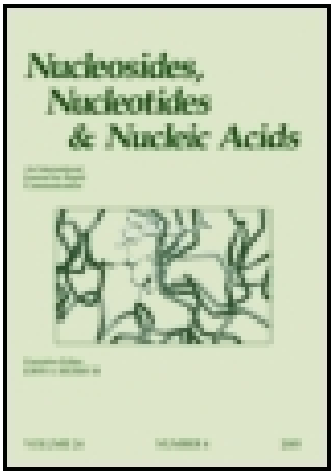

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Synthesis of Oligodeoxyribonucleoside Methylphosphonates Containing 2-Aminopurine

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SYNTHESIS OF OLIGODEOXYRIBONUCLEOSIDE METHYLPHOSPHONATES CONTAINING 2-AMINOPURINE

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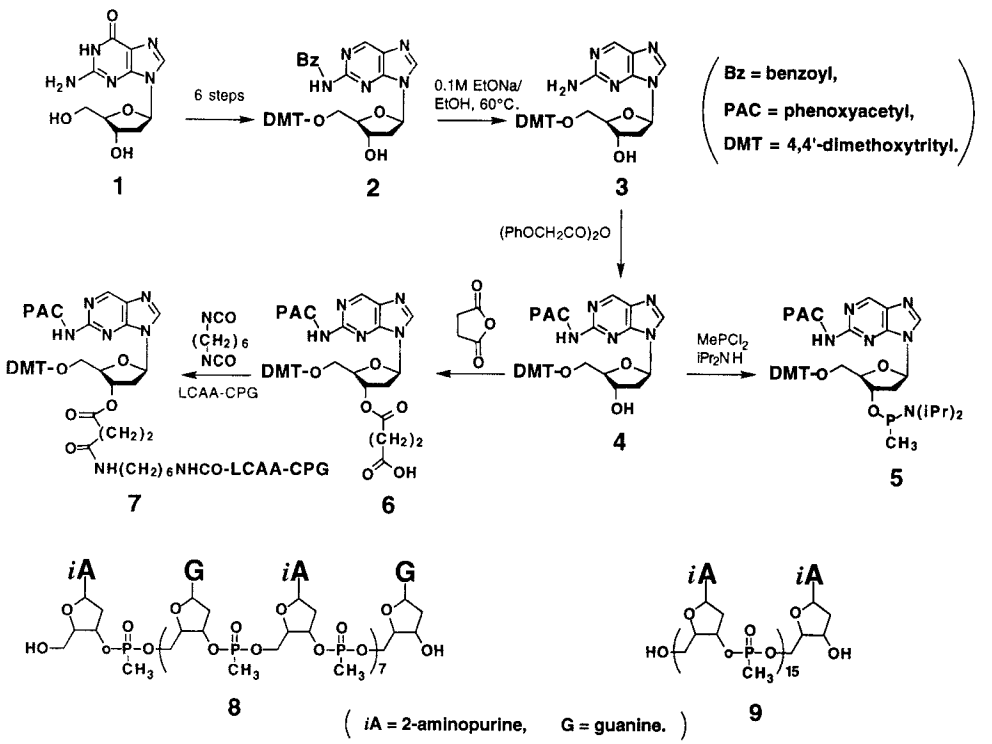
Abstract: Oligodeoxyribonucleoside methylphosphonates containing multiple 2-aminopurine bases were synthesized by solid-phase phosphonamidite chemistry for investigating their triple helix formation with natural DNA or other duplex targets and for cellular uptake studies of the methylphosphonate oligomers. The base-labile phenoxyacetyl group was used as the N^2-amino protecting group for 2-aminopurine, allowing the final deprotection of the oligomers to be performed under the standard ethylenediamine condition.

INTRODUCTION

Oligonucleoside methylphosphonates are of a class of backbone-modified non-ionic nucleic acid analogues in which one of the non-bridging oxygens of the normal phosphodiester linkage is replaced by a methyl group. Due to their strong binding to the natural nucleic acid target sequences, their resistance to cellular enzymatic degradation, and their effectiveness in regulating cellular gene function, their use as antisense and anticode therapeutics have been under extensive development.[1]

Until recently, most studies used methylphosphonate oligomers containing the four normal bases, either to bind single-stranded RNA sequences via duplex formation or to bind double-stranded DNA having homopurine target sequences via triplex formation. As part of our ongoing effort in designing effective antisense/anticode therapeutic agents, our group has proposed several triplex-binding motifs for recognition of single-stranded natural RNA target without sequence limitation, by the use of two strands of methylphosphonate oligomers to achieve higher affinity and higher selectivity in target recognition.[2,3] Each of the motifs is composed of four isomorphous base triads, participated by the four normal nucleosides together with some designed unusual ones, e.g., pseudouridine, pseudoisocytidine, and 2-aminopurine-2′-deoxyriboside (or isoadenosine, *i*A). We have recently succeeded in the synthesis of methylphosphonate oligomers containing pseudouridine[4] and pseudoisocytidine.[5] We wish to report here the solid-phase synthesis of oligodeoxyribonucleoside methylphosphonates containing 2-aminopurine.

Another motive for our effort in synthesizing 2-aminopurine containing methylphosphonate oligomers is due to the fluorescent properties of 2-aminopurine and its structural closeness to the normal purine bases. Oligonucleoside methylphosphonates comprised



Scheme 1

exclusively of or enriched in 2-aminopurine may be useful in cellular uptake studies of the methylphosphonate oligomers, without the need to use radioactively labeled oligomers or oligomers conjugated with the usually bulky fluorescent dyes, making interpretation of the oligomer uptake behavior straightforward.

RESULTS AND DISCUSSION

We used solid-phase phosphonamidite chemistry to incorporate 2-aminopurine into oligodeoxyribonucleoside methylphosphonates. The structures and the preparation of the designed 2-aminopurine methylphsphonamidite synthon (**5**) and the 2-aminopurine CPG support (**7**) are illustrated in Scheme 1.

To prepare the protected 2-aminopurine-2′-deoxyribosides we used the procedures of McLaughlin *et al* to convert the 6-keto oxygen atom of the guanine base to an aromatic hydrogen.[6] Thus, 2′-deoxyguanosine (**1**) was tribenzoylated, its *O*-6 was sulfonated and subsequently transformed to 6-hydrazino group. The 6-hydrazino group was then oxidized by silver (I) oxide to give tribenzoylated 2-aminopurine-2′-deoxyriboside. Selective removal

of the two benzoyl groups from the sugar residue followed by protection of the 5′-OH of the sugar with 4,4′-dimethoxytrityl group then gave 5′-*O*-(4,4′-dimethoxytrityl)-N^2-benzoyl-2-aminopurine-2′-deoxyriboside (**2**) with an overall yield of 36% from the starting material 2′-deoxyguanosine (**1**).

In principle, compound **2** could be phosphitylated directly to the corresponding methylphosphonamidite synthon and then used in solid-phase methylphosphonate oligomer synthesis, similarly to the reported incorporation of 2-aminopurine into oligodeoxyribonucleotides[6]. However, considering the instability of the methylphosphonate backbone under alkaline conditions relative to the phosphodiester backbone,[7] and the unknown lability of the benzoyl group on the N^2 position of 2-aminopurine under the methylphosphonate deprotection conditions, we decided to test the stability of compound **2** under the standard ethylenediamine deprotecting condition (the Genta one-pot condition[7]). This deprotecting condition involved an initial treatment of the oligomer-containing CPG with 10% ammonium hydroxide in 1:1 acetonitrile:ethanol (by volume) at room temperature for 30 minutes, followed by the addition of an equal volume of ethylenediamine and continuation of the treatment at room temperature.[7] The treatment was normally for six hours and the methylphosphonate backbone cleavage was kept at minimum. The oligomer was released from the CPG support and the base protecting groups were completely removed by this procedure, including the benzoyl on N^4 of cytosine and N^6 of adenine and the isobutyryl on the N^2 of guanine, but not the benzoyl group on the N^2 of 2-aminopurine. When we treated compound **2** under Genta one-pot condition,[7] we saw only about 15% benzoyl cleavage in 6 hours. Two days treatment was required for the deprotection going to completion. The complete removal of N^2-benzoyl from the 2-aminopurine base contained in an oligomer would take even longer. Such prolonged treatment would cause considerable cleavage of the methylphosphonate backbone. Thus this procedure for deprotection would be unacceptable for the synthesis of good yield of methylphosphonate oligomers in full length.

We realized, therefore, that it is necessary to replace the protective N^2-benzoyl group on the 2-aminopurine by a more base labile one. We chose phenoxyacetyl for this purpose since this labile group has been successfully used in many such applications.[8] The replacement was done as illustrated in Scheme 1. Compound **2** was reacted with 0.1 M EtONa in EtOH at 60°C for 2 hours to gave quantitatively compound **3** which has a free 2-amino group. Reaction of **3** with phenoxyacetic anhydride according to the previous method[8] produced the desired N^2-phenoxyacetyl derivative **4** in 73% yield. As was expected, the N^2-phenoxyacetyl group on 2-aminopurine is much more base-labile than the benzoyl group. When compound **4** was treated under Genta one-pot condition,[7] complete removal of phenoxyacetyl occurred in less than 30 minutes.

The observation of much higher stability of the benzoyl group on N^2 of 2-aminopurine relative to the benzoyl groups on N^6 of adenine and N^4 of cytosine and the isobutyryl group on N^2 of guanine corroborates with the results of several other findings. The final deprotection of oligodeoxyribonucleotides containing benzoyl protected 2-aminopurine base in McLaughlin′s study used concentrated ammonium hydroxide treatment at 50°C for two days,[6] compared to an overnight treatment required for deprotection of the normal oligodeoxyribonucleotides.[9] Schmidt and Cech also found that benzoyl protected 2-aminopurine-2′-deoxyriboside was only incompletely deprotected even after treatment with concentrated ammonium hydroxide at 70°C overnight. The isobutyryl group, on the other hand, could be quantitatively removed from N^2 of 2-aminopurine-2′-deoxyriboside during the ammonium

hydroxide treatment at 50°C overnight.[10] Thus, the commercially available 2-aminopurine-2′-deoxyriboside phosphoramidite synthon (Glen Research) used a rather labile dimethylformamidine as the N^2 protecting group, instead of the standard benzoyl group.

Phenoxyacetyl group on the N^2 of guanine residue has been reported to undergo some extent of transacylation by acetic anhydride during the capping steps in the solid-phase synthesis.[11] We therefore conducted a nucleoside model study on compound **4** by the treatment with 1:1 cap A (acetic anhydride/lutidine/THF) and cap B (4-dimethylaminopyridine/pyridine), Our results showed no detectable transacylation had taken place even after 2 hours treatment, indicating that the phenoxyacetyl protection of N^2 of 2-aminopurine is compatible with the standard capping condition. It is also compatible with the standard deblocking condition (2.5% dichloroacetic acid/dichloromethane) and the oxidation condition (iodine/lutidine/THF/H_2O) used in standard solid-phase synthesis of methylphosphonate oligomers.

The N^2-phenoxyacetyl protected 2-aminopurine derivative (**4**) was then converted to the corresponding phosphonamidite synthon (**5**) by the reactions involving methyldichlorophosphine and diisopropylamine, using a modified phosphitylation procedure based on those of Agrawal and Goodchild.[12] The synthon was purified by silica gel flash chromatography and hexane precipitation. Its identity and purity were confirmed by ^1H and ^{31}P NMR.

To incorporate a 2-aminopurine at the 3′ end of an oligomer, we also prepared the 2-aminopurine-2′-deoxyriboside CPG support (**7**). Thus, compound **4** was reacted with succinic anhydride to give the nucleoside 3′-succinate derivative (**6**), which was then reacted with 1,6-diisocyanatohexane and long chain aminoalkyl CPG by the procedure of Kumar *et al.*[13] The loading of 2-aminopurine-2′-deoxyriboside on the CPG was 40 μmole/g.

The 2-aminopurine methylphosphonamidite synthon (**5**) and the CPG support (**7**) were then used in the automated solid-phase synthesis of methylphosphonate oligomers. Stepwise coupling efficiency of the synthon (**5**) was 97-98%, as determined by the spectrophotometric assay of the deblocking fraction for the released DMT group.[14] The oligomers were deprotected by the Genta one-pot procedure[7] at room temperature for 6 hours, purified by preparative HPLC (reversed phase and/or polar-stationary phase). The purified oligomers were characterized by their UV spectra and analytical HPLC profiles both before and after digestion with 1 M piperidine-H_2O, and by their molecular weights as determined by pneumatically assisted electrospray mass spectrometry. Their extinction coefficients were calculated from the hyperchromicity due to piperidine digestion,[15] using the extinction coefficients of 2-aminopurine nucleosides[16,17] for the 2-aminopurine nucleoside species in the digest.

A 16mer oligodeoxyribonucleoside methylphosphonate (**8**) containing alternating 2-aminopurine and guanine residues was synthesized using the 2-aminopurine synthon (**5**) and 5′-*O*-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine-3′-diisopropylmethylphosphonamidite (JBL) on N^2-isobutyryl-2′-deoxyguanosine-3′-succinyl-LCAA-CPG support (Glen Research). After Genta one-pot deprotection, the oligomer (5′-DMT-off) was purified first by C18 reversed phase preparative HPLC, then by polar-stationary phase preparative HPLC[7]. It was then desalted and subsequently characterized. The HPLC profiles for the crude and purified oligomer **8** are shown in Figure 1. The UV spectra of the oligomer showed an intense absorption band around 310 nm region besides the other absorption bands below 300 nm (curve a, Figure 2), consistent with the presence of both guanine and 2-aminopurine residues.[16,17] After digestion with 1 M aqueous piperidine, the UV spectra of the digest was found essentially identical to that of an equimolar mixture of 2′-deoxyguanosine and 2-aminopu-

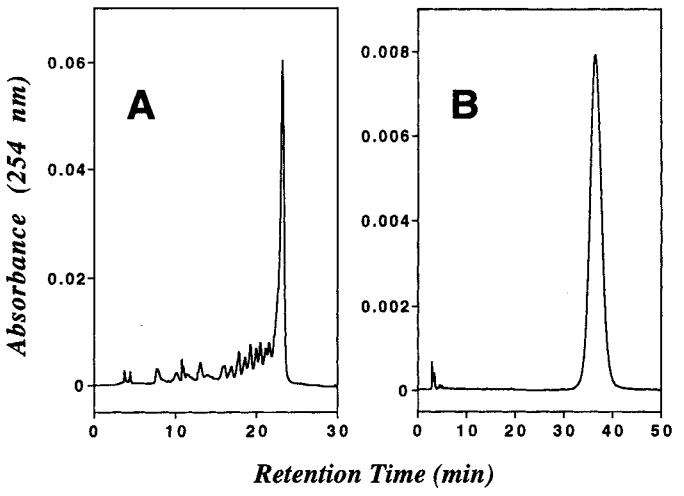


FIGURE 1. Polar stationary phase HPLC analysis of a 16mer methylphosphonate oligomer containing alternating 2-aminopurine and guanine bases (**8**). Column: Cyclobond 250 x 4.6mm. Detection: A254nm. (**A**): Crude **8**, linear 80% to 20% ACN gradient in 25 mM NH4OAc over 30min. (**B**): HPLC purified **8**, isocratic 55% ACN in 25mM NH4OAc.

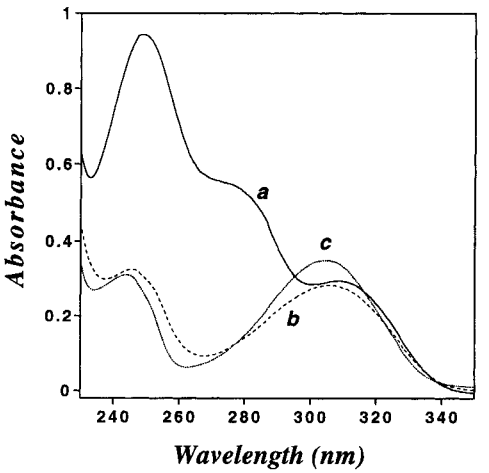


FIGURE 2. UV spectra of oligodeoxyribonucleoside methylphosphonates containing 2-aminopurine residues. (*a*): alternating 2-aminopurine-guanine 16mer**8** (6.25 μM strand). (*b*): 2-aminopurine 16mer**9** (3.125 μM strand). (*c*) 2-aminopurine-2′-deoxy-riboside (50 μM). Solvent: 10% ACN in 10 mM sodium phosphate, pH 7.

rine-2′-deoxyriboside. The extinction coefficients of the oligomer at both 260nm and 303nm were thus determined to be 107,000 and 46,000 $M^{-1}cm^{-1}$. Electrospray mass spectra revealed the molecular weight to be 5048.0 (theoretical 5047.8). This oligomer is now being investigated as the third strand in triplex formation with duplex targets containing alternating A and G bases on the second strand.

A 16mer oligodeoxynucleoside methylphosphonate (**9**) containing exclusive 2-aminopurine base was also synthesized, using the 2-aminopurine synthon (**5**) and the 2-aminopurine CPG (**7**). This oligomer was intended to be used in duplex and triplex studies, as well as in the cellular uptake studies of methylphosphonate oligomers. The oligomer was assembled by leaving the DMT group on the 5′-end. After deprotection by Genta one-pot procedure,[7] the DMT-on oligomer was then separated by C-18 reversed-phase preparative HPLC (Polar-stationary phase HPLC purification of this oligomer from the DMT-off synthesis was unsatisfactory). The DMT-on oligomer fractions were detritylated by 80% acetic acid-H_2O at room temperature for 30 min, repurified by reversed-phase HPLC, and then desalted. The HPLC analysis of the purified oligomer **9** is shown in Figure 3A. The UV spectra of the purified oligomer (curve b, Figure 2) and its piperidine digest were similar to the free 2-aminopurine-2′-deoxyriboside (curve c, Figure 2), showing a single band with maximum absorption at 305 nm and 303nm, respectively. HPLC analysis (at both 303nm and 260nm) of the piperidine digest showed 3 peaks (Figure 3B). The latest peak co-eluted with 2-aminopurine-2′-deoxyriboside, and the two earlier peaks were attributed to the 3′ and 5′-methylphosphonic acid species of the nucleoside, indicating that no modification had taken place on the 2-aminopurine base. The extinction coefficient at 303nm was determined to be 89,800 $M^{-1}cm^{-1}$.

The fluorescence emission and excitation spectra of the methylphosphonate 16mers **8** and **9** were determined in 10% acetonitrile containing 10 mM sodium phosphate buffered at pH 7. The emission spectra of the 2-aminopurine nucleosides and methylphosphonate oligomers are shown in Figure 4, with each sample containing the same amount of 2-aminopurine residues (5 μM). The emission spectra of the two oligomers (curves c and d, Figure 4) were similar in shape to the free 2-aminopurine-2′-deoxyriboside (curve b, Figure 4), all showing an emission maximum at 364 nm.[18] The excitation spectra (not shown) of the two oligomers were also similar in shape to the free nucleoside, although a slight red-shift (3 nm) in excitation maximum is observed for both oligomers (306 nm) relative to the free nucleoside (303 nm[18]). The most significant difference in the fluorescence properties between the oligomers and the nucleoside is in their fluorescence emission intensities. The molar strand fluorescence emission intensities at 364 nm (excitation at 303 nm) for both the oligomer **8** (containing 8 incorporated 2-aminopurine bases) and the oligomer **9** (containing 16 2-aminopurine bases) were only about 16% of the molar fluorescence emission of the free nucleoside. In other words, the fluorescence intensity of 2-aminopurine residue in the oligomer **8** and the oligomer **9** was only 2% and 1%, respectively, of that of the free nucleoside. This quenching in fluorescence emission of the oligomers can be attributed to the strong stacking interaction between the 2-aminopurine base and other adjacent purine bases, with the 2-aminopurine-2-aminopurine stacking being stronger than that of 2-aminopurine-guanine. In this regard, oligomers containing the fluorescent pyrimidine analogs should show much less fluorescence quenching, since the stacking interactions between pyrimidine bases is significantly weaker. This idea has been confirmed by our incorporation of the fluorescent 2-pyrimidinone base into methylphosphonate oligomers, which we will report in another publication.[19]

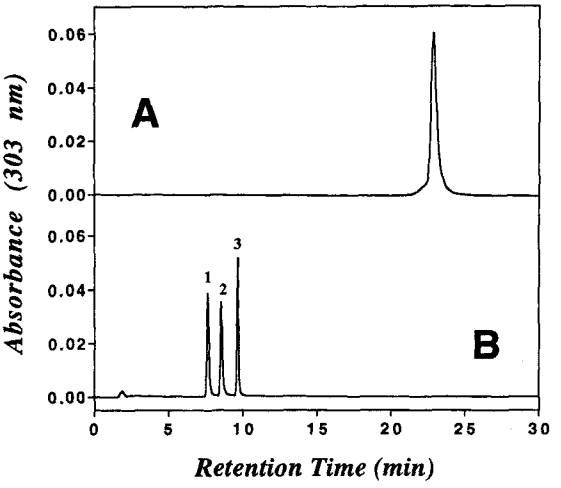


FIGURE 3. Reversed-phase HPLC characterization of the 16mer methylphosphonate containing exclusive 2-aminopurine base (**9**). Column: Microsorb C18 4.6x250mm. Detection: A303nm. Elution: linear 2-30% ACN gradient in 50 mM sodium phosphate, pH 7. (**A**): HPLC purified **9**. (**B**): piperidine-digested **9**. peak 1 and 2: 2-aminopurine-2′-deoxyriboside-3′(or 5′)-methylphosphonic acid. peak 3: 2-aminopurine-2′-deoxyriboside.

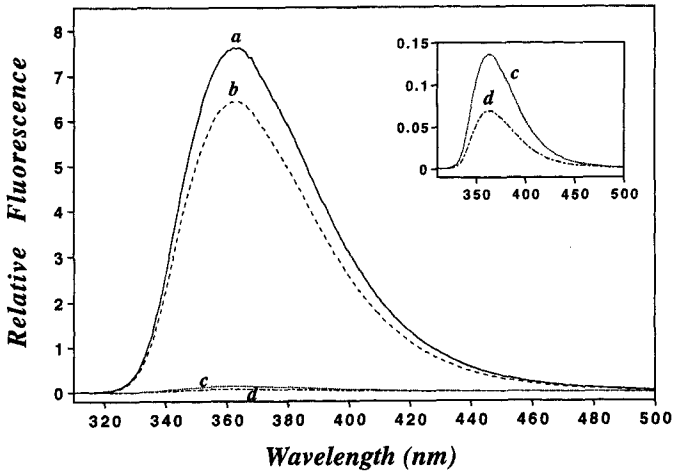


FIGURE 4. Fluorescence emission spectra of 2-aminopurine nucleosides and methylphosphonate oligomers. Each sample contains 5 μM 2-aminopurine residues. Solvent: 10% ACN in 10 mM sodium phosphate, pH 7. Excitation wavelength: 303nm. (*a*): piperidine digested **9**. (*b*): 2-aminopurine-2′-deoxyriboside. (*c*): alternating 2-amino- purine-guanine 16mer **8**. (*d*): 2-aminopurine 16mer **9**.

The fact that piperidine digestion recovered most of the fluorescence from the oligomer **9** suggested that quantitation of the amount of 2-aminopurine containing methylphosphonate oligomers uptaken by the cells could be achieved by the determination of the fluorescence emission of piperidine digested cell lysates. The digest is in fact more fluorescent than the free 2-aminopurine-2′-deoxyriboside, as shown in curve a, Figure 4. This could be explained by higher fluorescence quantum yields for the methylphosphonic acid derivatives of the nucleoside relative to the parent nucleoside. The possibility of using fluorescence microscopic methods to determine the intracellular distribution of the relatively weak-fluorescent 2-aminopurine methylphosphonate oligomers remains to be investigated.

In summary, we have successfully incorporated multiple 2-aminopurine residues into oligodeoxyribonucleoside methylphosphonates by solid-phase phosphonamidite chemistry, by the use of a labile protecting group, phenoxyacetyl, as the N^2-protecting group for 2-aminopurine. These oligomers can be used in the studies of triplex formation according to the base-triad motifs recently proposed by our laboratory. Due to their fluorescent properties, they are also potentially useful in the cellular uptake studies of methylphosphonate oligomers.

EXPERIMENTAL

Materials and Methods

2′-Deoxyguanosine was purchased from USB, Cleveland, OH. 4,4′-Dimethoxytrityl chloride, dimethylaminopyridine, anhydrous pyridine, anhydrous dichloromethane, succinic anhydride, 1,6-diisocyanatohexane were from Aldrich, Milwaukee, WI. Reagent grade diisopropylamine, triethylamine, *N,N*-diisopropylethylamine were purchased from Aldrich and dried by refluxing with and distilled from calcium hydride under nitrogen. Methyldichlorophosphine was purchased from Alfa Aesar, Ward Hill, MA. Long chain aminoalkyl controlled pore glass (LCAA CPG) was from CPG, Inc., Fairfield, NJ. Phenoxyacetic anhydride was prepared from phenoxyacetyl chloride and sodium phenoxyacetate according to the procedure of Smith *et al* for the preparation of phenylacetic anhydride.[20] Liquid chromatography was performed on columns packed with silica gel 60 (230-400 mesh) from Aldrich. Analytical TLC was performed on aluminum sheets coated with silica gel 60 F254 from EM Science, Gibbstown, NJ. Protected normal deoxyribonucleoside-3′-*O*-(*N,N*-diisopropyl)methylphosphonamidites were obtained from JBL Scientific, Inc., San Luis Obispo, CA. Other DNA synthesis reagents were from Glen Research, Sterling, VA.

Nuclear magnetic resonance spectra were recorded on a Bruker AMX300 (300 MHz) spectrometer at ambient temperature. Chemical shifts were measured relative to solvent signals as internal standards and expressed in ppm relative to tetramethylsilane. The exchangeable protons were confirmed by adding either deuterium oxide or deuteriated methanol. ^{31}P NMR were recorded in $CDCl_3$. Chemical shifts were obtained from the proton-decoupled experiments and were expressed relative to the external standard, 85% phosphoric acid. Pneumatically assisted electrospray mass spectra of oligonucleoside methylphosphonates were recorded on a Perkin Elmer SCIEX ATI3 instrument at Scripps Research Institute Mass Spectrometry Facility, La Jolla, CA. Fluorescence spectra were recorded on an Aminco•Bowman Series 2 luminescence spectrometer at 20°C. All spectra were instrument corrected.

5′-*O*-dimethoxytrityl-N^2-benzoyl-2-aminopurine-2′-deoxyriboside (2)

This compound was prepared from 2′-deoxyguanosine by converting the O^6 of protected guanosine to the hydrazino group followed by oxidation with silver (I) oxide, using a multi-step synthesis scheme published previously[6]. Overall yield: 36%. The purified **2** appeared as a single spot on TLC (Rf 0.45, 1:10 CH_3OH:CH_2Cl_2). ^1H NMR showed no detectable impurities and the data were consistent with those reported. ^1H NMR($CDCl_3$): δ 8.91(s, 2H, N^2H and H6), 8.20(s, 1H, H8), 7.10-7.81(m, 14H, aromatic DMT and Bz), 6.65-6.75(m, 5H, H1′ and aromatic DMT), 4.92 (m, 1H, 3′-OH), 4.29(m, 1H, H3′), 4.00(m, 1H, H4′), 3.72(s, 6H, DMT OCH_3), 3.30-3.50(m, 2H, H5′/H5′′), 2.8-2.91(m, 1H, H2′), 2.62-2.76(m, 1H, H2′′).

5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (3)

5′-*O*-dimethoxytrityl-N^2-benzoyl-2-aminopurine-2′-deoxyriboside (**2**, 1.0 g, 1.52 mmole) was suspended in 20 mL 0.1 M sodium ethoxide in ethanol and was heated to 60°C. A clear solution was obtained after 10 minutes. The reaction was allowed to proceed for 2 hours, when TLC indicated complete conversion of the starting compound to a slower moving blue fluorescent species (Rf 0.30, 1:10 CH_3OH:CH_2Cl_2). The reaction mixture was then cooled to room temperature. 40 mL of anhydrous pyridine were then added, followed by the addition of Dowex W50 (H^+ form) in small portions until the mixture reached pH 7. The Dowex resin was filtered and washed with 40 mL pyridine. The filtrates and the washes were combined and evaporated under reduced pressure. The residues were co-evaporated with toluene three times to remove the pyridine and then soaked with plenty amounts of hexanes to remove the benzoate. The residue was then dissolved in 1:10 CH_3OH:CH_2Cl_2, filtered, evaporated to dryness to give pure 5′-*O*-dimethoxytrityl-2-amino-purine-2′-deoxyriboside (**3**), which showed a single DMT-containing blue-fluorescent spot on analytical tlc. This material was used directly in the next reaction with phenoxyacetic anhydride. ^1H NMR($CDCl_3$): δ 8.66(s, 1H, H6), 7.86(s, 1H, H8), 7.16-7.42(m, 9H, aromatic DMT), 6.80(d, 4H, J = 8.9Hz, aromatic DMT), 6.34(t, 1H, J = 6.6Hz, H1′), 4.91(bs, 2H, 2-NH_2), 4.68(m, 1H, H3′), 4.14(m, 1H, H4′), 3.77(s, 6H, DMT OCH_3), 3.36(m, 2H, H5′/H5′′), 2.7-2.8(m, 1H, H2′), 2.4-2.5(m, 1H, H2′′).

5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (4)

To a solution of 5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (**3**, 1.52 mmole, obtained from the reaction of 1.0 g 5′-*O*-dimethoxytrityl-N^2-benzoyl-2-aminopurine-2′-deoxyriboside (**2**) with 0.1 M EtONa in EtOH) in 40 mL dry pyridine, was added 2.8 g (9.8 mmole) phenoxyacetic anhydride, and the mixture was stirred under nitrogen at room temperature. All of the phenoxyacetic anhydride went into solution in 10 minutes. After 2 hours, TLC indicated complete disappearance of the 5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (**3**). The reaction was stopped by adding 6 mL of water and stirring for another 10 minutes. The mixture was diluted with 300 mL of chloroform and washed twice with 5% aqueous sodium bicarbonate and once with saturated aqueous sodium chloride. The organic phase was evaporated under reduced pressure to an oil. The oil was co-evaporated with toluene and then put on vacuum overnight to give 5′-*O*-dimethoxytrityl-N^2,3′-*O*-bis (phenoxyacetyl)-2-aminopurine-2′-deoxyriboside (Rf = 0.8, 1:10 CH_3OH:CH_2Cl_2).

To selectively remove the 3′-*O*-phenoxyacetyl group, the diPAC compound was dissolved in 20 mL pyridine at 0°C, then mixed in cold with 20 mL triethylamine and 60 mL water. The mixture was stirred vigorously and allowed to warm to room temperature. The reaction went to completion in 0.5 to 1 hour. Tlc showed the formation of a major product

with Rf 0.60 and a minor species which co-migrated with 5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (**3**). The reaction mixture was diluted with 500 mL 5% aqueous sodium bicarbonate, and extracted with chloroform twice. The organic phase was washed with 5% aqueous sodium bicarbonate and water, dried over sodium sulfate, and then evaporated under reduced pressure. The residues were co-evaporated with toluene three times to remove traces of pyridine and then loaded on a silica gel column, and eluted with 0-10% methanol in chloroform. Fractions containing pure 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**) were pooled and evaporated to dryness to give 0.76 g (1.11 mmole) pure **4** as white solid. No impurities were detected by ^{1}H NMR. The two-step yield from **2** was 73%. ^{1}H NMR($CDCl_3$): δ 9.16(s, 1H, N^2H), 8.97(s, 1H, H6), 8.20(s, 1H, H8), 6.96-7.42(m, 14H, aromatic DMT and PAC), 6.77(d, 4H, J = 8.9Hz, aromatic DMT), 6.71(t, 1H, J = 6.6Hz, H1′), 4.85(m, 1H, H3′), 4.63(s, 2H, PAC CH_2), 4.25(m, 1H, H4′), 3.74(s, 6H, DMT OCH_3), 3.35-3.50(m, 2H, H5′/H5′′), 2.72-2.83(m, 1H, H2′), 2.63-2.71(m, 1H, H2′′).

Deprotecting conditions for Bz- and PAC-protected 2-aminopurine-2′-deoxyribosides

In two separate test tubes, each containing 0.5 mL ethylenediamine and 0.5 mL acetonitrile-ethanol-concentrated ammonium hydroxide (45:45:10) (Genta one-pot deprotecting condition[7]), were dissolved 5′-*O*-dimethoxytrityl-N^2-benzoyl-2-aminopurine-2′-deoxyriboside (**2**) and 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**), respectively. The test tubes were allowed to stand at room temperature, and the rates of cleavage of the benzoyl and the phenoxyacetyl groups from the protected nucleosides were assayed by analytical TLC (1:10 CH_3OH:CH_2Cl_2), by monitoring the disappearance of the starting materials and the formation of the amino-deprotected nucleoside, 5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (**3**). The experiments indicated that complete removal of benzoyl group required 2 days, whereas complete removal of phenoxyacetyl occurred within 30 minutes.

Stability of PAC-protected 2-aminopurine-2′-deoxyribosides under capping conditions

In two separate test tubes, each containing 0.5 mL cap A (acetic anhydride/lutidine/THF) and 0.5 mL cap B (4-dimethylaminopyridine/pyridine), were dissolved 5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (**3**) and 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**), respectively. The mixtures were allowed to stand at room temperature for 2-4 hours. The two reactions were analyzed by spotting samples alongside on the same TLC sheets. After developing (1:10 - 1:20 CH_3OH:CH_2Cl_2), the sheets were air-dried and heated to 120°C in the oven for 20 min. The sheets were visualized to detect if the capping reaction of 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**) had produced any DMT-containing species co-migrating with 5′-*O*-dimethoxytrityl-3′, N^2-diacetyl-2-aminopurine-2′-deoxyriboside, a product expected to be formed from 5′-*O*-dimethoxytrityl-2-aminopurine-2′-deoxyriboside (**3**), but not from 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**), unless transacylation of phenoxyacetyl by acetyl had taken place. The experiments indicated no formation of any detectable 5′-*O*-dimethoxytrityl-3′,N^2-diacetyl-2-aminopurine-2′-deoxyriboside from the capping reaction of 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**).

5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside-3′-*O*-diisopropylmethylphosphonamidite (5)

To a stirring solution of methyldichlorophosphine (271 μL, 3 mmole) in 20 mL anhydrous dichloromethane, was slowly injected 840 μL (6.03 mmole) of diisopropylamine.

The stirring was continued for 1 hour at room temperature. The solution was slowly transferred, through a stainless steel cannula via nitrogen pressure, to another stirring solution containing 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**, 66 mg, 0.96 mmole), diisopropylethylamine (1.15 mL, 6.6 mmole) and dichloromethane (10 mL). The reaction went to completion in 1.5 hour as revealed by TLC analysis. The excess amount of chlorophosphine was then quenched by the addition of 2 mL anhydrous methanol and the stirring was continued for an additional 5 minutes. The mixture was then diluted with 80 mL of dichloromethane and washed twice with 5% aqueous sodium bicarbonate and once with water. The organic phase was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure to give an off-white solid foam. The product was then purified by silica gel flash chromatography, eluted with ethylacetate:dichloromethane:triethylamine (10:10:1). Fractions containing the phosphonamidite **5** were pooled, evaporated, redissolved in 2 mL toluene, and precipitated by adding small drops of the toluene solution to 200 mL vigorously stirring hexanes at room temperature. The white solid precipitates were collected and dried under vacuum. Yield: 0.64 g (80%). Rf = 0.7 (ethylacetate:dichloromethane: triethylamine, 10:10:1). The synthon was found to be relatively unstable on analytical TLC sheets when exposed in the atmosphere, but it was stable when stored as dry solid as well as in anhydrous acetonitrile solution. The purity of the synthon was determined to be greater than 95% based on ^{1}H and ^{31}P analysis. ^{1}H NMR($CDCl_3$): δ 9.01(s, 1H, H6), 8.98(bs, 1H, N^2H), 8.18 and 8.19(2s, 1H, H8), 7.00-7.42(m, 14H, aromatic DMT and PAC), 6.77 and 6.78 (2d, 4H, J = 8.9Hz, aromatic DMT), 6.46-6.54(m, 1H, H1′), 4.74(s, 2H, PAC CH_2), 4.55-4.68 (m, 1H, H3′), 4.20-4.30(m, 1H, H4′), 3.76 and 3.77(2s, 6H, DMT OCH_3), 3.40-3.60(m, 2H, iPr CH), 3.30-3.40(m, 2H, H5′/H5′′), 2.52-2.81(m, 2H, H2′/H2′′), 1.04-1.28(m, 15H, P-CH_3 and iPr CH_3). ^{31}P NMR($CDCl_3$): δ 119.8 and 121.1 ppm.

5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside-3′-*O*-succinate (6)

In 4 mL of anhydrous 1,2-dichloroethane, was dissolved 344 mg (0.5 mmole) 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside (**4**). To this solution were added dimethylaminopyridine (31 mg, 0.25 mmole), triethylamine (69 μL, 0.5 mmole), and succinic anhydride (75 mg, 0.75 mmole). The mixture was stirred at room temperature overnight. Completeness of the reaction was confirmed by careful TLC analysis (1:10 methanol:ethylacetate). The starting nucleoside showed a normal spot with Rf = 0.4. The nucleoside succinate appeared as a slower moving smear band centered at about Rf = 0.2. The mixture was then diluted with dichloromethane, extracted twice with 10% aqueous citric acid buffered to pH 7 with triethylamine, and then once with water. The organic phase was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was redissolved in 3 mL dichloromethane and then precipitated from 100 mL 1:1 ethyl ether:hexanes. The white amorphous solids (**6**) were collected, washed with 1:1 ether:hexanes, and then dried under vacuum. ^{1}H NMR was consistent with the structure of **6** with no detectable impurities. ^{1}H NMR($CDCl_3$): δ 9.24(s, 1H, N^2H), 8.97(s, 1H, H6), 8.18(s, 1H, H8), 6.98-7.40(m, 14H, aromatic DMT and PAC), 6.79(d, 4H, J = 8.6Hz, aromatic DMT), 6.53(2d, 1H, J = 5.4 and 8.8Hz, H1′), 5.53(m, 1H, H3′), 4.70(s, 2H, PAC CH_2), 4.32(m, 1H, H4′), 3.77(s, 6H, DMT OCH_3), 3.37-3.53(m, 2H, H5′/H5′′), 2.99(q, 2H, J = 7.3Hz, Et_3NH^+ CH_2), 2.58-2.84(m, 6H, H2′/H2′′ and suc CH_2CH_2), 1.20(t, 3H, J = 7.3Hz, Et_3NH^+ CH_3).

5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside-3′-*O*-succinyl-LCAA-CPG (7)

To a solution containing 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside-3′-*O*-succinate (**6**, from the reaction of 0.5 mmole 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside with succinic anhydride), dimethylaminopyridine (61 mg, 0.5 mmole) and dichloromethane (12 mL) under the protection of a continuous flow of nitrogen, was injected 81 μL (0.5 mmole) of 1,6-diisocyanatohexane. The mixture was stirred at room temperature for 10 minutes. To this was then added 86 μL (0.5 mmole) diisopropylethylamine, and the resulting solution was transferred to a flask containing 2 g of TCA-activated LCAA-CPG.[21] The flask was then slowly rotated for 1 day. The CPG was then filtered, washed with dichloromethane, ether, and then dried. The CPG was then mixed with pyridine:water (8:2) for 2 hours to hydrolyze the unreacted isocyanate group. The CPG was filtered again, washed with pyridine, dichloromethane, ether, then dried. Unreacted free amino groups were then capped by mixing the CPG with a solution containing 18 mL dichloromethane, 1 mL pyridine, 3 g phenoxyacetic anhydride, 3 mL triethylamine, and 0.9 mL N-methylimidazole. After 40 min, the CPG was filtered, washed with pyridine, dichloromethane, ether, and then dried in vacuo. The loading of 2-aminopurine-2′-deoxyriboside was determined by mixing 5.4 mg derivatized CPG with 50.0 mL 5% dichloroacetic acid in dichloromethane and measuring the absorbance at 504 nm. Nucleoside loading was thus determined to be 40 μmole/g. Upon further treatment of the CPG with concentrated ammonium hydroxide, the supernatant containing the released nucleoside was analyzed by HPLC. The chromatogram showed a single peak which co-eluted with authentic 2-aminopurine-2′-deoxyriboside upon co-injection.

Solid-phase synthesis of 2-aminopurine-containing oligonucleoside methylphosphonates

Oligonucleoside methylphosphonates containing multiple 2-aminopurine residues were synthesized by the solid-phase phosphonamidite chemistry[22] on ABI 392-5 DNA synthesizer on 1 μmole scales. A 0.1 M solution of 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside-3′-*O*-diisopropylmethylphosphonamidite (**5**) in anhydrous acetonitrile was used for couplings. Sequences with a terminal 3′-2-aminopurine were assembled on the 2-aminopurine solid support, 5′-*O*-dimethoxytrityl-N^2-phenoxyacetyl-2-aminopurine-2′-deoxyriboside-3′-*O*-succinyl-LCAA-CPG (**7**). Other sequences were assembled on the commercially available nucleoside succinyl-CPGs purchased from Glen Research. The deblocking fractions were collected and were assayed for DMT to obtain the stepwise coupling efficiency. Average coupling efficiency for 2-aminopurine-2′-deoxyriboside methylphosphonamidite (**5**) is 97-98%.

Deprotection of the 2-aminopurine methylphosphonate oligomers were achieved according to the Genta one-pot procedures, i.e., treatment of the CPGs with 1 mL of 10% ammonium hydroxide in 1:1 acetonitrile:ethanol for half an hour followed by the addition of 1 mL ethylenediamine and continuing the treatment for an additional 6 hours at room temperature.[7] The mixtures were then neutralized and diluted to 10% organic by quickly mixing with an ice-cooled aqueous hydrochloric or acetic acid solution. The crude methylphosphonate oligomers were then purified by preparative HPLC. Reversed-phase preparative HPLC used a Microsorb C18 column (10x250mm, Rainin, Woburn, MA), eluting with linear acetonitrile gradients in 50 mM sodium phosphate buffer, pH 5.8. For reversed-phase analytical HPLC, a Rainin Microsorb C18 column (4.6x250mm) was used. When polar-stationary

phase HPLC purification was desired, a Cyclobond column (250x4.6mm, Advanced Separations Technologies, Whippany, NJ) was then used, employing a 30 min linear 80% to 20% acetonitrile gradient in 25 mM ammonium acetate, pH 7 buffer for analytical runs, and isocratic elution with acetonitrile composition between 50-60% in the same buffer system for preparative runs. The purified oligomers were then desalted on a C18 guard column, eluting with 50% acetonitrile-water. The desalted oligomers were diluted to 20% acetonitrile-water and stored in the freezer at -70°C until use.

The extinction coefficients of the oligodeoxyribonucleoside methylphosphonates containing 2-aminopurine were determined after digestion with 1 M aqueous piperidine at 37°C for 6 hrs, according to the same method used for the normal methylphosphonate oligomers.[15] The extinction coefficients of 2-aminopurine nucleosides[16,17] were used for the calculation of the extinction coefficients of the 2-aminopurine containing methylphosphonate oligomers from the observed hyperchromicity due to piperidine digestion.

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