, -OH), 5.57 (d, 1H), 5.76 (d, 1H), 7.56 (d, 1H), 8.14 (s, 1H), 8. 22 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 65.0, 70.4, 73.3, 83.2, 87.2, 102.2, 111.1, 140.2, 147.7, 150.9, 152.9, 153.0, 160.8, 163.3. High-resolution MS (positive ion FAB) calcd for $C_{14}H_{16}N_7O_8PNa$ 464.2859, found 464.0699.

Uridine 5′-Phosphoro-3-methyladeninium (3-CH$_3$adenpU) (5d). Compound **5d** was obtained as a colorless solid (0.177 g, 77.8%) and was shown to be 74% pure by HPLC on a reverse phase column. Compound **5d** hydrolyzed during the purification process using the preparative reverse phase chromatography and as a result was not purified further. ^{1}H NMR (DMSO-d_6): δ 3.80 (s, 3H), 3.83 (m, 2H), 4.05 (m, 1H), 4.28 (m, 1H), 4.50 (m, 1H), 5.20 (bs, -OH), 5.45 (bs, -OH), 5.67 (d, 1H), 5.75 (d, 1H), 7.54 (d, 1H), 8.53 (s, 1H), 8.66 (s, 1H).

General Procedure for the Clay-Mediated Oligomerization Reaction of Activated Nucleotides. A stock solution (2.5 mL) of activated nucleotide (0.015 M) was prepared from the sodium chloride (0.2 M)/magnesium chloride (0.075 M) electrolyte solution (pH 8). This solution (1 mL) was added to Na^+ montmorillonite (50 mg), the reaction mixture was vortexed for 30 s, the pH was again adjusted to 8, and the mixture was kept at room temperature for 7 days. A control reaction which did not contain montmorillonite was allowed to stand under the same conditions. The reaction with montmorillonite was centrifuged, and the supernatant was removed from the clay and filtered through a 0.45 mm pore filter. Ammonium acetate (0.1 M, 1 mL) was added to the montmorillonite, vortexed, and allowed to stand for 24 h. The ammonium acetate clay mixture was centrifuged, and the supernatant was removed. The supernatant was filtered and combined with the first supernatant.

Effect of pH on the Oligomerization of 2-CH$_3$adenpA (4b) on Montmorillonite. Solutions of compound **4b** (0.015 M, 3 × 2.5 mL) were prepared in the buffer solutions A [MES (2-[*N*-morpholino]-ethanesulfonic acid) (0.1 M), NaCl (0.2 M), and $MgCl_2$ (0.075 M), pH 6], B [HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid) (0.1 M), NaCl (0.2 M), and $MgCl_2$ (0.075 M), pH 7], and C [HEPES (0.1 M), NaCl (0.2 M), and $MgCl_2$ (0.075 M), pH 8]. One milliliter of each solution was added to Na^+ montmorillonite (50 mg), and the reaction mixture was vortexed for 30 s. The reactions proceeded as described in the general procedure for the clay-mediated oligomerization of activated nucleotides. The products were analyzed by anion exchange HPLC.

General Procedure for the Analysis of Oligomers Formed from the Reaction of Compounds 4a−c on Na$^+$ Montmorillonite. The reaction mixture (50 μL) was separated using the anion exchange HPLC, and the monomer, dimer, trimer, and tetramer fractions were collected separately. The collected fractions (200 μL) were reinjected on the anion exchange HPLC to check the homogeneity of the collected samples. The monomer, dimer, and trimer fractions were analyzed by reverse phase HPLC and the presence of pA$^{3'}$pA, pA$^{2'}$pA, pA$^{3'}$pA$^{2'}$pA, pA$^{3'}$pA$^{3'}$pA, and $\overline{{}^{3'}\text{pA}^{3'}\text{pA}^{3'}\text{pA}}$ were determined form their retention times and by coinjecting with the authentic samples.

The proportion of oligomers containing a terminal phosphate group was determined by the APH hydrolysis of the individual fractions. An aliquot (600 μL) was treated with APH (0.33 units) at 37 °C for 4 h. The reaction mixtures were analyzed by HPLC on the reverse phase and anion exchange columns.

The percent 3′,5′-phosphodiester linkages formed in the oligomerization reaction was determined by hydrolysis of the separated oligomeric fractions with RNase T_2. They were also determined by hydrolyses with RNase T_2 followed by APH hydrolysis. The hydrolysates were analyzed by HPLC on the anion exchange and reverse phase columns. The proportion of pyrophosphate-derived oligomers was determined from the sequential hydrolysis by RNase T_2 and APH and analysis of products on the reverse phase HPLC. No oligomers containing the A$^{5'}$ppA$^{2'}$pA structural unit were detected.

Analysis of Oligomers Formed from the Reactions of 1-CH$_3$adenpA (4a), 2-CH$_3$adenpA (4b), and AdenpA (4c) on Na$^+$ Montmorillonite. The RNase T_2 hydrolyses of dimer and trimer fractions (600 μL) from the reaction of 1-CH$_3$adenpA (**4a**) require the use of 1.5 units of enzyme at 37 °C for 5 h and 0.6 unit of enzyme at 37 °C for 4 h for the tetramer fraction (600 μL). The RNase T_2 hydrolysates (600 μL) were adjusted to pH 8 and then treated with APH (0.33 unit) at 37 °C for 4 h. The RNase T_2 hydrolysis of dimer fraction (600 μL) from the reaction of 2-CH$_3$adenpA (**4b**) was carried out with 0.6 unit of RNase T_2 at 37 °C for 4 h and the trimer fraction (600 μL) with 1.0 unit of RNase T_2 at 37 °C for 5 h. The RNase T_2 hydrolysates (600 μL) were adjusted to pH 8 and then treated with APH (0.33 unit) at 37 °C for 4 h. The RNase T_2 hydrolysis of dimer fraction from the reaction of AdenpA (**4c**) was carried out with an aliquot amount of dimer fraction (600 μL) and RNase T_2 (0.6 unit) at 37 °C for 3 h. The RNase T_2 hydrolysis of the corresponding trimer fraction was carried out with an aliquot of trimer fraction (600 μL) and RNase T_2 (1.0 unit) at 37 °C for 5 h. The RNase T_2 hydrolysates (600 μL) were adjusted to pH 8 and then treated with APH (0.33 unit) at 37 °C for 4 h.

General Procedure for Determining the Binding Reaction of 1-CH$_3$adenpA (4a), 2-CH$_3$adenpA (4b), ImpA (6), and 4-(CH$_3$)$_2$NpypA (7) on Na$^+$ Montmorillonite. A solution of activated nucleotide (2 mL, 0.0025 M) was prepared in 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES buffer solution (pH 8) at 4 °C. One milliliter of this solution was added to Na^+ montmorillonite (50 mg), and the reaction mixture was vortexed for 30 s. The reaction mixture was centrifuged at regular intervals and an aliquot (10 μL) was analyzed by HPLC on the anion exchange HPLC, and the extent of binding and oligomer formation was determined from peak areas.

Effect of Concentration on the Oligomerization of 1-CH$_3$adenpA (4a), ImpA (6), and 4-(CH$_3$)$_2$NpypA (7) on Na$^+$ Montmorillonite. Solutions of various concentrations (0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 mM) of activated nucleotide (2 mL) were prepared using sodium chloride (0.2 M), magnesium chloride (0.075 M), and HEPES (0.1 M) buffer solution (pH 8). Each solution (1 mL) was added to Na^+ montmorillonite (50 mg). The reactions were performed as explained in the general procedure for clay-mediated oligomerization of activated nucleotides. The reaction mixtures were analyzed by HPLC on the anion exchange HPLC, and the plot of total yields of oligomers versus concentration is given in Figure 2.

Results and Discussion

In initial studies of the effects of the phosphate-activating group on the oligomerization 5′-AMP on montmorillonite, it was concluded that the nucleophilic displacement of amine-activating group proceeds in a concerted process.[9] The reactivity of the amine as an activating group correlated with its pK_a and with the extent of its stabilization by charge delocalization. In the course of this initial study it was shown that the 4-aminopyridines are more efficient phosphate-activating groups in the montmorillonite-catalyzed oligomerization of activated monomers of 5′-AMP than is imidazole.[5] It appears unlikely that 4-aminopyridines or imidazoles were present in high concentrations on the primitive earth so alternative bases were investigated. Since purines and pyrimidines are essential for the prebiotic synthesis of RNA monomers and they contain the same or similar functionality present in imidazole and 4-aminopyridine, they were investigated as possible activating groups for RNA formation. Previous studies suggested that the most effective activating groups for use in the montmorillonite-catalyzed synthesis of RNA oligomers have pK_a values in the 6−9 range,[9] so purines and pyrimidines with these pK_a values were investigated first. These include 1-methyladenine (pK_a 7.2), 3-methyladenine (pK_a 6.1), 3-methylcytosine (pK_a 7.4),[15] 4-(dimethylamino)pyrimidine (pK_a 6.35), 4-(methylamino)-pyrimidine (pK_a 6.12), and 4-aminopyrimidine (pK_a 5.71).[16]

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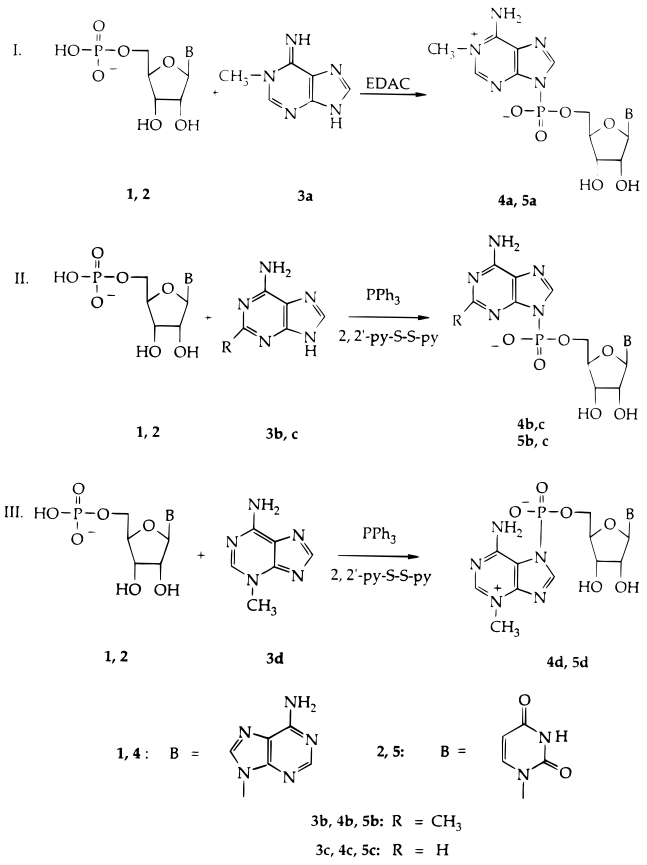
Scheme 1



Later studies were extended to 2-methyladenine (pK_a 5.1), adenine (pK_a 4.12), and 8-methyladenine.[17]

Synthesis and Purification of Activated Nucleotides (4,5). The activated nucleotides **4a** and **5a** were synthesized by a modified procedure of Ivanovskaya *et al.* in which EDAC was used as the condensing agent (Scheme 1).[12] An excess of 1-methyladenine was avoided in the syntheses of **4a** and **5a**, since the separation of activated nucleotides (**4a** and **5a**) from the partially soluble, unreacted 1-methyladenine is difficult. Compounds **4a** and **5a** were separated from the urea derivative formed by the hydrolysis of EDAC by passing the reaction mixture through a Na^+ Dowex 50 cation exchange column and washing the column with water. Compound **4a** showed poor solubility in water and gave a clear solution when its concentration was <0.005 M. Compounds **4b**−**d** and **5b**−**d** were synthesized by using diphenylphosphine and dipyridyl disulfide as condensing agents.[13] Since the free bases of 2-methyladenine and adenine are insoluble in most organic and aqueous solvents, the more soluble hemisulfate salts were used to synthesize the compounds **4 b,c** and **5 b,c**. Unsuccessful attempts were made to synthesize the *N*-phosphoryl-5′-derivatives of the other purine derivatives 8-methyladenine and N^6-(methylamino)purine. The pyrimidine derivatives, 3-methylcytosine, 4-aminopyrimidine, 4-(methylamino)pyrimidine, and 4-(dimethylamino)pyrimidine were used in attempts to synthesize their respective *N*-phosphoryl-5′-derivatives but only diadenosine 5′,5′-pyrophosphate ($A^{5'}$ppA) was obtained as the product. The *N*-phosphoryl-5′-derivatives of pyrimidines may have formed in the reaction mixture which then reacted rapidly with the nucleoside 5′-monophosphate to yield $A^{5'}$ppA.

The activated nucleotides **4** and **5** were purified on a preparative reverse phase column at 0−4 °C. They were eluted with water followed by a water−acetonitrile mixture. The eluent pH was adjusted to 8−9 by adding a few microliters of triethylamine to minimize the acid hydrolysis of the activated nucleotide. The strong binding of adenine to the free silanol groups of the reverse phase column caused special problems in the purification of **4c**. The peak for adenine tailed and overlapped with that of **4c** so it was not possible to purify **4c** by reverse phase HPLC,[18] and addition of dimethylformamide as a reverse phase modifier did not eliminate tailing.[19] It was possible to remove the adenine from **4c** by its selective precipitation by dissolution of **4c** in DMF, centrifugation to remove the adenine, and then reprecipitation of **4c** by acetone addition to the supernatant. This procedure was repeated twice to obtain the pure **4c**. Compounds **4d** and **5d** hydrolyzed during the preparative reverse phase column chromatography, therefore the oligomerization of **4d** was performed on sample which was 75% pure.

Structural Characterization of Activated Monomers (4, 5). The structures of **4a**−**d** were determined by NMR and high-resolution FAB mass spectrometry. The phosphorylation of the adenines could have taken place at positions 9, 7, and N^6, but reverse phase HPLC and silica gel TLC indicated the presence of only one phosphorylated adenine derivative. To our knowledge, the phosphorylation of adenine derivatives has not been reported in the literature. Initial NMR studies of **4a**−**d** in DMSO and D_2O did not reveal the site of phosphorylation because these compounds have two adenine rings, consequently it was not possible to distinguish the ^{1}H and ^{13}C NMR peaks of the adenine-activating group from the nucleobase adenine moiety. Thus, the corresponding derivatives of uridine 5′-monophosphate (**5a**−**d**) were synthesized, and the ^{1}H and ^{13}C NMR spectra were measured. Comparison of NMR chemical shifts for the adenine protons and carbons in **4a**−**d** with those of the carbons and hydrogens in the adenine-activating groups in **5a**−**d** made it possible to assign the NMR peaks of the phosphorylated adenine of compounds **4a**−**d** (Tables 1 and 2).

The isomers of acyladenines were identified from the differences between the chemical shifts of H-2 and H-8 of adenine protons ($\Delta\delta$).[20] Attempted determination of the site of phosphorylation of the adenine ring by correlation of the difference in chemical shifts of the H-8 and H-2 ring protons with those of the acyladenines was not successful.

It was possible to establish the sites of the phosphorylation of the adenines by comparison of the ^{13}C NMR chemical shifts of the phosphorylated adenine with that of the nonphosphorylated derivative.[21,22] The peak upfield in **5c** (δ 111.1) was assigned to C-5 since the highest π-electron density is predicted to be at that carbon. The other carbons have signals down field at δ 160.8, 153.0, 152.9, and 147.7, which are assigned to C-6, C-2, C-4, and C-8 respectively.[23] Phosphorylation resulted in the shift of the C-5 signal upfield from δ 120 to 111 and the downfield shift of C-8 from δ 139 to 147 due to the presence of 9-phosphoryl substitution. The C-6 resonance value was

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Table 1. ^{1}H NMR Chemical Shift Values for the Aglycon Protons of the Activated Nucleotides

	DMSO-d_6					D_2O				
	activating group			nucleobase		activating group			nucleobase	
	H-2	H-8	$\Delta\delta$	H-2	H-8	H-2	H-8	$\Delta\delta$	H-2	H-8
					Adenosines					
4a	8.12	8.32	0.2	8.54	8.40	8.15	8.18	0.03	8.07	8.03
4b		8.14		8.32	8.09		8.22		7.97	7.93
4c	8.19	8.31	0.12	8.11	8.09	8.00	8.35	0.35	8.00	7.9
4d	8.45	8.58	0.13	8.17	8.08	8.30	8.44	0.14	8.08	8.06
					Uridines					
5a	8.14	8.33	0.19	7.78[a]	5.69[b]	8.30	8.37	0.07	7.54[a]	5.80[b]
5b		8.13		7.61[a]	5.61[b]		8.41		7.3[a]	5.66[b]
5c	8.14	8.22	0.08	7.56[a]	5.57[b]	8.24	8.48	0.24	7.35[a]	5.70[b]
5d	8.53	8.66	0.13	7.54[a]	5.67[b]	8.51	8.62	0.11	7.45[a]	5.77[b]

[a] H-4. [b] H-5.

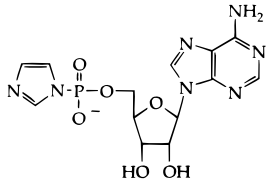
Table 2. 13C NMR Chemical Shift Values for the Aglycon Carbons of the Activated Nucleotides

	activating group						nucleobase				
	C-2	C-4	C-5	C-6	C-8	-CH_3	C-2	C-4	C-5	C-6	C-8
						Adenosines					
4a	148.6	147.0	120.1	150.7	146.9	37.6	152.8	149.7	118.9	156.1	139.6
4b	156.2	152.6	109.3	160.9	147.5	25.4	152.8	149.7	119.4	161.6	139.4
4c	153.0	152.9	111.1	160.7	147.8		156.2	149.7	119.1	162.5	139.4
						Uridines					
5a	148.4	147.1	120.4	151.0	146.7	37.5	150.8	140.9	102.2	163.2	
5b	160.9	152.6	109.2	161.6	147.5	25.4	150.9	140.2	102.2	163.2	
5c	153.0	152.9	111.1	160.8	147.7		150.9	140.2	102.2	163.3	

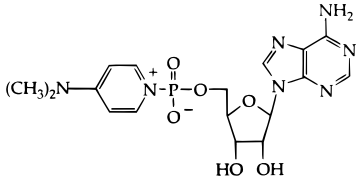
unaffected from the parent adenine chemical shift value which proved that the phosphoryl substitution did not occur at N-7 and N^6 positions. Thus the ^{13}C NMR spectral data confirms that compounds **5c** and **4c** are 9-phosphorylated adenine derivatives. The same reasoning established that 2-methyladenine is also phosphorylated at the 9-position in **4b** and **5b**. The molecular weights determined by high-resolution FAB mass spectroscopy are consistent with structures **4b,c** and **5b,c**.

The conclusion that 1-methyladenine is phosphorylated at the 9-position to give **4a** and **5a** is consistent with the observation that 1-methyladenine alkylated at the 9-position.[24] What remained to be determined is whether the phosphorylated adenine exists in the 6-imino (**8**) or the zwitterionic 6-amino form (**4a** and **5a**).[25,26] The zwitterionic structure was suggested by the high-resolution mass spectra of **4a** and **5a** in which the molecular ions do not indicate the presence of Na^+ counterions. The ^{13}C NMR chemical shift for the carbons of the phosphorylated adenine are comparable to those reported for protonated 1,9-dimethyladenine in which the 1-amino group is protonated.[27]

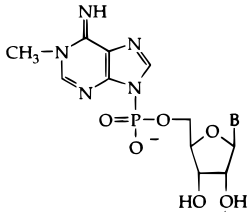
Proof of the zwitterionic structures for the **4a** and **5a** was obtained from UV spectral data. The UV spectra of **5a** was measured at pH 4, 7, and 10 in 0.01 M solutions of sodium acetate, HEPES, and CAPS buffers, respectively, and a maximum was observed at 257 nm at each pH. This result is consistent with reported 257 nm absorbance of protonated 1,9-disubstituted adenine derivatives.[27,28] No absorption would be expected at 257 nm at pH 10 if it was not a zwitterion, since simple adenines are not protonated at pH 10 and thus will not exhibit absorption at 257 nm.



6



7



8

Since 3-methyladenine is alkylated to 3,7-dimethyladenine,[29] it is assumed that **4d** and **5d** are 7-phosphoryl derivatives. These compounds are expected to have positive charges at the 3-position of the adenine ring.[29] It was not possible to purify compounds **4d** and **5d** because they were readily hydrolyzed so they could not be characterized by ^{13}C NMR and FAB mass spectroscopy.

Oligomerization of Activated Nucleotides 4a−d in the Presence of Na^+ Montmorillonite. The oligomerization reactions of activated nucleotides **4a−d** were carried out in NaCl−$MgCl_2$ electrolyte solution at pH 8 in the presence of Na^+ montmorillonite. Control reactions were performed in the absence of montmorillonite. Both the clay and control reactions were analyzed by HPLC using an anion exchange HPLC column (Figure 1).[30] The yields of reaction products formed from the reaction **4a−d, 6**, and **7** are given in Table 3. Montmorillonite-catalyzed reactions of **4a** and **4d** yielded oligomers containing

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Table 3. Percent of Oligomeric Products Formed from the Reactions of **4a**−**d**, **6**, and **7** on Na^+ Montmorillonite

	1	2	3	4	5	6	7	8	9	10	11
4a	32	30	17	11	4	2.2	1.1	0.6	0.3	0.13	0.03
4b	51	26	8	4	0.4	0.1	0.04				
4c	58	32	7	1.8	0.1						
4d	41	27	18	9	3	0.5	0.3	0.05	0.02	*a*	*a*
6	33	24	18	11	4.5	2.3	1.0	0.6	0.3	0.1	0.03
7	26	23	18	15	7	4	1.5	1.0	0.6	0.4	0.06

[a] Peak detected but not integrated.

Table 4. RNase T_2 Hydrolyses of the Oligomers

	dimer			trimer			tetramer		
nucleotide	**4a**	**4b**	**4c**	**4a**	**4b**	**4c**	**4a**	**4b**	**4c**
A	25	18	18	20	14	16	21	*c*	*c*
pA2′pA	10	15	24	*a*	*a*	*a*	*a*	*c*	*c*
A2′pA	*a*	*a*	*a*	18	21	30	3	*c*	*c*
Ap2′Ap	*b*	*b*	*b*	*b*	*b*	*b*	9	*c*	*c*
Ap	41	52	40	27	19	18	46	*c*	*c*
pAp	23	16	18	33	47	38	16	*c*	*c*
AppAp	1	*b*	*b*	2	*b*	*b*	3	*c*	*c*
pAppAp	*a*	*a*	*a*	1	*b*	*b*	2	*c*	*c*

[a] Not expected. [b] Not found. [c] Not determined.

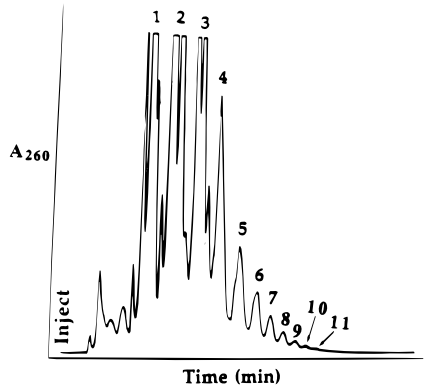

Figure 1. Anion exchange HPLC of the reaction products of **4a** on Na^+ montmorillonite

up to 11 monomer units, while the control reactions, carried out in the absence of montmorillonite, yielded dimers. The montmorillonite-catalyzed reaction of **4b** and **4c** yielded oligomers as long as heptamers and pentamers, respectively. The 1- and 3-methyladenine-activating groups of **4a** and **4d** have pK_a values of 7.2 and 6.1, respectively, which are in the optimal range for the formation of longer oligomers. 2-Methyladenine and adenine have pK_a values of 5.1 and 4.1, respectively, pK_a values which are below 6, the lowest pK_a for the formation of oligo(A)s as long as 10-mers.[9] This lower reactivity suggests that the leaving group was not protonated by acidic sites on the montmorillonite. Reducing the pH of the reaction solution from 8 to 7 and to 6 in an attempt to facilitate the protonation of the adenine leaving group did not enhance the yield of the oligomers from **4b**.

Oligomer Structure and Regioselectivity of Phosphodiester Bond Formation. The structures of the oligomers formed by the montmorillonite-catalyzed reaction of **4a**−**c** were characterized by selective enzymatic hydrolysis. The monomer through tetramer fractions from **4a** were collected from the anion exchange HPLC. The proportion of oligomers having terminal phosphate groups were determined by treating oligomeric fractions individually with APH followed by HPLC analysis on anion exchange and reverse phase columns.[31] The analysis of monomer fraction and its APH hydrolysate on the reverse phase HPLC showed that A5′ppA (5%) was a product along with pA. Since the montmorillonite-catalyzed reactions of **6** and **7** yield cyclic oligomers it is expected that components of the dimer, trimer, and tetramer fractions formed from **4a**, which are not cleaved by APH contain both cyclic and A5′ppA-derived oligonucleotides.

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Table 5. RNase T_2 and APH Sequential Hydrolyses Products of the Oligomers

	dimer			trimer			tetramer		
nucleotide	**4a**	**4b**	**4c**	**4a**	**4b**	**4c**	**4a**	**4b**	**4c**
AppA	3	b	b	3	*b*	*b*	8	*c*	*c*
A	86	84	79	78	78	77	75	*c*	*c*
A2′pA	11	16	22	19	22	23	17	*c*	*c*

[a] Not expected. [b] Not found. [c] Not determined.

The proportion of 3′,5′-phosphodiester linkages was determined by treating the oligomeric fractions with RNase T_2 (Table 4) and subsequently treating the RNase T_2 hydrolysates with APH (Table 5). The hydrolysates were analyzed by HPLC on the anion exchange and reverse phase columns. The amount of A5′ppA formed by the sequential enzymatic hydrolyses indicated the amount of A5′ppA incorporated in each oligomeric fractions. From these results, the composition of the oligomers present in each of the fractions was determined, and the results are given in Table 6 along with the compositions of the oligomers formed in the reactions of compounds **6** and **7**.[5,9]

The reaction of **4a** on montmorillonite yields oligomers which contain high percentages of 3′,5′-phosphodiester linkages. The slower rate of elongation at a 2′,5′-phosphodiester bond was shown by the release of A2′pA on hydrolysis of oligomers with RNase T_2.[5] Some cyclic oligonucleotides were shown to have a 2′,5′-phosphodiester bonds by the release of A2′pAp on RNase T_2 hydrolysis which in turn was hydrolyzed to A2′pA by APH.

The 1-methyladenine-activating group in **4a** gave the longest oligomers and greater percentage of 3′,5′-phosphodiester bonds. It is second only to 4-(dimethylamino)pyridine grouping in **7** in its efficiency in forming long RNA oligomers (Table 6). The yield of cyclic oligomers reached a maximum of 32% in the dimer fraction (actually cyclic trimers). The yield of A5′ppA-contained oligomers increased in the order 3, 8, and 19% going from the dimer, trimer, and tetramer fractions, respectively. The increase with chain length reflects the greater rate of reaction

Table 6. Composition of Oligomers Formed from the Reactions of 1-(CH_3)adpA (**4a**), 2-(CH_3)adpA (**4b**), AdpA (**4c**), ImpA (**6**), and 4-$(CH_3)_2$NpypA (**7**)

product	4a	4b	4c	6[6]	7[9]
	Dimer Fraction				
pA3′pA	45	28	33	52	23
pA2′pA	20	32	43	33	14
pA3′PA3′pA3′	32	40	24	13	63
AppA3′pA	3	*b*	*b*	3	*b*
	Trimer Fraction				
pA3′pA3′A	37	37	43	28	23
pA3′pA2′A	50	56	55	47	64
pA3′pA3′pA3′pA3′	5	5	2	*b*	8
pA3′pA3′pA2′pA3′	*b*	1	*b*	*b*	5
A5′ppA3′pA3′pA	5	b	b	15	*b*
A5′ppA3′pA2′pA	*b*	*b*	*b*	4	*b*
$(Ap)_mA^{5'}ppA(pA)_n$ $(m+n=2)$	3	*b*	*b*	2	*b*
	Tetramer Fraction				
pA3′pA3′pA3′pA	50	*c*	*c*	9	34
pA3′pA3′A2′pA	12	*c*	*c*	22	4
$(pA)_4$ isomers	*b*	*c*	*c*	37	*b*
pA3′pA3′pA3′pA3′pA3′	4	*c*	*c*	*b*	11
pA3′pA3′pA3′pA2′pA3′	15	*c*	*c*	*a*	43
A5′ppA(pA)3	11	*c*	*c*	1	*b*
$(Ap)_mA^{3'}ppA(pA)_n$ $(m+n=3)$	8	*c*	*c*	32	*b*

[a] Not expected. [b] Not found. [c] Not determined.

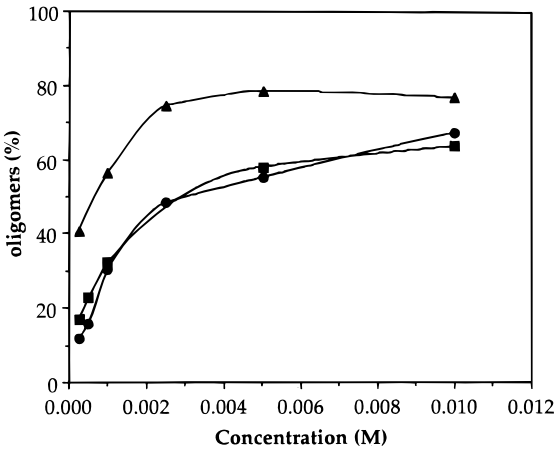

Figure 2. Reactions of **4a, 6** (ImpA), and **7** on Na^+ montmorillonite. Total oligomers formed from **4a** (■), **6** (●), and **7** (▲) at various concentrations. The product yields are not corrected for hyperchromicity.

activated nucleotides with A5′ppA than with another activated monomer.[32]

The time courses of the reactions of different concentrations of compounds **4a**, **6**, and **7** on montmorillonite were studied and plots of total yields of oligomers versus concentration are given in Figure 2. Higher yields of oligomers were obtained from compound **7** than from **4a** and **6**.

Studies on the binding of compounds **4a**, **4b**, **6**, and **7** were performed on the montmorillonite clay at 4 °C (Figures 3 and 4). Most of compound **4a** (85%) binds to montmorillonite in a 15 min time period. This result is similar to the binding of compound **7** to montmorillonite where 95% binds in 15 min. The oligomerization reaction starts as soon as the compound is mixed with clay. More oligomers were formed from the

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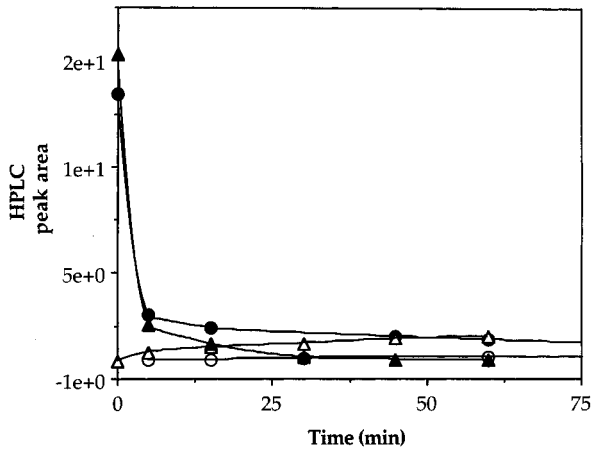

Figure 3. Loss of **4a** (●) and **7** (▲) (0.0025 M) in the presence of Na^+ montmorillonite at pH 8 and 4 °C measured by HPLC peak area. Oligomers formed from **4a** (○) and **7** (△). The oligomer yields are not corrected for hyperchromicity.

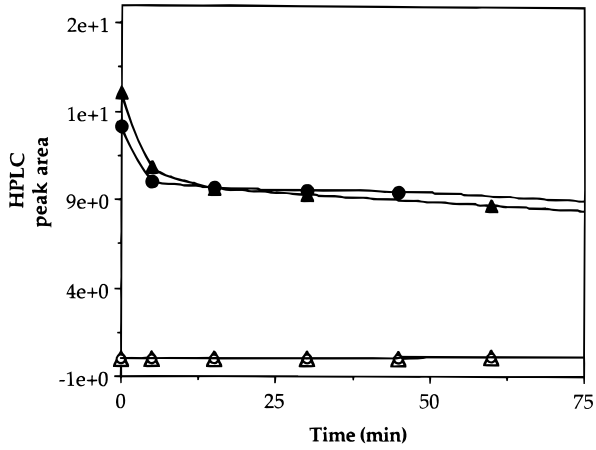

Figure 4. Loss of **4b** (●) and **6** (▲) (0.0025 M) in the presence of Na^+ montmorillonite at pH 8 and 4 °C measured by HPLC peak area. Oligomers formed from **4b** (○) and **6** (△). The oligomer yields are not corrected for hyperchromicity.

Table 7. Oligomer Structural Data (% of 3′,5′-Phosphodiester Bonds Formed on the Reactions of Activated Nucleotides)

fraction	4a	4b	4c	6[6]	7[6]
dimer	88	82	71	68	94
trimer	76	73	73	72	77
tetramer	88	*a*	*a*	∼67	94

[a] Not determined.

reaction of compound **7** on montmorillonite than were observed from the reaction of **4a** (Figure 3). The rates of reaction of **4b** and **6** on montmorillonite are much less than those of **4a** and **7**. They bind less strongly (30 and 45%, respectively), and no measurable amounts of oligomers were observed after a 1 h reaction time.

The average regioselectivity of 3′,5′-phosphodiester bond formation is highest with **7** and **4a** at 88 and 84%, respectively (Table 7). This suggests that leaving groups containing resonance stabilized positive charges are most effective in forming 3′,5′-linked oligomers. The rates of reaction of ImpA (**6**), **4b**, and **4c** are slower, and the average 3′,5′-regioselectivity is in the 70% range.

The compositions of the oligomers formed from **4b** and **4c** were determined as described for the oligomers produced from **4a** and the results are given in Tables 5 and 6. No A5′ppA was found by the the sequential RNase T_2 and APH hydrolysates of dimer and trimer fractions of **4b** and **4c** confirming that no

A[5′]ppA-derived oligomers were formed and the cyclic oligonucleotides were the only APH unhydrolyzed products present in the dimer and trimer fractions. This finding differs from that observed in the oligomers formed from compound **4a** and **6** in which both cyclic and A[5′]ppA-derived oligomers were observed. The percent 3′,5′-phosphodiester linkages in the products of **4c** is lower than in the **4b** reaction products, but comparable that of ImpA (**6**).

Conclusions

Good yields of oligomers are obtained in the condensation reaction of 5′-AMP activated by methylated adenines on Na^+ montmorillonite. In addition, greater than 80% of the phosphodiester bonds contain 3′,5′-linkages, a regioselectivity that is markedly higher than the 67% observed with the imidazole activating group.[5] The 1-methyladenine-activating group forms up to 11-mers and has a efficiency for regioselectivity for oligomer formation that is comparable to that of 4-(dimethylamino)pyridine. Adenine derivatives whose pK_a values are between 6 and 9, such as 1-methyladenine (pK_a 7.2) and 3-methyladenine (pK_a 6.1), yield longer oligomers than adenine (pK_a 4.1) and 2-methyladenine (pK_a 5.2) whose pK_a values are <6. These results support our previous conclusion that the pK_a values of the amines used as a phosphate-activating groups should be in the 6−9 range for the formation of long oligomers.[9] In addition, these studies confirm a previous conclusion that most effective activating groups contain a delocalized positive charge. Thus, compounds **4a** and **7** are are more efficient than ImpA (**6**), **4b**, and **4c** in the regioselective formation of 3′,5′-phosphodiester linkages.

These studies show that purines which contain a 4-aminopyridine type structural unit are efficient phosphate-activating groups and these adenine derivatives could have served as the phosphate-activating groups for the synthesis of RNA on the primitive earth. Since adenine and other purines are required for the prebiotic synthesis of RNA-like monomers, it is likely that adenine or its derivatives would be present in sufficient amounts on the primitive earth to also serve as phosphate-activating groups. 1-Methyladenine is one the reaction products of methylamine with adenine[33] while the corresponding 5′-phosphoro-1-methyladeninium derivative (**4a**) could have been formed by the reaction of 1-methyladenine with ATP.[34]

Acknowledgment. This research was supported by NSF Grant CHE 9619149. HPLC equipment was purchased on NASA Grant NGR-38-018-1148. The Unity 500 NMR was purchased with funds from NSF Grant CHE-9105906 and PHS Grant 1 SIORR6245-01.

JA9700764

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