Effect of Phosphate Activating Group on Oligonucleotide Formation on Montmorillonite: The Regioselective Formation of 3′,5′-Linked Oligoadenylates

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Abstract: The effects of amine structure on the montmorillonite-catalyzed oligomerization of the 5′-phosphoramidates of adenosine are investigated. 4-Aminopyridine derivatives yielded oligoadenylates as long as dodecamers with a regioselectivity for 3′,5′-phosphodiester bond formation averaging 88%. Linear and cyclic oligomers are obtained and no $A^{5'}$ppA-containing products are detected. Oligomers as long as the hexanucleotide are obtained using 2-aminobenzimidazole as the activating group. A predominance of $pA^{2'}pA$ is detected in the dimer fraction along with cyclic 3′,5′-trimer; no $A^{5'}$ppA-containing oligomers were detected. Little or no oligomer formation was observed when morpholine, piperidine, pyrazole, 1,2,4-triazole, and 2-pyridone are used as phosphate-activating groups. The effects of the structure of the phosphate activating group on the oligomer structure and chain lengths are discussed.

Introduction

The observation that RNA can catalyze reactions as well as store genetic information suggests that it may have been the most important biopolymer in the earliest life on earth.[1,2] This scenario implies that the organic precursors to RNA-like molecules formed spontaneously on the primitive earth and condensed to form oligonucleotides. The information in these oligomers was preserved by template-directed synthesis of the complementary RNA oligomers. Problems with this scenario include the failure to find plausible prebiotic synthesis of mononucleotides and polynucleotides and the limited capability of template-directed synthesis.[3,4] Recently, studies from our laboratory emphasized the central role of catalysis in prebiotic chemistry[5] and have demonstrated the formation of oligoadenylates by the condensation of 5′-phosphorimidazolide of adenosine (ImpA) on montmorillonite in aqueous solution.[6,7] The condensation of the dinucleotide pyrophosphate of the adenosine ($A^{5'}$ppA) with ImpA and ImpU results in the formation of oligomers in which over 80% of the phosphodiester bonds are 3′,5′-linked.[5,8,9] Oligomers synthesized by these and other catalytic processes may have been replicated and elongated by template directed processes.[10,11]

The role of the phosphate activating group in oligonucleotide formation on RNA templates or on montmorillonite has not been investigated in a systematic fashion. Studies on the nonenzymatic template directed synthesis of oligonucleotides utilize imidazole or substituted imidazoles as leaving groups because nucleoside di- and triphosphates react slowly and undergo hydrolysis.[12] We have shown that $A^{5'}$ppA and the di- and triphosphates of adenosine do not condense to form oligomers on montmorillonite.[7] More recently, while this research was in progress, Sawai and co-workers reported the use of 2-methylbenzimidazole, triazole, and 3-nitrotriazole as 5′-phosphate activating groups for the UO_2^{2+}-catalyzed formation of 2′,5′-linked oligonucleotides.[13] The oligomers formed using these leaving groups were shorter than those formed using imidazole.

In the present study we report the effect of phosphorus leaving group on oligomer formation and on the regioselectivity of the phosphodiester bond formation in the presence of montmorillonite.

Experimental Section

General. ^{1}H NMR spectra were recorded on Varian XL-200 and Varian Unity 500 instruments. ^{13}C NMR and ^{31}P NMR were recorded on Varian Unity 500 instrument operating at 125 and 202 MHz, respectively. NMR spectra were obtained as D_2O solutions using the following references; 3-(trimethylsilyl)-1-propanesulfonic acid (Tsp) for ^{1}H NMR, DMSO-d_6 solvent peaks for ^{13}C NMR, and 85% H_3PO_4 for ^{31}P NMR spectra. The chemical shifts are reported in ppm. High-resolution mass spectra (FAB; matrix of dithiothreitol and dithioerythritol) were obtained at the School of Chemical Sciences, University of Illinois, Urbana. The C_{18} Bondapak reverse-phase gel 100 Å (mesh 15-20 μm) was purchased from Waters for use in the preparative reverse-phase column. The Dowex 50 W-X8 cation exchange resin (mesh 15-20 μm) was purchased from Bio-rad laboratories and activated by Cooper's procedure.[14] Adenosine 5′-monophosphate (5′-AMP), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDAC), and ribonuclease T_2 (RNase T_2) were obtained from Sigma. Bacterial alkaline phosphatase (APH) was obtained from Sigma and Worthington Biochemical Corp. 2,2′-Dipyridyl disulfide, triphenylphosphine, 4-(dimethylamino)pyridine, 4-aminopyridine, 2-aminobenzimidazole, morpholine, piperidine, and 2-pyridone were obtained from Aldrich. DMF and DMSO were purchased from Fisher, and ether, acetone, and triethylamine were obtained from Mallinckrodt. 4-(Methylamino)-pyridine was synthesized and had a mp of 123-124 °C (lit. mp 124.5-

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125 °C).[15] Montmorillonite 22A clay was obtained from Wards Natural Science Establishment,[16] and the homoionic montmorillonite 22A was prepared by the saturation method.[17]

HPLC analyses were performed on a Waters HPLC system equipped with Lamda-Max model 481 UV detector operating at 260 nm, 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 in 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 at isocratic mode of buffer A 98% and buffer B 2% for 6 min followed by a regular gradient elution mode.

General Procedure for the Preparation of Activated Nucleotides 3a−f.[18] A mixture of 5'-AMP·H_2O (free acid) (0.365 g, 1 mmol) and heterocyclic base **2a−f** (3 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 a mixture of DMF (10 mL) and DMSO (10 mL) and stirred with 2,2'-dipyridyl disulfide (0.666 g, 3 mmol), triphenylphosphine (0.786 g, 3 mmol), and triethylamine (1 mL) under an argon atmosphere for 2 h. The resulting clear yellow reaction mixture was added dropwise to a 1 L flask containing a solution of anhydrous sodium perchlorate (2 g) in a mixture of ether (200 mL), acetone (125 mL), and triethylamine (15 mL) with stirring under argon atmosphere. The stirring was continued for 2 h, and a colorless, flocculant solid separated. The stirring was stopped and the solid allowed to settle for 15 min. The supernatent was drained and the remaining portion centrifuged. The resulting colorless pellet was washed twice with 50% acetone−ether mixture, centrifuged, and dried under vacuum.

General Procedure for the Preparation of Activated Nucleotides (3g−h).[19] A mixture of 5'-AMP·H_2O (free acid) (0.365 g, 1 mmol), heterocyclic base **2g−h** (3 mmol), and 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide hydrochloride (EDAC) (0.955 g, 5 mmol) was dissolved in water (3 mL). The solution was adjusted to pH 5−5.5 with sodium hydroxide solution and stirred at room temperature for 4 h. During the reaction, the EDAC was hydrolyzed to a substituted urea. The activated nucleotide was separated from the urea derivative by passing the reaction mixture through a Dowex 50 W-X8 cation-exchange column. The column was eluted with water (150 mL), and the collected fraction was lyophilized to yield a colorless solid.

General Procedure for the Purification of Activated Nucleotides (3). The activated nucleotides **3a−h** were purified using preparative μ-Bondapak reverse-phase column. The column was eluted with water and 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 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 a colorless solid. The activated nucleotides were obtained as sodium salts.

Adenosine 5'-Phosphoro-4-(dimethylamino)pyridinium (4-$(CH_3)_2$-NpypA) (3a). Compound **3a** was shown to be 95% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.35 g, 77.6%). ^{1}H NMR (D_2O): δ 3.05 (s, 6H), 4.35 (m, 2H), 4.52 (m, 1H), 4.7 (t, 1H), 4.8 (merged with HOD peak), 5.9 (d, 1H), 6.4 (d, 2H) 7.95 (m, 2H), 8.15 (s, 1H), 8.17 (s, 1H). ^{13}C NMR (D_2O): δ 157.9, 156.5, 154.1, 150.0, 141.9, 119.8, 107.5, 89.2, 84.4, 74.3, 71.1, 68.5, 40.9. ^{31}P NMR (D_2O): δ −5.21. Compound **3a** was not sufficiently stable at room temperature to submit for FAB MS analysis. It was stable for 3 weeks at −20 °C.

Adenosine 5'-Phosphoro-4-(methylamino)pyridinium (4-CH_3-NHpypA) (3b). Compound **3b** was shown to be 97% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.33 g, 76.5%). ^{1}H NMR (D_2O): δ 2.8 (s, 3H), 4.3 (m, 2H) 4.55 (m, 1H), 4.85 (merged with HOD peak), 5.9 (d, 1H), 6.15 (m, 2H), 7.8 (m, 1H), 7.9 (m, 1H), 8.05 (s, 1H), 8.10 (s, 1H). ^{13}C NMR (D_2O): δ 159.6, 156.7, 154.3, 150.2, 143.8, 141.87, 141.22, 119.9, 110.6, 105.2, 89.1, 84.4, 74.4, 71.2, 68.4, 30.4. ^{31}P NMR (D_2O): δ −1.07. Compound **3b** was not sufficiently stable at room temperature to submit for FAB MS analysis. It was stable for 3 weeks at −20 °C.

Adenosine 5'-Phosphoro-4-aminopyridinium (4-NH_2pypA) (3c). Compound **3c** was shown to be 76% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.30 g, 70.9%). ^{1}H NMR (D_2O): δ 4.25 (m, 2H), 4.5 (m, 1H), 4.9 (merged with HOD peak), 5.85 (d, 1H), 6.35 (d, 2H), 7.9 (m, 2H), 8.15 (s, 1H), 8.2 (s, 1H). ^{31}P NMR (D_2O): δ −1.07. Compound **3c** was not sufficiently stable to submit for FAB MS analysis. It was stable for 2 weeks at −20 °C.

Adenosine 5'-Phosphoro-2-aminobenzimidazolide (2-NH_2-benzimpA) (3d). Compound **3d** was shown to be 98% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.375 g, 81.1%). ^{1}H NMR (D_2O): δ 4.05 (m, 2H), 4.25 (m, 1H), 4.28 (t, 1H), 4.65 (t, 1H), 5.9 (d, 1H), 6.7 (t, 1H), 6.9 (t, 1H), 7.0 (d, 1H), 7.2 (d, 1H), 7.99 (s, 1H), 8.0 (s, 1H). ^{13}C NMR (D_2O): δ 157.4, 157.3, 154.0, 150.0, 141.1, 134.0, 124.6, 122.3, 120.4, 115.3, 114.2, 88.2, 84.8, 75.1, 71.7, 67.0. ^{31}P NMR (D_2O): δ −7.36. High-resolution MS (positive-ion FAB): calcd for $C_{17}H_{20}N_8O_6P$ [as acid form, M + H] 463.1243, found 463.1250.

Adenosine (5'-Phosphoro-4-morpholinide) (MorpA) (3e).[18] Compound **3e** was shown to be 99% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.36 g, 86.7%). ^{1}H NMR (D_2O): δ 2.9 (t, 2H), 3.55 (t, 2H), 4.05 (m, 2H), 4.35 (m, 1H), 4.5 (t, 1H), 4.8 (merged with HOD peak), 6.1 (d, 1H), 8.25 (s, 1H), 8.45 (s, 1H). ^{13}C NMR (D_2O): δ 156.9, 154.6, 150.47, 141.2, 119.9, 88.6, 85.5, 75.7, 71.9, 68.4, 65.3, 46.3. ^{31}P NMR (D_2O): δ 8.05. High-resolution MS (positive-ion FAB): calcd for $C_{14}H_{21}N_6O_7PNa$ [MH] 439.1107, found 439.1111.

Adenosine (5'-Phosphoro-1-piperidinide) (PiperpA) (3f). Compound **3f** was shown to be 98% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.31 g, 74.8%). ^{1}H NMR (D_2O): δ 1.4 (bs, 6H), 2.85 (bs, 4H), 4.05 (m, 2H), 4.37 (m, 1H), 4.55 (t, 1H), 4.8 (merged with HOD peak), 6.1 (d, 1H), 8.22 (s, 1H), 8.45 (s, 1H). ^{13}C NMR: (D_2O) δ 159.7, 154.2, 150.3, 141.2, 119.8, 88.8, 85.5, 75.9, 71.9, 65.3, 27.4, 25.6. ^{31}P NMR (D_2O): δ 9.69. High-resolution MS (positive-ion FAB): calcd for $C_{15}H_{23}N_6O_6PNa$ [MH] 437.1314, found 437.1323.

Adenosine 5'-Phosphoropyrazolide (PyrapA) (3g). Compound **3g** was shown to be 96% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.25 g, 62.9%). ^{1}H NMR (D_2O): δ 4.12 (m, 2H), 4.3 (m, 1H), 4.38 (t, 1H), 4.7 (t, 1H), 6.0 (d, 1H), 6.35 (bs, 1H), 7.71 (bs, 1H), 7.91 (bs, 1H), 8.11 (s, 1H), 8.19 (s, 1H). ^{13}C NMR (D_2O): δ 157.0, 154.3, 150.5, 145.6, 141.1, 136.3, 120.1, 108.6, 88.6, 85.0, 75.7, 72.0, 67.2. ^{31}P NMR (D_2O): δ −6.91. High-resolution MS (positive-ion FAB): calcd for $C_{13}H_{16}N_7O_6PNa$ [MH] 420.0797, found 420.0810.

Adenosine 5'-Phosphoro-1,2,4-triazolide (TriazpA) (3h). Compound **3h** was shown to be 98% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.26 g, 62.5%). ^{1}H NMR (D_2O): δ 4.2 (m, 2H), 4.3 (m, 1H), 4.45 (t, 1H), 4.75 (t, 1H), 6.05 (d, 1H), 8.0 (s, 1H), 8.22 (s, 1H), 8.3 (s, 1H), 8.6 (s, 1H). ^{13}C NMR (D_2O): δ 157.2, 154.7, 150.6, 150.0, 141.4, 120.2, 88.2, 84.8, 75.4, 71.8, 67.7. ^{31}P NMR (D_2O): δ −9.33. High-resolution MS (positive-ion FAB): calcd for $C_{12}H_{15}N_8O_6PNa$ [MH] 421.0750, found 421.0751.

Adenosine (5'-Phosphoroxy-2-pyridinide) (2-OxypyripA) (3i). Compound **3i** was shown to be 99% pure by HPLC on a μ-Bondapak column. It was obtained as a colorless solid (0.25 g, 56%). ^{1}H NMR (D_2O): δ 4.25 (m, 2H), 4.38 (m, 1H), 4.5 (t, 1H), 4.85 (1H, merged with HOD peak), 6.05 (d, 1H), 7.0 (m, 2H), 7.55 (m, 1H), 8.05 (m, 1H), 8.10 (s, 1H), 8.25 (s, 1H). ^{31}P NMR (D_2O): δ −5.32. High-resolution MS (positive-ion FAB): calcd for $C_{15}H_{17}N_6O_7PNa$ [MH] 447.0794, found 447.0806.

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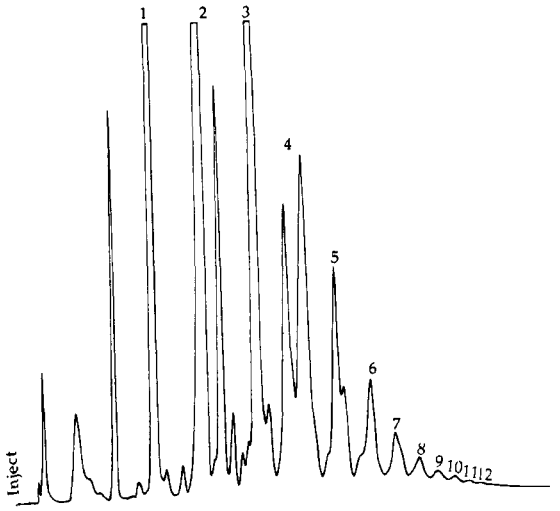


Figure 1. Anion-exchange HPLC of the reaction products of **3a** on Na^+ montmorillonite 22A.

General Procedure for the Clay-Mediated Oligomerization Reaction of Activated Nucleotides. An electrolyte solution (50 mL) was prepared from a mixture of sodium chloride (0.2 M) and magnesium chloride (0.075 M), and the pH was adjusted to 8. A stock solution (2.5 mL) of activated nucleotide (0.015 M) was prepared from the sodium chloride–magnesium chloride electrolyte. The activated nucleotide solution (1 mL) was added to Na^+ montmorillonite 22A clay (0.05 g); the reaction mixture was vortexed for 30 s and the pH adjusted to 8. The reaction mixture was kept at the room temperature for 7 days. The activated nucleotide solution (1 mL) which did not contain montmorillonite was kept under the same conditions as a control reaction. The reaction with the clay was centrifuged, and the supernatant was removed from the clay and filtered through a 0.45 μm pore filter. The montmorillonite clay was vortexed with ammonium acetate (1 mL, 0.1 M) solution and allowed to stand for 24 h. The reaction mixture was centrifuged. The supernatant was filtered and combined with the first supernatant solution.

Analysis of Oligomers Formed from the Reaction of 4-$(CH_3)_2$-NpypA (3a) on Na^+ Montmorillonite. The product mixture from the reaction of **3a** (45 μL) were analyzed by anion-exchange HPLC (Figure 1); the monomer, dimer, trimer and tetramer fractions were collected and stored in the freezer. An aliquot of each fractions was reinjected in the anion-exchange column to determine the homogeneity of the collected fractions. The collected monomer, dimer, and trimer fractions were analyzed by reverse-phase HPLC. The pA, $pA^{2'}pA$, $pA^{3'}pA$, $pA^{3'}$-$pA^{3'}pA$, and $pA^{3'}pA^{2'}pA$ were identified from their retention times in the reverse-phase HPLC profile and the presence of these isomers further confirmed by co-injecting the authentic samples along with the collected fraction in the reverse-phase column by HPLC.

The ratio of oligomers containing terminal phosphate were determined by APH. An aliquot amount of each fraction (300 μL) was treated with 0.166 units of APH at 37 °C for 4 h. The monomer and dimer hydrolysis products were analyzed by reverse-phase HPLC, and trimer and tetramer hydrolysis products were analyzed by anion-exchange chromatography.

The ratios of 3′,5′- and 2′,5′-linkages in the monomer through tetramer fractions were determined by ion-exchange HPLC analysis of the RNase T_2 and RNase T_2 followed by APH reaction products of the collected fractions.

(i) The monomer fraction (300 μL) was treated with 0.3 units of RNase T_2 at 37 °C for 4 h and analyzed by reverse-phase HPLC.

(ii) The dimer fraction (300 μL) was treated with 0.3 units of RNase T_2, incubated at 37 °C for 3 h, and analyzed on the reverse-phase and anion-exchange HPLC.

(iii) The dimer fraction RNase T_2 reaction mixture (300 μL) was adjusted the pH to 8, treated with 0.166 units of APH at 37 °C, and analyzed by reverse-phase and anion-exchange HPLC.

(iv) The trimer fraction (300 μL) was treated with 0.8 units of RNase T_2 at 37 °C for 4 h and analyzed by anion exchange and reverse-phase HPLC.

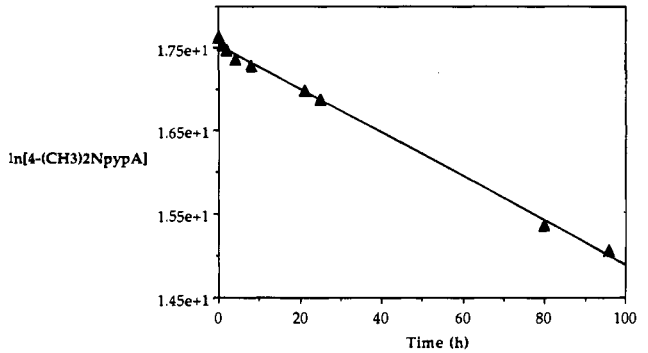


Figure 2. Pseudo-first-order plot of the kinetic data from the hydrolysis **3a** in 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at pH 8.1 at 23–25 °C.

(v) The trimer RNase T_2 reaction mixture (300 mL) was adjusted to pH 8, treated with 0.166 units of APH at 37 °C for 2 h, and analyzed using anion-exchange and reverse-phase chromatography.

(vi) The tetramer fraction (300 μL) was treated with 0.3 units of RNase T_2 at 37 °C for 2 h and analyzed in the anion-exchange chromatography.

(vii) The tetramer fraction RNase T_2 reaction mixture (300 μL) was adjusted the pH to 8, treated with 0.166 units of APH at 37 °C for 2 h and analyzed by anion-exchange and reverse-phase chromatography.

Isolation of Cyclic Trimer from the Reaction Products of 4-$(CH_3)_2$NpypA (3a) by HPLC. The reaction products of 4-$(CH_3)_2$-NpypA **3a** (45 μL) were injected on the anion-exchange column and eluted the column at isocratic mode of buffer A 98% and buffer B 2% for 6 min followed by a regular gradient elution mode. The dimer fraction was resolved into three peaks, the first peak was collected as $pA^{2'}pA$ (fraction A), and the remaining two peaks of the dimer fraction were eluted close together and collected together as a mixture of cyclic trimer and $pA^{3'}pA$ (fraction B). The presence of $pA^{2'}pA$ in the fraction A and cyclic trimer and $pA^{3'}pA$ in fraction B was confirmed by analyzing the collected fractions by reverse-phase HPLC. The cyclic trimer was characterized by HPLC analysis of the RNase T_2 and the RNase T_2 plus APH hydrolysis products.

Isolation and Characterization of Dimer Fraction from the Reaction Products of 2-NH_2benzimpA (3d) on Na^+ Montmorillonite. The reaction products of **3d** (45 μL) were separated by the anion-exchange HPLC, and the dimer fraction was collected. The dimer fraction was reinjected in the anion-exchange column to determine its homogeneity and then was injected on the reverse-phase column, and the $pA^{2'}pA$, cyclic trimer, and $pA^{3'}pA$ were identified by their HPLC retention times. The products were characterized by HPLC analysis of the RNase T_2 and the RNase T_2 plus APH hydrolysis products.

Hydrolysis of 4-$(CH_3)_2$NpypA (3a) at pH 8. A buffer solution of 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M of (*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) was prepared, and the pH was adjusted to 8; 1 mL of 4-$(CH_3)_2$NpypA **3a** solution (0.015 M) was prepared using HEPES buffer and the hydrolysis reaction was carried out at 23–25 °C. The hydrolysis of **3a** was monitored at regular intervals by analyzing the reaction mixture (10 μL) by reverse-phase HPLC. A linear plot of ln [4-$(CH_3)_2$NpypA] vs time (Figure 2) was obtained.

General Procedure for the Binding Studies of 3a and ImpA on Na^+ Montmorillonite. A solution of activated nucleotide (2 mL, 0.015 M) was prepared in 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES (pH 8) buffer at 4 °C; 1 mL of this solution was added to Na^+ montmorillonite (50 mg) and vortexed for 30 s. The reaction mixture was centrifuged at regular intervals, and 5 μL was analyzed by anion-exchange HPLC. A plot of HPLC peak area of the activated nucleotide vs time is given in Figures 3 and 4.

Molecular Modeling Study of 4-$(CH_3)_2$NpypApApA. The structure of 4-$(CH_3)_2$NpypApApA was modeled in the CAChe molecular modeling work system (release 3.5) running on a Macintosh Centris 650, and the molecular mechanics calculation was carried out using MM2 force field parameters. Conjugate gradient was used to locate the energy minimum, and all atoms were moved at once. Van der Waals interactions between atoms separated by greater than 9.00 Å

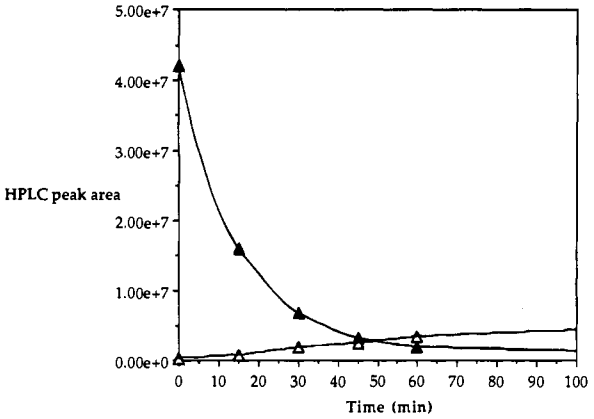


Figure 3. Loss of **3a** (0.015 M) in the presence of Na^+ montmorillonite 22A at pH 8 and 4 °C measured by HPLC peak area at 260 nm (▲). Total oligomer formation not corrected for hyperchromicity measured at 260 nm (△). The intensity of the of the UV absorption of **3a** is greater than that of the oligomers because of the contribution of the DMAP activating group to the absorption of **3a**.

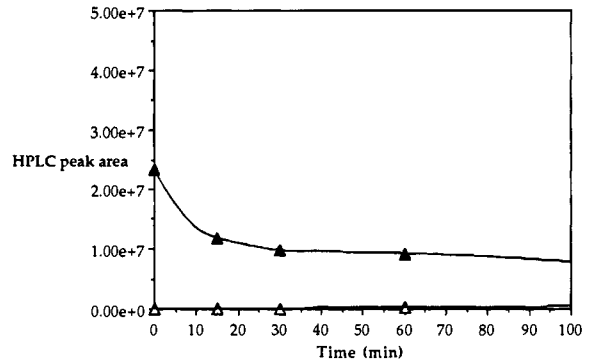


Figure 4. Loss of ImpA (0.015 M) in the presence of Na^+ montmorillonite 22A at pH 8 and 4 °C measured by HPLC peak area at 260 nm (▲). Total oligomer formation not corrected for hyperchromicity measured at 260 nm (△).

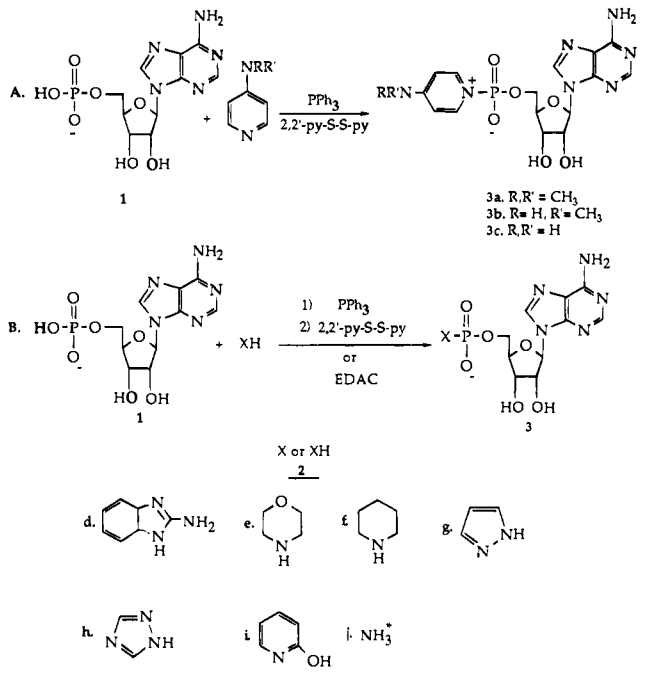
were excluded. The optimization was continued until the energy change was less than 0.001 kcal/mol.

Results and Discussion

Synthesis and Purification of Activated Nucleotides 3a–f. Activated nucleotides **3a–f** (Scheme 1) were synthesized by the procedure of Mukaiyama et al.[8,18] The products were separated from the reaction mixture by precipitating as sodium salts using a solution of sodium perchlorate in acetone–ether. Although the synthesis of **3h** was reported using the procedure of Mukaiyama et al., we were not able to prepare it or **3g** and **3i** by this route.[13] The latter compounds were synthesized in aqueous solution using 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride (EDAC) as condensing agent.[19] The optimal reaction time was determined by monitoring the reaction using reverse-phase HPLC. The activated nucleotide was 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 with water. The water was lyophilized to yield the activated nucleotide as sodium salt. It was not possible to prepare *N*-phosphoryl-5'-derivatives of 2-aminopyridine, 2-(dimethylamino)pyridine and 1,1,3,3-tetramethylguanidine by either of the above procedures. Steric effects of the 2-substituent may explain our failure to isolate *N*-phosphoryl-5'-derivatives of 2-amino- and 2-(dimethylamino)-pyridine.[20]

Compounds **3a–h** were shown to be 70–98% pure by reverse-phase HPLC, and further purification was carried out

Scheme 1



*3j was purchased from Sigma.

using preparative μ-Bondapak reverse-phase chromatography. To minimize hydrolysis to pA during purification, chromatography was performed at 4 °C under 5–10 psi argon pressure with the flow rate of 7–10 mL/min. The water used as eluent was adjusted to pH 9 with a trace of triethylamine to minimize the hydrolysis of the activated nucleotides. Use of an excess of triethylamine was avoided since this resulted in the formation of triethylammonium salts of the activated nucleotides.

Compounds **3a–i** were characterized by ^1H NMR, ^{13}C NMR, and ^{31}P NMR spectroscopy. Compounds **3d–i** were also characterized by FAB high-resolution mass spectrometry. Mass spectra of compounds **3a–c** were not obtained because they are not stable at room temperature and are only stable for 2–3 weeks at −20 °C.

Oligomerization Reaction of Activated Nucleotides 3a-j on Na^+ Montmorillonite. The oligomerization reactions of the activated nucleotides **3a–j** were carried out in NaCl–$MgCl_2$ electrolyte solution at pH 8 on montmorillonite 22A clay. The reactions of the activated nucleotides **3a–j** in the absence of montmorillonite were carried out as controls. The course of each reaction was monitored by reverse-phase HPLC over a period of 7 days. The analysis of reaction of compounds **3a–c** in the absence of montmorillonite by reverse-phase and anion-exchange HPLC showed hydrolysis was the main reaction pathway. Diadenosine pyrophosphate (A^5'ppA) (6%) and dimer and trimer isomers were also detected as products. Compound **3d** was very stable in the absence of montmorillonite; only 7% hydrolyzed to pA over 7 days. Compounds **3e–j** hydrolyzed to pA in the absence of montmorillonite and no oligomers or A^5'ppA were detected as hydrolysis products.

Products from the reaction of compounds **3a–c** on montmorillonite were analyzed by anion-exchange and reverse-phase HPLC. Oligomers containing up to 12 monomer units were detected by anion-exchange HPLC (Figure 1).[21] Oligomers containing up to six monomer units were obtained by the reaction of compound **3d** in the presence of montmorillonite Hydrolysis to pA was the main reaction pathway of **3e–j** on

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Table 1. Products from the Reaction of **3e–j** in the Presence of Montmorillonite[a]

activated nucleotides	pA	A$^{5'}$ppA	pA$^{2'}$pA	pA$^{3'}$pA
MorpA (**3e**)	58	1.8	1.0	0
PiperpA (**3f**)	65	0	1.5	0.8
PyrazpA (**3g**)	88	2.6	3.9	1.8
TriazpA (**3h**)	96	4.0	0	0
2-OxypyripA (**3i**)	75	1.0	0.1	0.8

[a] Determined from the products of HPLC analysis on reverse-phase after APH hydrolysis.

montmorillonite. Low yields of A$^{5'}$ppA and dinucleotides were also detected (Table 1).

The Effect of Phosphate Activating Group on Nucleotide Reactivity. The nucleophilic displacement of amines from phosphoramidates proceeds in a concerted process.[22,23] The position of the transition state depends on the relative nucleophilicities of the nucleophile and leaving group. The rate of hydrolysis of the monoanions of phosphoramidates decrease in the order shown for the following amines: pyridine > aniline > ammonia > imidazole > *n*-butylamine > 4-(dimethylamino)-pyridine (DMAP).[24] Linear correlations of the log of the rate constant with amine pK_a are observed only within groups of structurally related amines. The substantially greater stability of imidazole and DMAP phosphoramidates as compared to the corresponding pyridine derivative reflects the greater delocalization of the positive charge on the amino grouping in the former compounds.[25] The charge is delocalized to the second nitrogen by resonance interaction and by solvation.[26]

A variety of amine structural types were investigated in the search for new phosphate activating groups because of the absence of a general correlation between hydrolytic reactivity and amine basicity. The reaction of the phosphorimidazolide of adenosine is believed to be initiated by protonation of the imidazole (pK_a = 6.0)[27] grouping.[8] Since the pK_a of phosphoramidate is approximately 1 pK_a unit less than the pK_a of the corresponding amine, (e.g., the pK_a of imidazole is 7.0)[28] it was expected that phosphoramidates of strongly basic amines such as morpholine (**3e**) and piperidine (**3f**) would be protonated more readily than the phosphorimidazolide grouping and undergo rapid reaction.[24] Facile hydrolysis of **3e** and **3f** but no oligomer formation was observed. The 5′-phosphoramidate of adenosine (**3j**) is resistant to hydrolysis, a finding consistent with the low basicity of ammonia and the reported slow hydrolysis of the phosphoramidate of ammonia.[25] Pyrazole and triazole are weak bases (pK_as 2.5 and 2.3, respectively),[28] so the corresponding phosphoramidates **3g** and **3h**, respectively, are not basic and are not protonated by montmorillonite. They undergo rapid hydrolysis because pyrazole and triazole are good leaving groups even in the absence of acid catalysts.[29] The 2-oxypyridinyl activated nucleotide **3i** is formally a phosphate ester, but its displacement may be energetically favored because of 2-pyridone formation. The reaction proceeded, but only hydrolysis to pA was observed.

2-Aminobenzimidazole was studied as a leaving group because it contains the imidazole moiety which is known to yield oligomers in the presence of montmorillonite. It was postulated that it may be more effective than imidazole because the 2-amino grouping enhances the basicity of the imidazole by generating a substituted guanidine derivative (pK_a 7.5).[28] The corresponding phosphoramidate would be protonated more extensively by the acidic montmorillonite and either undergo facile oligomer formation or hydrolysis. Oligomers which contain almost comparable amounts of 2′,5′- and 3′,5′-phosphodiester bonds are formed using **3d** (see below), but in yields lower than those observed with imidazole itself. These low yields may reflect the different steric requirements of **3d** as compared to ImpA. For example, small changes in the substituents present on the imidazole ring can result in major changes in the structures of the oligo(G)s formed by template directed synthesis on poly(C).[30] Alternatively, the low yields may reflect the inefficient formation of 2′,5′-linked phosphodiester bond from adenine nucleotides on montmorillonite. For example, the presence of 2′,5′-linked unit on the end of the growing oligomer chain inhibits chain elongation in the oligomerization of ImpA.[5] Similarly, binding UO_2^{2+} to montmorillonite inhibits the formation of longer 2′,5′-linked oligomers; longer oligomers were formed when the same amount of UO_2^{2+} was used as a catalyst in the absence of montmorillonite.[8]

The 4-aminopyridine activated derivatives of 5′-AMP (**3a–c**) are the most effective compounds for the oligo(A) formation on montmorillonite. Chain lengths as high as the dodecamer (Figure 1) and 3′,5′-phosphodiester bond regioselectivity of about 88% were observed (see below) using the (dimethylamino)pyridine (DAMP) derivative **3a**. The successful synthesis of oligomers with ImpA[6,7] and **3a** may reflect the greater stability of the activated monomers in aqueous solution, and their enhanced reactivity on the montmorillonite surface due to the lower concentration of water and the higher concentration of monomer. Acid catalysis by montmorillonite may also contribute to oligomer formation. Compound **3a** was used in the studies on oligomer formation because DMAP is commercially available and the activated nucleotide is more stable than the 4-aminopyridine derivative **3c**.[31]

Our success in forming oligonucleotides when imidazoles or 4-aminopyridines are the activating groups of 5′-AMP, and our failure with other activating groups makes it possible to draw some conclusions concerning the optimal properties of activated nucleotides. First, nucleotides with positively charged leaving groups bind more efficiently to the catalytic sites on the negatively charged faces of montmorillonite.[32] Binding studies with **3a** demonstrate its rapid and almost complete binding to montmorillonite (Figure 3). ImpA, which must be protonated by acidic sites on the montmorillonite, binds less strongly. Second, leaving groups consisting of resonance stabilized cations, such as those present on **3a–d** and protonated ImpA initiate oligomer formation. The absence of oligomer formation when **3e** and **3f** react on montmorillonite indicates that when the positive charge is not delocalized oligomers are not formed from the activated monomer. Third, the ratio of the rate of oligomer formation to the rate of hydrolysis of the activated monomer is a better indicator of the extent of oligomer formation than simply the rate of monomer hydrolysis.[33] Compound **3a** has a half-life of hydrolysis of 1 day (see below) while that of

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Table 2. Differentiation of the Cyclic and Linear Oligomers by APH Hydrolysis

oligomer fraction	linear oligomers (%)	chain length	cyclic oligomers (%)	chain length
	A. Products from **3a**			
"dimers"	37	2	63	3
"trimer"	86	3	14	4
"tetramers"	38	4	62	5
"pentamers"	65	5	35	6
	B. Products from **3d**			
"dimers"	59	2	41	3

Table 3. Hydrolysis Products of the Oligomers from **3a**

	"dimers"		"trimers"		"tetramers"	
product	RNase T_2[a]	RNase T_2 + APH[a]	RNase T_2[b]	RNase T_2 + APH[a]	RNase T_2[b]	RNase T_2 + APH[a]
A	11	89	9.5	62	15	76
$A^{2'}pA$	*c*	11	35	38	2	24
Ap	73	*c*	9.5	*c*	45	*c*
$pA^{2'}pA$	8	*c*	*c*	*c*	*c*	*c*
$A^{2'}pAp$	*c*	*c*	2	*c*	18	*c*
pAp	8	*c*	44	*c*	13	*c*
$pA^{2'}pAp$	*c*	*c*	*c*	*c*	7	*c*

[a] Determined by reverse-phase HPLC. [b] Determined by anion-exchange HPLC. [c] Not expected. [d] Not detected.

ImpA when measured under comparable reaction conditions is 9.3 days,[34] yet **3a** gives higher yields of longer oligomers than ImpA because its rate of oligomerization on montmorillonite is much faster than that of ImpA (Figures 3 and 4). Half the oligomers are formed from **3a** in about 1 h, while essentially no oligomers are formed from ImpA in that time period (Figures 3 and 4).

Effect of Monomer Activating Group on Oligonucleotide Structure. The oligomers formed by the reaction of **3a** and **3d** on montmorillonite were characterized by selective enzymatic hydrolyses. The dimer through pentamer fractions from the reaction of **3a** were collected after separation by ion-exchange HPLC. Each fraction was treated with APH, and the reaction mixture was analyzed by HPLC to determine the presence of isomers with terminal phosphate groupings (Table 2).[5] The isomers which contained 3′,5′-phosphodiester bonds are hydrolyzed by RNase T_2,[6,7] while those with 2′,5′-links are not cleaved. The extent of cleavage by RNase T_2 was determined by HPLC analysis before and after hydrolysis of the RNase T_2 products with APH.

Appreciable yields of unreacted oligomers were detected by the HPLC analysis of the APH hydrolysis products (Table 2). These were shown to be cyclic nucleotides by their degradation to dinucleotides and smaller products on enzymatic hydrolysis with RNase T_2 (Table 3). No adducts of $A^{5'}ppA$ were present as shown by the absence of $A^{5'}ppA$ by successive reaction of the oligomers with RNase T_2 and APH. This was an unexpected finding in view of the observation of adducts of $A^{5'}ppA$ in the reaction of ImpA in the presence of montmorillonite.[5,7]

Further evidence for the presence of cyclic trimer in the dimer fraction was obtained by collection of the presumed cyclic trimer from the reverse-phase HPLC column together with $pA^{3'}pA$.[21] Several attempts to resolve these closely eluting compounds were unsuccessful. A mixture of $pA^{3'}pA$ and the cyclic trimer was treated with RNase T_2, and the hydrosylate was shown to contain pAp, Ap, and A when analyzed by reverse-phase HPLC. Adenosine and a 3% yield of a peak with the same retention time as $A^{2'}pA$ (Table 4) were the only products observed after treating the RNase T_2 hydrolysate with APH. From these data

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Table 4. Hydrolysis Products of the Cyclic Trimer−$pA^{3'}pA$ Mixture from **3a** (%)

products	RNase T_2	RNase T_2 + APH
A	16	97
$A^{2'}pA$	2[a]	3[a]
Ap	71	*b*
$pA^{2'}pA$	*b*	*b*
pAp	11	*b*

[a] The source of the $A^{2'}pA$ is unknown. [b] Not expected as a reaction product.

Table 5. Comparison of Oligomers formed from 4-$(CH_3)_2$NpypA (**3a**) and ImpA

Product	**3a**	ImpA[c]	3′,5′-links (%) **3a**	3′,5′-links (%) ImpA[c]
"Dimers"			94	68
$pA^{2'}pA$	14	33		
$pA^{3'}pA$	23	52		
$\lfloor pA^{3'}pA^{3'}pA^{3'}\rfloor$	63	13		
$AppA^{3'}pA$	a	2.6		
"Trimers"			77	72
$pA^{3'}pA^{2'}A$	64	47		
$pA^{3'}pA^{3'}A$	23	28		
$\lfloor pA^{3'}pA^{3'}pA^{3'}pA^{3'}\rfloor$	8	a		
$\lfloor pA^{3'}pA^{3'}pA^{2'}pA^{3'}\rfloor$	5	a		
$A^{5'}ppA^{3'}pA^{3'}pA$	a	15		
$A^{5'}ppA^{3'}pA^{2'}pA$	a	4		
$(Ap)_mA^{5'}ppA(pA)_n$ (m+n=2)	a	2		
"Tetramers"			94	b
$pA^{3'}pA^{3'}A^{3'}pA$	34	9		
$pA^{3'}pA^{3'}A^{2'}pA$	4	22		
$(pA)_4$ isomers	a	37		
$\lfloor pA^{3'}pA^{3'}pA^{3'}pA^{3'}pA^{3'}\rfloor$	11	a		
$\lfloor pA^{3'}pA^{3'}pA^{3'}pA^{2'}pA^{3'}\rfloor$	43	a		
$A^{5'}ppA(pA)3$	a	1		
$(Ap)_mA^{5'}ppA(pA)_n$ (m+n=3)	a	32		

[a] Oligomer not detected. [b] Not determined. [c] Reference 7.

it was concluded that cyclic trimer, in which virtually all the bonds are 3′,5′-linked, is the main product present in the dimer fraction.

The proof of the presence of cyclic trimer in the dimer fraction, coupled with the absence of $A^{5'}ppA$ containing oligomers in the trimer to pentamer fractions, led to the conclusion that cyclic oligomers are present in these fractions also. The presence of a mixture of all 3′,5′-linked cyclic tetramer and a cyclic tetramer containing one 2′,5′-linkage was deduced from the failure to cleave these isomers with APH and the formation of $A^{2'}pAp$ as a reaction product on treatment with RNase T_2. A similar approach was used to determine the proportion of all 3′,5′-linked cyclic pentamer and cyclic pentamer with only one 2′,5′-linkage in the tetramer fraction (Table 5). The observation of 35% unreacted material on treatment of the pentamer fraction with APH led to the conclusion that it contains 35% cyclic hexamer.

The dramatic differences in the structures of the reaction products observed when DMAP (**3a**) and imidazole (ImpA) are used as activating groups are illustrated in Table 5. Reasons for the absence of pyrophosphate groupings in the products formed from **3a** and their presence in the oligomers formed from ImpA was investigated. As noted previously, $A^{5'}ppA$ is formed in about 6% yield in control reactions of **3a** carried out in the absence of montmorillonite, but the rate of $A^{5'}ppA$ formation

is slower than oligomer formation. Kinetic analysis of the hydrolysis of **3a** under the oligomerization conditions but in the absence of montmorillonite gave a pseudo-first-order rate constant of 2.9×10^{-2} h^{-1}; a rate equivalent to a half-life of about 24 h (Figure 2). As noted above, the half time for oligomer formation is about 1 h, so hydrolysis in aqueous solution is about 24 times slower than oligomer formation. These data demonstrate that oligomer formation on montmorillonite is complete before appreciable $A^{5'}$ppA can form in aqueous solution. The absence of $A^{5'}$ppA-containing oligomers may also be due to the efficiency with which **3a** binds to montmorillonite. About 75% binds within 30 min (Figure 3) while 55% of the ImpA binds in the same time period (Figure 4). The binding and rapid reaction of **3a** continues in the subsequent 30 min time interval while the concentration of ImpA in the solution phase stays almost constant. The much more rapid reaction of **3a** results in its conversion to oligomers well before the reaction of ImpA is completed. $A^{5'}$ppA forms in the aqueous phase of the ImpA reaction during the relatively slow reaction of ImpA and the dinucleoside pyrophosphate binds to the montmorillonite and is incorporated into the oligomers.

The second structural difference observed between the oligomers formed from ImpA and **3a** is the high proportion of cyclic nucleotides obtained from **3a** (Tables 2 and 5). Cyclic trimer, tetramer, pentamer, and hexamer were observed with an overall yield of 18%. Cyclic dimers and trimers have been reported[35] as products from the oligomerization of ImpC and ImpU on UO_2^{2+}, and we observed cyclic trimer from ImpA on montmorillonite (Table 5).[7] Formation of cyclic oligomers requires the close proximity of the 5'- and 3'-terminii of the oligomer chain. Molecular modeling studies (CAChe) indicates that an extended form of DMAPpApApA is of lower energy than a conformer in which the 3'- and 5'-ends are proximate. The solution-phase cyclization of activated linear nucleotides is also considered to be an unlikely possibility because of the absence of a progressive decrease in the yields of cyclic oligomers with increasing nucleotide chain length (Table 2).[36] The linear oligomer conformation in which the 3'- and 5'-ends are proximate may be a low-energy conformation on the surface of montmorillonite which results in the formation of cyclic oligomers.

The third structural difference between the oligomers formed from **3a** and ImpA is the greater regioselectivity for 3',5'-phosphodiester bond formation observed using **3a** (Table 5). About 88% of the phosphodiester bonds formed with the (dimethylamino)pyridinium activating group are 3',5'-linked while about 67% are 3',5'-linked when imidazole is the activating group. This regioselectivity of phosphodiester bond formation is greater than the highest previous value of 80% observed in the reaction of 9:1 ImpA, $A^{5'}$ppA mixtures.[7]

The 2-aminobenzimidazole-activated 5'-AMP (**3d**) reacted in the presence of montmorillonite to give oligomers containing up to six monomer units. The yield of the oligomers longer than dimer was very low so it was only possible to characterize the dimer fraction. HPLC analysis on a reverse-phase column separated the mixture into 49% $pA^{2'}pA$, 10% $pA^{3'}pA$, and 41% 3',5'-cyclic trimer. The presence of cyclic trimer and the absence of $A^{5'}$ppA-containing oligomers was confirmed by the failure to observe $A^{5'}$ppA as a product after the sequential treatment of the dimer fraction with RNase T_2 followed by APH. The excess of $pA^{2'}pA$ over $pA^{3'}pA$, a result that is markedly different from that observed with ImpA, suggests that the higher oligomers are mainly 2',5'-linked.

It is concluded from this study that the best nitrogen-containing leaving groups for nucleotide oligomerization in aqueous solution should have a positive charge or be capable of being protonated on montmorillonite. These positively charged groupings are stabilized by delocalization and solvation. The DMAP group has proven to be the most effective activating group to date for the formation of longer oligomers which have the highest proportion of 3',5'-linkages.

Conclusions

This investigation uncovered a new class of phosphate activating groups, the 4-aminopyridine derivatives (**3a–c**), which are more effective than imidazole derivatives for the regiospecific formation of 3',5'-linked oligoadenylates on montmorillonite in aqueous solution. Chain lengths as high as dodecamers are formed with a regioselectivity for 3',5'-phosphodiester bond formation averaging 88%. The most effective nitrogen-containing activating groups for the formation of oligonucleotides in aqueous solution contain either a positively charged nitrogen attached to the phosphorus or a nitrogen that is sufficiently basic so that it is protonated by the acidic sites on the montmorillonite. If this positive charge is stabilized by delocalization and solvation, then oligomer formation competes successfully with the hydrolytic cleavage of the activating group and oligomers are formed. The presence of the positive charge is important since this attracts the nucleotide to the negative sites on the montmorillonite resulting in the binding of the activated nucleotide to the catalytic sites there. It should be noted that purines and pyrimidines also contain 4-aminopyridine-type structural units. Some of these compounds, which can be formed from HCN or cyanoacetylene in prebiotic simulation experiments,[37] may have served as phosphate-activating groups.

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