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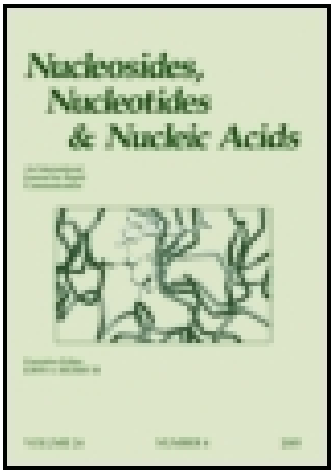

Nucleosides and Nucleotides
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lncn19

Rapid and Efficient Syntheses of Phosphorylated Dinucleotides

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Published online: 24 Sep 2006.

To cite this article: James P. Ferris & John R. Peyser (1994) Rapid and Efficient Syntheses of Phosphorylated Dinucleotides, Nucleosides and Nucleotides, 13:5, 1087-1111, DOI: 10.1080/15257779408011881

To link to this article: http://dx.doi.org/10.1080/15257779408011881

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NUCLEOSIDES & NUCLEOTIDES, 13(5), 1087-1111 (1994)

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RAPID AND EFFICIENT SYNTHESES OF PHOSPHORYLATED DINUCLEOTIDES

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Abstract: Nine or ten-step solution phase syntheses of the dimers dpApA, dApAp, dpTpT and dTpTp in 0.1-0.5 g amounts, in overall yields of 49%, 45%, 32%, and 20% respectively, are described. The synthetic intermediates were characterized by ^{1}H and ^{31}P NMR and the structures of the final products were established by hydrolysis with alkaline phosphatase and comparison of the products with authentic samples.

Oligodeoxynucleotides are potentially useful therapeutic agents[1] and have been useful in research in biomedical areas[2] and in studies of metal complexes.[3] Their diverse functionality and ability to associate via hydrogen bonding and stacking interactions makes them attractive for investigations of weak interactions in molecular systems. A major problem with the widespread use of oligonucleotides in chemical and biological studies is that current synthetic methodology only gives microgram amounts of the desired product. We became aware of this problem when we attempted to obtain deoxyribonucleic acid dimers of the general formulas dpNpN and dNpNp for the investigation of the oligomerization of deoxyribonucleotides on montmorillonite.[4,5] Solid phase methodology[6] for their synthesis did not generate sufficient material for binding and reaction studies on montmorillonite clay.

Methodology for the preparation of dpApA, dpTpT in nine steps and dApAp and dTpTp in ten steps, starting from **1** or **2** in yields of 49%, 32%, 45% and 20%, respectively, on a 0.25-0.5 g scale is described. A solution phase approach was selected so as to generate sufficient supply for our studies of montmorillonite catalysis. The phosphoramidite procedure[6] was adopted because the reactions proceed rapidly, in high yield and because it is currently used extensively in the solid phase synthesis of deoxyoligoribonucleotides. In addition, some of the phosphoramidite starting materials are available commercially. Procedures were developed in which the use of chromatographic separations were minimized, and when chromatography was required it was for relatively simple purifications. The high yield, preparative scale (0.25-0.5 g)

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syntheses developed in this research will also make these compounds more readily available as synthetic intermediates.

The 200 MHz ^1H NMR spectra of the reaction intermediates and final products are consistent with the proposed structures (see Experimental). Only the ^1H NMR spectrum of of dTpTp (**27**) has been reported previously.[11]

The reverse phase HPLC analysis (35% CH_3CN-0.1 M NH_4Ac, pH 7)[8] of the diasteriomeric reaction intermediates reflected the isomeric nature of the of product mixtures as shown by multiple overlapping peaks. These chromatograms were not useful for assessing product purity. The signals in the ^{31}P NMR spectra were better resolved and provided more useful criteria for product purity. The HPLC analysis (details given in Experimental) of the deblocked products **21, 22, 23, 24, 26,** and **27** consisted of sharp discrete peaks, a finding consistent with the presence of pure reaction products.

Efficient synthetic procedures were developed for starting materials **1-7** listed in Scheme 1 but are not included in this manuscript because the compounds have already been reported in the literature. The procedures used are given in the Ph. D. thesis of J. R. Peyser.[9]

Dinucleotides were obtained by reaction of the 3'-OH of **1** or **2** with phosphine **3**[10] to give nucleoside phosphines **4** and **5** (Scheme 1). Compounds **4** and **5** were then reacted with protected nucleosides **6** or **7** and the adducts were oxidized with iodine to give the fully protected dimers **8** and **9**[11] respectively. Optimal yields of **8** and **9** were obtained when a 10% molar excess of **4** and **5** respectively, were used. Best yields of coupled products were obtained when the phosphite group was oxidized by titration with a equivalent amount of freshly prepared iodine solution. Both products were purified by flash chromatography on silica gel[25] and obtained as amorphous solids. The selective deprotection outlined in Scheme 1 yielded dinucleotides **10-13**[12] with a deblocked 3'- or 5'-OH. Compounds **11, 12,** and **13** each exhibited two ^{31}P NMR signals of approximately equal intensity consistent with the presence of two diasteriomeric phosphate derivatives.

A reagent was required for the addition of a terminal phosphate group to **10-13** in which the phosphorus blocking group could be removed in the final step of the synthesis. Addition of a terminal phosphate group in the 3'- or 5'-position was accomplished using p-nitrophenethylphosphine derivative **14**.[13] This reagent yielded a penultimate reaction product containing a nonpolar p-nitrophenethyl (PNPE) protecting group which facilitated the purification of this dinucleotide adduct. Reaction of **14** with **10-13**, followed by iodine oxidation of the phosphorus, gave **15-18** respectively, which were purified by flash chromatography on silica gel (Scheme 2).

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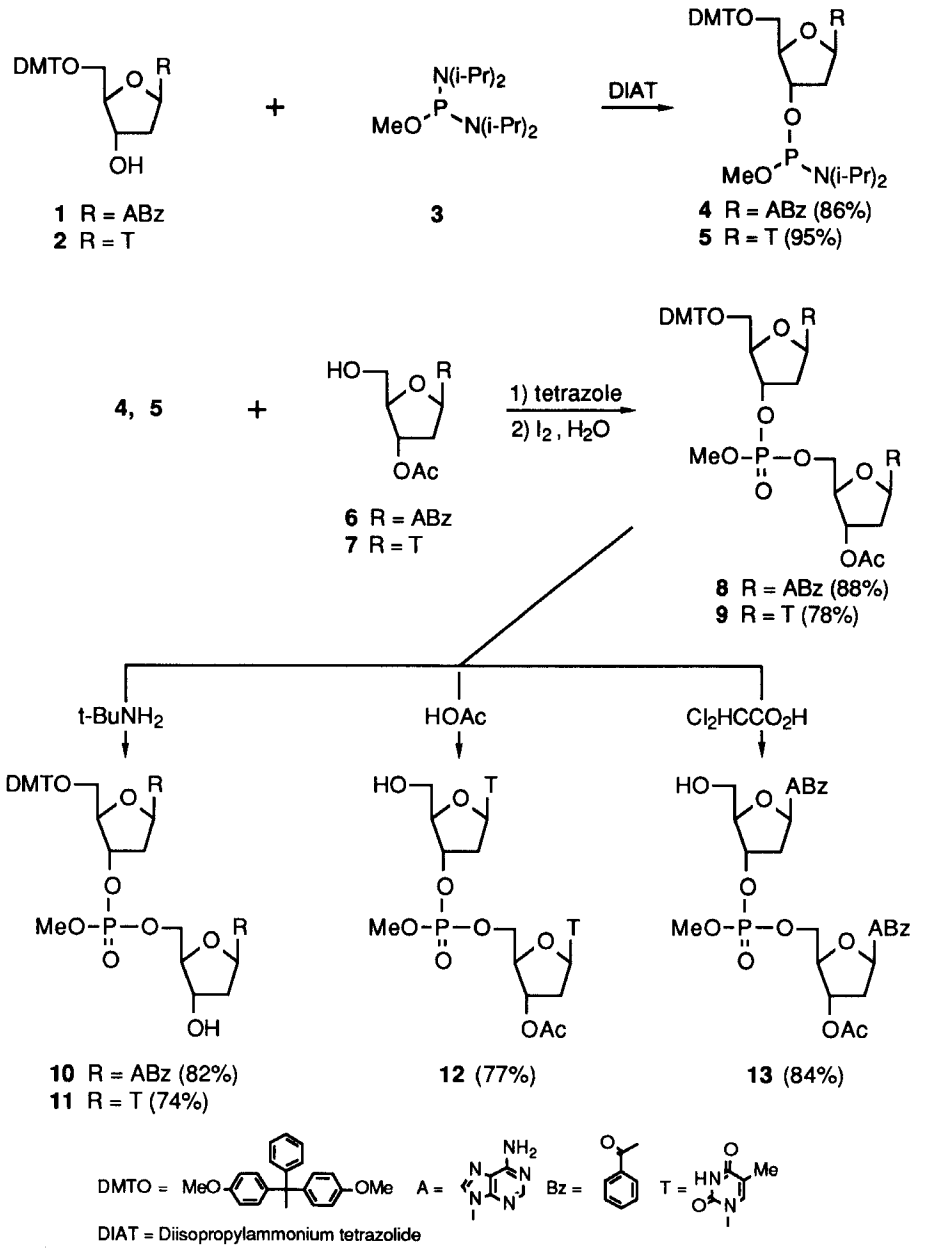

Scheme 1

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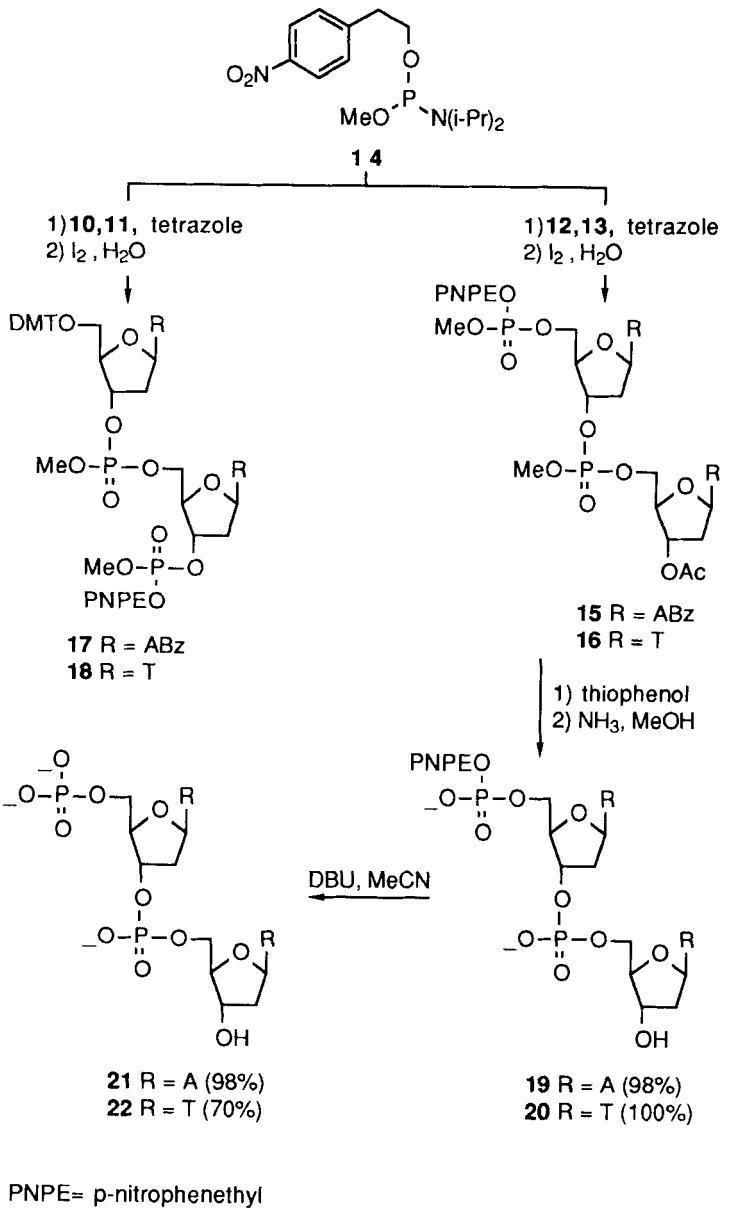

PNPE= p-nitrophenethyl

Scheme 2

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Compounds **16-18** exhibited 7 or 8 signals in their ^{31}P NMR spectra. Four diastereomers are expected due to the presence of two asymmetric phosphate groupings. Eight signals are expected so the "missing" signal, when only seven are observed, must be due to the overlap of two signals with very similar chemical shifts.

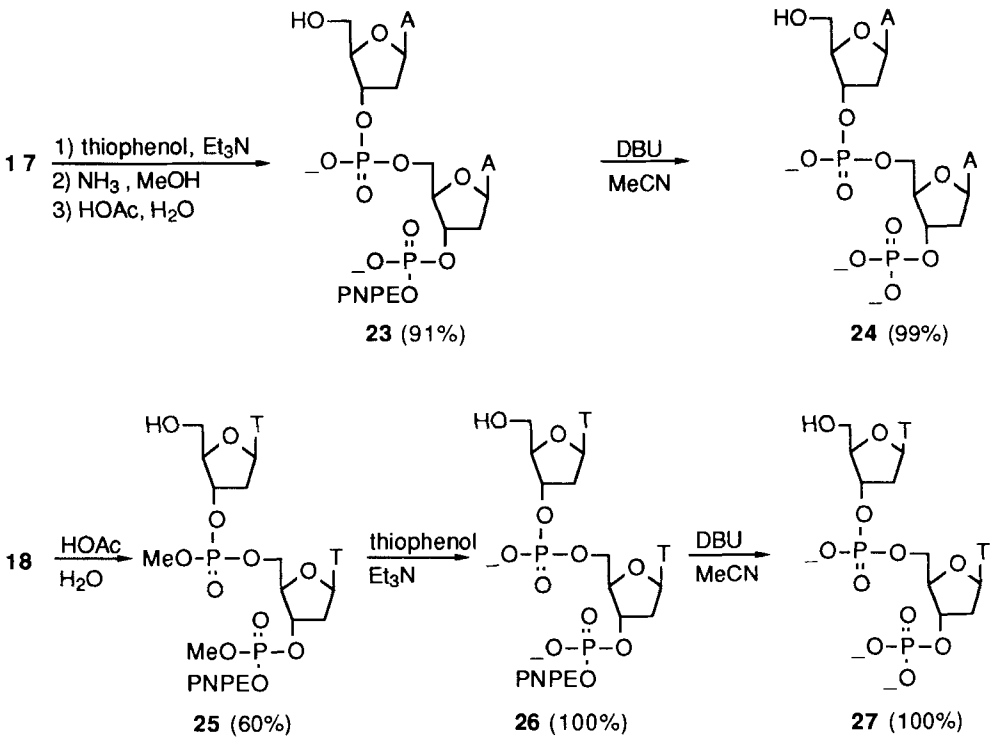
The O-CH_3 and acetate blocking groups on **15** and **16** were removed by thiophenol and NH_3 respectively. Compound **19** was purified by preparative reverse phase chromatography as the triethylamine salt while **20** was used directly after conversion to the Na^+ salt. Both **19** and **20** were converted to their disodium salts on a Na^+-Dowex 50 resin for NMR analyses. The Na^+ salt of **19** was too insoluble in acetonitrile so the more soluble triethylamine salt was used in the subsequent elimination reaction. DBU catalyzed elimination of the PNPE groupings[13] yielded **21** and **22** which were converted to the Na^+ salts by ion exchange chromatography and were purified by reverse phase chromatography. The pApA and pTpT were purified by crystallization. The ^{31}P spectra of **19, 21,** and **22** exhibited two peaks of equal intensity as expected for the presence of two phosphate groupings in each structure.

The 3'-phosphorylated dimers, **24** and **27**, were prepared by procedures similar to those used for **21** and **22** (Scheme 3) except acid hydrolysis was required to remove the 5'-DMT group. Both products were converted to their sodium salts and purified by preparative reverse phase chromatography. The ^{31}P NMR spectrum of **25** exhibited seven of the expected eight peaks while each of the spectra of **23, 24,** and **27** had two ^{31}P signals of approximately equal intensity consistent with the presence of two diastereomeric phosphate derivatives.

The isomeric dinucleotides of adenosine, **21** and **24**, have different HPLC retention times but were both converted to dApA by cleavage of the terminal phosphate group with alkaline phosphatase (APH). The dApA formed had the same HPLC retention time as an authentic sample when coinjected with it. The structures of **22** and **27** were established similarly by APH hydrolysis to dTpT.

EXPERIMENTAL

General: ^1H NMR spectra were recorded on Varian XL-200, with chemical shifts reported in parts per million (ppm) from tetramethylsilane (TMS) and 31P NMR spectra were recorded on Varian UNITY 500 MHz spectrometer in $CDCl_3$ or D_2O in HEPES buffer at pH 7-7.5 with 85% phosphoric acid as an external standard. In some instances it was necessary to add 0.01 M EDTA to the 31P NMR solvent mixture to obtain sharp signals.[14] All glassware used in synthetic reactions and all glassware used to contain reaction products were base washed in a solution of sodium hydroxide and ethanol, rinsed thoroughly with distilled water, and air dried. Reaction solvents were of



Scheme 3

reagent grade and were used without further purification unless otherwise noted. Methylene chloride was high performance liquid chromatography grade (Baker). Pyridine and acetonitrile were continuously distilled from calcium hydride under a nitrogen atmosphere in apparatus fitted with rubber septa to allow removal of the solvent by syringe. Water used to dissolve nucleotide products and for chromatographic work was double distilled from an all glass distillation apparatus. All nucleoside and nucleotide starting materials and reaction products were handled at temperatures below 40 °C, and all reactions were conducted in flasks fitted with rubber septa under an atmosphere of argon at room temperature unless otherwise noted. Crude reaction mixtures were ordinarily dried by treatment with saturated sodium chloride solution and azeotropic removal of residual water by distillation of the methylene chloride used for product extractions. Unless otherwise noted, analytical thin layer chromatography (TLC) was done on silica gel (Kieselgel 60 F254, 0.2 mm, Merck EM) using a developer of 5% methanol in methylene chloride, and preparative column chromatography was done using Merck EM Kieselgel 230-400 mesh packed in methylene chloride. High

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performance liquid chromatography (HPLC) was performed on a Waters HPLC system with a model 680 programmable automated gradient controller and a model 481 variable wavelength spectrophotometric detector. Output was recorded on a Spectral Physics model SP 4290 integrator. The HPLC system employed either a Waters μBondpak octadecyl silica gel (C18) 0.2 x 30 cm analytical column (P/N 27324), a Synchropak AX 100 0.2 x 6 cm ion exchange column for analytical work, or a Waters μBondpak C_{18} 0.18 x 30 (P/N 84176) preparative column for preparative separations. Sample elution for both the analytical and preparative C18 columns was performed employing a solvent gradient of buffers "A" and "B". Buffer "A" was a 5% (v/v) methanol-water solution which was 5.00 mM in KH_2PO_4 (Fisher). The buffer was adjusted to a pH of 3.5 with a 0.25 M solution of phosphoric acid. Buffer "B" was a 40% (v/v) methanol-water solution which was 10.0 mM in KH_2PO_4. The buffer was adjusted to a pH of 4.0 with a 1 M solution of 0.25 M phosphoric acid. Nucleotide products were converted to their sodium salts by elution through a 2 x 25 cm column of Dowex 50W-X8 cation exchange resin (16-40 mesh). A preparative intermediate pressure reverse phase (C-18) was employed for final nucleotide product purification. The column was prepared by dry-packing an Altex 15 x 200 mm glass column with octadecyl silica gel (Waters, 37-55 micron, P/N 30632). The column was fitted to a Fluid Metering Inc. (FMI) solvent delivery pump model RP5Y. Product elution was monitored with an LKB Uvicord type 4701A optical detector equipped with a LKB Uvicord model 4701A control unit. Output was recorded on a Sargent-Welsh model SRLG chart recorder. The column was prepared by sequential rinsing of the dry-packed gel with 500 mL portions of water, methanol, acetonitrile, methlyene chloride, acetonitrile, methanol, and water. In typical use, the column was operated at 100 PSI. Three gradients were routinely used in the operation of the analytical C-18 column. Gradient "1" consisted of an initial mixture of 100% buffer "A" in "B" which decreased to 60% in 30 min. From 30 to 35 min the concentration of buffer "A" further decreased to 40% and from 35-37 min, the concentration of buffer "A" was increased to 100% following a linear curve. A second gradient (gradient "3") was employed for confirmation that all materials had eluted from the column. This gradient was identical to gradient 1 except that from 35-45 min, the concentration of buffer "A" was decreased to 0% following a linear curve, and the buffer composition was maintained for 10 min. From 55-57 min the composition of buffer "A" was restored to 100% following a linear curve.

THE SYNTHESIS OF d(pApA) (21)

Fully Protected 2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine (8)

In a 250 mL round-bottomed flask were placed 2.222 g (2.717 mmol)5'-dimethoxytrityl-N-benzoyl-2'-deoxyadenosine-3'-(methoxydiisopropylamino)-

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phosphite (**4**) and 0.971 g (2.44 mmol, 0.9 eq) of 3'-acetyl-N-benzoyl-2'-deoxyadenosine (**6**). The mixture was dried by coevaporation with three 100 mL portions of benzene on a rotary evaporator, and the system was restored to atmospheric pressure each time with argon. 600 mg (8.5 mmol) of freshly sublimed tetrazole (Aldrich gold label) were added, and reaction flask was evacuated to 0.01 torr for 4 h. The flask was filled with argon, stoppered with a rubber septum, and 20 mL of continuously distilled acetonitrile were added by syringe. The reaction flask was swirled for 5 min during which time all solids dissolved to yield a yellow solution which was allowed to stand at room temperature overnight. The pale yellow crude reaction mixture was oxidized by titration to a persistent pale orange color with a 1.0 M I_2 solution in 2,6-lutidine, tetrahydrofuran, and water (2:2:1). The resulting pale orange reaction mixture was quenched after 2 min by the addition of *ca.* 10 mL of an aqueous solution of saturated sodium thiosulfate. The colorless two phase mixture was treated with 50 mL of a saturated sodium bicarbonate solution, and the mixture was extracted with four 25 mL portions of methylene chloride in a separatory funnel. The organic phases were combined, dried with a single 25 mL portion of saturated sodium chloride solution, and reduced in volume on a rotary evaporator to yield *ca.* 10 mL of yellow liquid which smelled strongly of lutidine. The residue was coevaporated at oil pump pressure on a rotary evaporator with three 20 mL portions of acetonitrile to yield a yellow oil which smelled faintly of lutidine. The oil was dissolved in 5 mL of methylene chloride and purified by flash chromatography on a 2.6 x 25 cm silica gel column using a 0-4% gradient of methanol in methylene chloride in 0.5% steps to a total solvent volume of *ca.* 2 L. 50 mL fractions were collected and analyzed by TLC, and solvent removal from fractions 14-20 yielded 2.41 g (88%) of **8** as a brittle yellow foam.

^{1}H NMR ($CDCl_3$) d 9.30-9.17 (m, 2, CONH), 8.73 (2s, 1, H_8), 8.61 (2s, 1, H_{8a}), 8.32 (2s, 1 H_2), 8.13 (2s, 1, H_{2a}), 8.07-7.92 (m, 4, aryl), 7.65-7.13 (m, 15, aryl), 6.78 (d, J=8.2 Hz, 4, aryl), 6.58-6.40 (m, 2, $H_{1'}$,$_{1a'}$), 5.55-5.45 (m, 1, $H_{3'}$), 5.32-5.22 (m, 1, $H_{3a'}$), 4.47-4.25, (m, 4, $H_{5'}$,$_{5''}$,$_{5a'}$,$_{5a''}$), 3.77 (2d J= 11.3 Hz, 3, $POCH_3$) 3.74 (s, 6, OCH_3), 3.50-3.30 (m, 2, $H_{4'}$, $_{4a'}$,), 3.28-2.85 (m, 2, $H_{2'}$,$_{2a'}$), 2.85-2.55 (m, 2, $H_{2''}$,$_{2a''}$), 2.13 (s, 3, $COCH_3$).

5'-Hydroxy Fully Protected 2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine (13)

In a dry 100 mL round-bottomed flask fitted with a magnetic stirring bar was placed a solution of 1112 mg (0.100 mmol) of fully protected 2'-deoxyadenyl-(3',5')-2'-deoxyadenosine (**8**) dissolved in 100 mL of dry methylene chloride. To the vigorously stirred pale yellow solution were added in one portion 200 mL of a 2% (v/v) solution of freshly distilled dichloroacetic acid (Aldrich) dissolved in methylene chloride. The blood red solution was stirred for 20 min, and quenched with 25 mL of a saturated solution of sodium bicarbonate. The resulting pale yellow two phase mixture

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was transferred to a separatory funnel, the organic phase separated, and the aqueous phase extracted with three 25 mL portions of methylene chloride. The organic portions were combined and washed with a single 25 mL portion of saturated sodium chloride solution, and the solvent was removed on a rotary evaporator to yield a yellow gum. The gum was purified by flash chromatography on a 2.5 x 15 cm. silica gel column. The column was eluted with a 0-8% gradient of methanol in methylene chloride in 1% steps to a total solvent volume of 1.5 liters. 100 mL fractions were collected and analyzed by TLC. Fractions 12-14 were combined, and the solvent was removed on a rotary evaporator to yield 68.1 mg (84%) of **13** as a white, brittle foam.

^{1}H NMR ($CDCl_3$) d 9.48-9.30 (m, 2, CONH), 8.73 (2s, 1, H_8), 8.68 (2s, 1, H_{8a}), 8.38 (2s, 1 H_2), 8.23 (2s, 1, H_{2a}), 7.99 (d J=6.91 Hz, 4, aryl), 7.68-7.38 (m, 6, aryl), 6.62-6.50 (m, 1, $H_{1'}$), 6.50-6.32 (m, 1, $H_{1a'}$), 6.01-5.83 (m, 1, OH), 5.58-5.48 (m, 1, $H_{3'}$), 5.33-5.18 (m, 1, $H_{3a'}$), 4.48-4.30, (m, 4, $H_{5',5'',5a',5a''}$), 3.90-3.78 (m, 2, $H_{4'}$, 4a',),3.80 (d, J=11.3 Hz, 3, $POCH_3$), 3.28-2.80 (m, 2, $H_{2',2a'}$), 2.80-2.52 (m, 2, $H_{2'',2a''}$), 2.15 (s, 3, $COCH_3$)

^{31}P NMR ($CDCl_3$) -0.094, -0.305.

Fully Protected 5'-Phosphoryl-2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine (15)

In a dry 100 mL round-bottomed flask were placed 488.6 mg. (0.605 mmol) of 5'-hydroxy-fully protected 2'-deoxyadenyl-(3',5')-2'-deoxyadenosine (**13**) and 169.3 mg (2.4 mmol, 4.0 eq) tetrazole (Aldrich gold label). The reaction flask was evacuated with an oil pump for 30 min, filled with argon, and stoppered with a rubber septum. 10 mL of continuously distilled acetonitrile were added by syringe, and 396 mg (1.21 mmol, 2.0 eq) of 4-nitrophenethylmethoxydiisopropylaminophosphine (**14**)[26] were introduced with swirling. The resulting yellow solution was allowed to stand at room temperature for 4 h, and the solution was oxidized by titration to a persistent pale orange color with a solution of 1.0 M I_2 dissolved in a mixture of 1,6-lutidine, tetrahydrofuran, and water (2:2:1). The reaction mixture was quenched with 10 mL of a saturated solution of sodium thiosulfate after 2 min. 20 mL of saturated sodium bicarbonate was added slowly with stirring to the pale yellow solution, and the two phase mixture was transferred to a separatory funnel. The aqueous phase was extracted with three 25 mL portions of methylene chloride. The organic extracts were combined, washed with a single 25 mL portion of saturated sodium chloride solution, and the solvent was removed on a rotary evaporator to yield a yellow oil which smelled strongly of lutidine. The oil was coevaporated at oil pump pressure on a rotary evaporator with three 20 mL portions of acetonitrile to yield a yellow oil which smelled faintly of lutidine. The oil was dissolved in 5 mL of methylene chloride and purified by flash chromatography on a 3x30 cm. silica gel column using a 0-10% gradient of methanol in

methylene chloride in 1% steps to a total solvent volume of 2 L. 100 mL fractions were collected and analyzed by TLC. Solvent removal from fractions 14-18 on a rotary evaporator yielded 509.0 mg. (80%) of **15** as a pale yellow foam. (It is essential that the product be virtually free of impurities as indicated by TLC before use in subsequent steps).

^{1}H NMR ($CDCl_3$) d 9.60-9.25 (m, 2, CONH), 8.78-8.62 (m, 2, H_8), 8.48 (2s, 1, H_2), 8.23 (2s, 1, H_{2a}), 8.18-7.93 (m, 6, aryl) 7.65-7.28 (m, 8, aryl), 6.62-6.38 (m, 2, $H_{1'}$,$_{1a'}$), 5.58-5.45 (m, 1, $H_{3'}$), 5.33-5.15 (m, 1, $H_{3a'}$), 4.52-4.10, (m, 8, CH_2-CH_2,$H_{5'}$,$_{5''}$,$_{5a'}$,$_{5a''}$), 3.80 (d, J=11.4 Hz, 3, $POCH_3$), 3.65 (2d, J=11.7 Hz, 3, $POCH_{3a}$), 3.25-2.80 (m, 4, $H_{4'}$,$_{4a'}$, $H_{2'}$,$_{2a'}$), 2.80-2.52 (m, 2, $H_{2''}$,$_{2a''}$), 2.15 (3s, 3, $COCH_3$).

5'-(4-Nitrophenethyl)phosphoryl-2'-Deoxy-adenyl-(3',5')-2'-Deoxyadenosine (19)

In a magnetically stirred, 100 mL round-bottomed flask fitted with an ammonia gas inlet tube and a mineral oil bubbler were placed 509 mg (0.485 mmol) of purified fully protected 5'-phosphoryl-2'-deoxyadenyl-(3',5')-2'-deoxyadenosine (**15**) and 10 mL of a solution of thiophenol, triethylamine, and dioxane (1:2:2 v/v). The resulting yellow solution was allowed to stir at room temperature during which time a dark yellow oil separated. 30 mL of dry methanol were added to the two phase mixture and the resulting colorless solution was cooled to -78 ^{o}C. Anhydrous ammonia was introduced until the flask was *ca.* 2/3 filled with liquid, and the colorless solution was allowed to warm to room temperature and stir overnight. The colorless solution was reduced in volume with a water aspirator to yield *ca.* 10 mL of a strongly smelling pale yellow liquid. The liquid was dissolved in 10 mL double distilled water, and the aqueous phase was washed with seven 75 mL portions of ether until the smell of thiophenol was gone. The pale yellow aqueous solution was transferred to a separatory funnel with the aid of an additional 15 mL of double distilled water, and the solution was washed with five 50 mL portions of methylene chloride to yield a colorless aqueous phase. The aqueous solution was reduced in volume on a rotary evaporator fitted to an oil pump to a volume of *ca.* 2 mL. The solution was adjusted to a pH of 6.8 with a solution of 0.1 M HCl and applied to a 1.5 x 20 cm column of octadecyl silica gel (Waters). The column was washed with 250 mL of double distilled water to remove short retention time impurities, followed by elution with *ca.* 250 mL of a 50% solution of methanol in water. Elution was monitored with a UV detector and the product was collected as a single fraction. The solvent was removed on a rotary evaporator to yield 477 mg (98%) of the triethylamine salt of **19** as a colorless glass.

A 20 mg sample of the glass was removed for NMR analysis and applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). Elution of the column

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with water, solvent removal from the product containing fractions, and solvent removal on a rotary evaporator gave the sodium salt of the product as a colorless glass.

1H NMR (D2O) d 8.02 (s, 1, H8), 7.75 (s, 1, H8a), 7.69 (s, 1, H2), 7.58 (s, 1, H2a), 7.46 (d, J=8.3 Hz, 2, aryl), 6.77 (d, J=8.3 Hz, 2, aryl), 5.98 (t, J=6.7 Hz,1, H1',), 5.82 (m, 1, H1a'), 4.9-4.5 (m, 2,), 4.12-3.82 (m, 2), 3.82-3.37 (m, 2, H2',2a'), 2.67-2.07 (m, 2, H2",2a").

^{31}P NMR (D_2O, HEPES) 0.562, -0.558

5'-Phosphoryl-2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine Trisodium Salt (21)

466 mg (0.470 mmol) of 5'-(4-nitrophenethyl)phosphoryl-2'-deoxyadenyl-(3',5')-2'-deoxyadenosine triethylamine salt (**19**) were dissolved in 50 mL of a 0.5 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile containing 2.5 mL water in a magnetically stirred 100 mL round-bottomed flask fitted with a reflux condenser. The yellow solution was held at reflux temperature for 90 min. The resulting yellow solution containing a trace of solid material was cooled to room temperature, and the solvent was removed on a rotary evaporator evacuated with an oil pump. The yellow residue was dissolved in 20 mL of double distilled water, and the aqueous solution was washed with ten, 100 mL portions of methylene chloride to yield a colorless aqueous solution. The solvent was removed from the aqueous portion on a rotary evaporator at oil pump pressure to yield 331 mg of brittle white foam. The foam was dissolved in 5 mL of double distilled water and applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). The column was eluted with 200 mL of double distilled water, and elution was monitored with a UV detector connected to a chart recorder. The product was collected in a single 100 mL fraction and adjusted to a pH of 6.8 with a solution of 0.1 M HCl. The solvent was removed on a rotary evaporator at oil pump pressure to yield a pale yellow glass. The glass was dissolved in 2 mL of double distilled water and applied to a 1.5 x 20 cm column of octadecyl silica gel (Waters). The column was eluted with 250 mL of double distilled water followed by elution with *ca.* 500 mL of a 6% solution of methanol in water. Elution was monitored with a UV detector and 100 mL fractions were collected and analyzed by HPLC. Fractions containing product in excess of 98% purity as measured by the integrated peak areas were combined and the solvent was removed on a rotary evaporator to yield 324 mg (98%) of the product as an amorphous white solid. The product was dissolved in 5 mL of methanol and rendered anhydrous by coevaporation with three 25 mL portions of absolute ethanol. The white powdery residue was dissolved in 5 mL of dry methanol and ethanol was added dropwise to yield a slightly turbid solution at reflux temperature. The solution was allowed to cool slowly to room temperature and stored at -10 ^{o}C overnight. The resulting white

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powdery precipitate was collected on a small sintered glass filter funnel and rinsed with three 1 mL portions of absolute ethanol. The powder was dissolved in 10 mL of double distilled water, and the solvent was removed on a rotary evaporator at oil pump pressure. The residue was coevaporated at oil pump pressure with three 25 mL portions of double distilled water to remove ethanol. The resulting colorless glass was dissolved in 10 mL of double distilled water and freeze-dried to yield **21** as a white powder.

^{1}H NMR (D_2O) d 7.96 (s, 1, H_8), 7.89 (s, 1, H_{8a}), 7.60 (s, 1, H_2), 7.47 (s, 1, H_{2a}), 5.91 (t, J=6.4 Hz,1, $H_{1'}$,), 5.80 (dd, J=8.7 Hz, $H_{1a'}$), 4.73-4.50 (m, 1,), 4.61 (s, D_2O), 4.60-4.45 (m, 1), 4.15-4.05 (m, 1), 4.05-3.85 (m, 2,), 3.85-3.65 (m, 2), 2.58-1.95 (m, 4).

^{31}P NMR (D_2O, PIPES) 0.577, -0.547

THE SYNTHESIS OF d(ApAp) (24)

3'-Hydroxy Fully Protected-2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine (10)

1.112 g (1.0 mmol) of fully protected 2'-deoxyadenyl-(3',5')-2'-deoxyadenosine (**8**) were dissolved in 50 mL of dry methanol, and the colorless solution was cooled to -78 °C. 100 mL of a 0.15 M solution of freshly distilled t-butylamine were added to the turbid mixture to yield a colorless solution. The reaction mixture was warmed to -20 °C in a constant temperature bath and allowed to react 24 h. The solvent was removed from the cold solution on a rotary evaporator at oil pump pressure taking care not to allow the distillation residue to exceed a temperature of 0 °C. The cold, yellow, gummy, residue was dissolved in 50 mL of methanol precooled in a dry ice bath, and the solvent was removed to yield a yellow gum. The gum was dissolved in 10 mL of methylene chloride and purified on a 3 x 30 cm silica gel column using a step gradient of 2-8% methanol in methylene chloride in 1% steps to a solvent volume of 2 L. 100 mL fractions were collected and analyzed by TLC. Product fractions were combined, and the solvent was removed on a rotary evaporator to yield 873.4 mg (82%) of **10** as an off-white foam.

^{1}H NMR ($CDCl_3$) d 9.37-9.23 (m, 2, CONH), 8.69 (2s, 1, H_8), 8.60 (2s, 1, H_{8a}), 8.23 (2s, 1 H_2), 8.16 (2s, 1, H_{2a}), 8.08-7.91 (m, 4, aryl), 7.52-7.10 (m, 15, aryl), 6.75 (d, J=8.5 Hz, 4, aryl), 6.52-6.32 (m, 2, $H_{1'}$,$_{1a'}$), 5.32-5.16 (m, 1, $H_{3'}$), 4.91-4.70 (m, 1, $H_{3a'}$), 4.42-4.15, (m, 4, $H_{5'}$,$_{5''}$,$_{5a'}$,$_{5a''}$), 3.77 (2d J= 11.3 Hz, 3, $POCH_3$) 3.72 (s, 6, OCH_3), 3.48-3.25 (m, 2, $H_{4'}$,$_{4a'}$), 3.28-2.45 (m, 4, $H_{2''}$,$_{2a''}$,$_{2'}$,$_{2a'}$).

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3'-Phosphoryl- Fully Protected 2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine (17)

In a 100 mL round-bottomed flask were placed 488.6 mg (0.605 mmol) of 3'-hydroxy-fully protected 2'-deoxyadenyl-(3',5')-2'-deoxyadenosine (**10**) and 170 mg (2.4 mmol, 4.0 eq) of tetrazole (Aldrich, gold label). The flask was evacuated on an oil pump for 30 min, filled with argon, and stoppered with a rubber septum. 10 mL of continuously distilled acetonitrile were added by syringe followed by 396 mg (1.21 mmol, 2.0 eq) of 4-nitrophenethylmethoxydiisopropylaminophosphine.[26] The crude reaction mixture was swirled for 5 min to yield a pale yellow solution containing a granular precipitate. The mixture was allowed to stand at room temperature for 4 h and oxidized by titration to a persistent pale orange with a 1.0 M solution of I_2 dissolved in 2,6-lutidine, THF, and water (2:2:1). After 2 min, the pale orange solution was quenched with 10 mL of a saturated sodium thiosulfate solution. 20 mL of a saturated solution of sodium bicarbonate and 25 mL methylene chloride were added, and the mixture was transferred to a separatory funnel. The aqueous phase was extracted with three 25 mL portions of methylene chloride, and the combined extracts were washed with a single 25 mL portion of a saturated solution of sodium chloride. The solvent was removed from the resulting pale yellow solution to yield a yellow oil which smelled strongly of lutidine. The oil was coevaporated at oil pump pressure on a rotary evaporator with three 20 mL portions of acetonitrile to yield a yellow gum which smelled faintly of lutidine. The gum was dissolved in 10 mL of methylene chloride and purified by chromatography on a 3 x 30 cm column of silica gel. The column was eluted with a 0-10% gradient of methanol in methylene chloride in 1% steps to a total solvent volume of *ca.* 2 L. 100 mL fractions were collected and analyzed by TLC. Product containing fractions were combined, and the solvent was removed on a rotary evaporator to yield 509 mg (80%) of **17** as a pale yellow brittle foam.

^1H NMR ($CDCl_3$) d 9.20-9.05 (m, 2, CONH), 8.74 (2s, 1, H_8), 8.60 (2s, 1 H_{8a}), 8.24 (2s, 1, H_2), 8.14 (2s, 1, H_{2a}), 8.14 (d, J=7.9 Hz, 2, aryl), 8.08-7.91(m, 2, aryl), 7.79-7.05 (m, 17, aryl), 6.82-6.70 (d, J=8.3 Hz, 4, aryl) 6.52-6.38 (m, 2, $H_{1'}$,$_{1a'}$), 5.32-5.18 (m, 2, $H_{3'}$,$_{3a'}$), 4.50-4.18, (m, 6, CH_2,$H_{5'}$,$_{5''}$,$_{5a'}$,$_{5a''}$), 3.80-3.70 (m, 12, OCH_3,$POCH_3$), 3.50-3.30 (m, 2, $H_{4'}$,$_{4a'}$), 3.20-2.90 (m, 4, CH_2, $H_{2'}$,$_{2a'}$), 2.83-2.60 (m, 2, $H_{2''}$,$_{2a''}$).

^{31}P NMR ($CDCl_3$) -0.155, -0.162, -0.200, -0.230, -0.268, -0.287, -0.317.

3'-(4-Nitrophenethyl)phosphoryl-2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine (23)

In a magnetically stirred 100 mL round-bottomed flask fitted with an ammonia gas inlet tube and a mineral oil bubbler were placed 637.9 mg (0.485 mmol) of

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carefully purified 3'-phosphoryl-fully protected 2'-deoxyadenyl-(3',5')-2'-deoxyadenosine (**17**) and 10 mL of a solution of thiophenol, triethylamine, and dioxane (1:2:2 v/v). The resulting yellow solution was allowed to stir at room temperature for 30 min. 30 mL of dry methanol were added to the yellow emulsion, and the resulting colorless true solution was cooled to -78 ^{o}C. Anhydrous ammonia was introduced until the flask was *ca.* 2/3 filled with liquid, and the colorless solution was allowed to warm to room temperature and stir overnight. The colorless solution was reduced in volume with a water aspirator, and the remaining volatiles were removed on a rotary evaporator fitted to an oil pump to yield a yellow highly scented oil. The oil was treated with 30 mL of 80% acetic acid at room temperature to yield a pale yellow solution containing a white solid. The mixture was allowed to stir at room temperature for 30 min during which time the white solid completely dissolved to yield an orange solution. The solvent was removed on a rotary evaporator at oil pump pressure to yield an odoriferous pale orange solid. 20 mL of water were added, and the mixture was washed with ten 50 mL portions of ether until the smell of thiophenol was gone. The pale yellow aqueous solution was transferred to a separatory funnel and washed with five 50 mL portions of methylene chloride to yield a colorless aqueous solution. The solvent was removed from the aqueous phase on a rotary evaporator at oil pump pressure to yield a pale yellow gum. The gum was dissolved in 2 mL of double-distilled water and the pH was adjusted to 6.8 with a solution of 0.1 M HCl, and applied to a 1.5 x 20 cm column of octadecyl silica gel (Waters). The column was washed with 250 mL of double distilled water to remove short retention time impurities, followed by elution with *ca.* 250 mL of a 50% solution of methanol in water. Elution was monitored with a UV detector and the product was collected as a single fraction. The solvent was removed on a rotary evaporator to yield 437 mg (91%) of the triethylamine salt of the product as a colorless glass. 20 mg of the glass were dissolved in 1 mL of double distilled water for NMR analysis, and the solution was applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). Elution of the column with distilled water followed by solvent removal from the product containing fraction gave the sodium salt of **23** as a colorless glass.

^{1}H NMR(D_2O) d 8.74 (s, 0.5, H_8), 7.65-7.50 (m, 1.5 H_{8a}), 7.31-7.20 (m, 1, H_2), 7.02-6.90 (m, 1, H_{2a}), 5.72-5.60 (m, 1, $H_{1'}$), 5.60-5.48 (m, 1, $H_{1a'}$) 4.65 (s, D_2O), 4.65-4.40 (m, 2, $H_{3',3a'}$), 4.02-3.70, (m, 6, CH_2,$H_{5',5'',5a',5a''}$), 3.50-3.38 (m, 2, $H_{4',4a'}$), 2.73-2.61 (m, 2, CH_2), 2.20-1.95 (m, 2, $H_{2',2a'}$), 1.95-1.70 (m, 2, $H_{2'',2a''}$).

^{31}P NMR (D_2O, HEPES) -0.245, -0.660

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3'-Phosphoryl-2'-Deoxyadenyl-(3',5')-2'-Deoxyadenosine Trisodium Salt (24)

466 mg (0.470 mmol) of 3'-(4-nitrophenethyl)-phosphoryl-2'-deoxyadenyl-(3',5')-2'-deoxyadenosine triethylamine salt **(23)** were dissolved in 50 mL of a 0.5 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile containing 2.5 mL water in a magnetically stirred 100 mL round-bottomed flask fitted with a reflux condenser. The solution was heated to reflux temperature for 2 h, and the resulting yellow solution was cooled to room temperature. The solvent was removed on a rotary evaporator evacuated with an oil pump. The yellow residue was dissolved in 20 mL of double distilled water, and the aqueous solution was washed with ten 100 mL portions of methylene chloride to yield a colorless aqueous solution. The solvent was removed from the aqueous portion on a rotary evaporator at oil pump pressure to yield a brittle white foam. The foam was dissolved in 5 mL of double distilled water and applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). The column was eluted with 200 mL of double distilled water, and elution was monitored with a UV detector. The product was collected in a single 150 mL fraction and adjusted to a pH of 6.8 with a solution of 0.1 M HCl. The solvent was removed on a rotary evaporator at oil pump pressure to yield a pale yellow glass. The glass was dissolved in 2 mL of double distilled water and applied to a 1.5 x 20 cm column of octadecyl silica gel (Waters). The column was eluted with 250 mL of double distilled water followed by elution with *ca.* 500 mL of a 6% solution of methanol in water. Elution was monitored with a UV detector and 100 mL fractions were collected and analyzed by HPLC. Fractions containing product in excess of 98% purity as measured by the integrated peak areas were combined, and the solvent was removed on a rotary evaporator to yield 331 mg (99%) of the product as an amorphous white solid. The product was dissolved in 5 mL of methanol and rendered anhydrous by coevaporation with three 25 mL portions of absolute ethanol. The white powdery residue was dissolved in 15 mL of dry methanol and ethanol was added dropwise to yield a slightly turbid solution at reflux temperature. The product which appeared to exhibit greater solubility at lower temperatures was precipitated by solvent evaporation from a 90 ^{o}C water bath of a period of 2 h on a slowly turning rotary evaporator at atmospheric pressure. The resulting white powdery precipitate was collected on a small sintered glass filter funnel and rinsed with three 1 mL portions of absolute ethanol. The powder was dissolved in 10 mL of double distilled water, and the solvent was removed on a rotary evaporator at oil pump pressure. The residue was coevaporated at oil pump pressure with three 25 mL portions of double distilled water to

remove ethanol. The resulting colorless glass was dissolved in 10 mL of double distilled water and freeze-dried to yield **24** as a flocculant white powder.

^{1}H NMR (D_2O) d 8.13 (s, 1, H_8), 7.76 (s, 1, H_{8a}), 7.72 (s, 1, H_2), 7.59 (s, 1, H_{2a}), 6.12-5.95 (m, 1, $H_{1'}$,), 5.95-5.71 (m, 1, $H_{1a'}$), 4.73-4.62 (m, 1, $H_{3'}$), 4.65 (s, D_20), 4.65-4.50 (s, 1, $H_{3a'}$), 4.25-4.15 (m, 1,$H_{5a'}$), 4.05-3.85 (m, 3, $H_{5'}$,5",5a'), 3.55-3.45 (m, 2, $H_{4'}$,4a'), 2.70-2.40 (m, 2, $H_{2'}$,2"), 2.30-2.13 (m, 1, $H_{2a'}$), 1.95-1.72 (m, 1, $H_{2a"}$).

^{31}P NMR (D_20, HEPES) -0.268, -0.686.

THE SYNTHESIS OF d(pTpT) (22)

Fully Protected Thymidyl-(3',5')-Thymidine (9)

In a 250 mL round-bottomed flask was placed 4.59 g (6.5 mmol) 5'-dimethoxytritylthymidine(methoxydiisopropylamino)phosphite (**5**), 1.43 g (5.05 mmol, 0.8 eq) of 3'-thymidine acetate (**7**), and 1.12 g (16.8 mmol, 3.0 eq) of tetrazole (Aldrich gold label). The flask was evacuated to a pressure of 5 x 10^{-3} torr overnight, filled with argon, and stoppered with a rubber septum. 20 mL of continuously distilled acetonitrile was added by syringe to yield a colorless true solution which precipitated a granular solid in *ca.* 5 min. The reaction mixture was allowed to stir overnight at room temperature after which TLC on silica gel with a developing solution of hexane, acetone, and triethylamine (45:45:10) indicated the complete consumption of the phosphoramidite starting material. (Note: It is necessary to treat the TLC plate with the developing solution, and to dry the plate prior to spotting with sample). The colorless reaction mixture which contained a granular precipitate was oxidized by titration to a persistent pale orange color with a 1.0 M I_2 solution in 2,6-lutidine, tetrahydrofuran, and water (2:2:1). The resulting pale orange reaction mixture was quenched after 2 min by the addition of *ca.* 10 mL of an aqueous solution of saturated sodium thiosulfate. The colorless two phase mixture was treated with 150 mL of a saturated sodium bicarbonate solution and extracted with four 50 mL portions of methylene chloride. The organic fractions were combined and washed with a single 50 mL portion of water followed by a single 50 mL portion of saturated sodium chloride solution. The pale yellow solution was reduced in volume on a rotary evaporator to yield *ca.* 10 mL of yellow liquid which smelled strongly of lutidine. The residue was coevaporated at oil pump pressure on a rotary evaporator with three 20 mL portions of acetonitrile to yield a yellow oil which smelled faintly of lutidine. The oil was dissolved in 30 mL of methylene chloride and purified by flash chromatography on a 4 x 30 cm silica gel column using a 0-4% gradient of methanol in methylene chloride in 0.5% steps to a total solvent volume of *ca.* 4 L. 200 mL fractions were collected and analyzed

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by TLC, and solvent removal from fractions containing the product yielded 3.56 g (78%) of **9** as a brittle yellow foam.

^{1}H NMR ($CDCl_3$) d 10.10-9.95(m, 2, CONH), 7.63-7.52 (m, 2, $H_{6,6a}$), 7.50-7.18 (m, 9, aryl), 6.84 (d, J=8.9 Hz, 4, aryl), 6.50-6.30 (m, 2, $H_{1',1a'}$), 5.30-5.12 (m, 2, $H_{3'3a'}$), 4.40-4.12, (m, $H_{5',5',5a',5a''}$), 3.79 (s, 6, OCH_3), 3.60-3.32 (m, 2, $H_{4'}$, $_{4a'}$), 2.73-2.10 (m, 4, $H_{2',2'',2a',2a''}$), 2.10 (2s, 3, $COCH_3$), 1.90 (s, 1.5, CH_3), 1.40 (s, 1.5, CH_3).

5'-Hydroxy Fully Protected Thymidyl-(3',5')-Thymidine (12)

3.56 g (3.94 mmol) of fully protected thymidyl-(3',5')-thymidine (**9**) were dissolved in 50 mL of 80% acetic acid in a 250 mL round-bottomed flask. The orange solution was stirred at room temperature for 2 h, and the solvent was removed on a rotary evaporator at oil pump pressure to yield a yellow gum. The gum was dissolved in 10 mL of a 10% solution of methanol in methylene chloride, and the product was purified by flash chromatography on a 3 x 30 cm silica gel column using a 0%-8% step gradient of methanol in methylene chloride in 1% steps to a total solvent volume of *ca.* 2.5 L. 125 mL fractions were collected and analyzed by TLC. The product fractions were combined, and the solvent was removed on a rotary evaporator to yield 1.82 g (77%) of **12** as a colorless foam which was freely soluble in methylene chloride following addition of a few drops of methanol.

^{1}H NMR($CDCl_3$+1 drop CD_3OD) d 10.01-9.83(m, 2, CONH), 7.60 (s, 1, $H_{6,}$), 7.41 (2s, 1, H_{6a}), 6.40-6.20 (m, 2, $H_{1',1a'}$), 5.35-5.25 (m, 1, $H_{3'}$), 5.20-5.05 (m, 1, $H_{3a'}$), 4.40-4.28 (m, 2, $H_{5',5'}$), 4.28-4.15 (m, 2, $H_{5a',5a''}$), 3.90-3.80 (m, 2, $H_{4'}$, $_{4a'}$), 3.84 (d, J=11.4Hz, 3, POCH3), 3.47 (s, 1, CD_3OH), 2.65-2.20 (m, 6, $H_{2',2'',2a',2a''}$), 2.12 (s, 3, $COCH_3$), 1.92 (2s, 3, CH_3).

^{31}P NMR ($CDCl_3$ + 1 drop CD_3OD) -0.151, -0.441.

5'-Phosphoryl Fully Protected Thymidyl-(3',5')-Thymidine (16)

602 mg (1.0 mmol) of 5'-hydroxy-fully protected thymidyl-(3',5')-thymidine (**12**) and 490 mg (1.5 mmol, 1.5 eq) of p-nitrophenethyldiisopropylammoniummethoxyphosphine (**14**)[13] were dried in a 100 mL round-bottomed flask by coevaporation with 20 mL of continuously distilled acetonitrile on a rotary evaporator at oil pump pressure. Argon was bled into the system when evaporation was complete, and the reaction vessel was quickly stoppered with a rubber septum. 20 mL of additional acetonitrile were added with a syringe, and the mixture was swirled 5 min to yield a colorless solution. 280 mg (4.0 mmol, 4.0 eq) of tetrazole (Aldrich gold label) were added, and the flask was quickly restoppered and swirled to yield a colorless solution which began to precipitate a colorless granular material in a few minutes. The reaction mixture was allowed to stand overnight at room temperature after which TLC of the

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crude mixture on a silica gel plate pretreated with a developing solution of hexane, acetone and triethylamine, (9:9:2) showed complete consumption of the nucleotide starting material. The colorless reaction mixture was oxidized by titration with a 1 M solution of I_2 dissolved in 2,6-lutidine, THF, water (2:2:1) to a persistent pale orange color, and after 2 min 5 mL of a saturated solution of sodium thiosulfate were added. The colorless emulsion was reduced in volume on a rotary evaporator at oil pump pressure to *ca.* 10 mL to remove acetonitrile. The residue was dissolved in 75 mL of methylene chloride, and the organic layer was washed with a single 50 mL portion of a saturated solution of sodium bicarbonate followed by a single 50 mL portion of saturated sodium chloride solution. Solvent removal from the organic layer on a rotary evaporator yielded a colorless gum which smelled strongly of lutidine. The gum was coevaporated at oil pump pressure on a rotary evaporator with three 20 mL portions of acetonitrile to yield a colorless oil which smelled faintly of lutidine. The oil was dissolved in 5 mL of methylene chloride, and the product was purified by flash chromatography on a 3 x 30 cm silica gel column with a 0%-6% gradient of methanol in methylene chloride in 1% steps to a total solvent volume of 2 L. Fractions were collected and analyzed by TLC. The fractions containing pure product were combined, and the solvent was removed on a rotary evaporator to yield 671 mg (80%) of **16** as a brittle white foam (Note: It is imperative that the product be virtually free of impurities before use in the next step).

^1H NMR ($CDCl_3$) d 9.55-9.30 (m, 2, CONH), 8.22-8.13 (m, 2, aryl), 7.48-7.32 (m, 4, aryl, $H_{6,6a}$), 6.40-6.25 (m, 2, $H_{1',1a'}$), 5.38-5.23 (m, 1, $H_{3'}$), 5.15-5.00 (m, 1, $H_{3a'}$), 4.42-4.12 (m, 8, CH_2,$H_{5',5',5a',5a'',4',4a'}$), 3.79 (2d, J=11.3 Hz, 6, $POCH_3$), 3.12 (t, J=6.6 Hz, 2, CH_2), 2.65-2.00 (m, 4, $H_{2',2'',2a',2a''}$), 2.12 (s, 3, $COCH_3$), 1.92 (2s, 3, CH_3).

^{31}P NMR ($CDCl_3$) 0.603, 0.569, 0.483, 0.445, 0.026, -0.332, -0.351.

5'-(4-Nitrophenethyl)phosphorylthymidyl-(3',5')-thymidine (20)

In a magnetically stirred 100 mL round-bottomed flask fitted with an ammonia gas inlet tube and a mineral oil bubbler, 671.1 mg (0.8 mmol) of fully protected 5'-phosphorylthymidyl-(3'-5')-thymidine (**16**) were dissolved in 20 mL of a solution of thiophenol, triethylamine, and dioxane (1:2:2 v/v). The yellow solution was stirred for 30 min at room temperature, and 30 mL of dry methanol was added. The almost colorless resulting solution was cooled to -78 ^oC, and anhydrous ammonia was introduced through a bubbler until the flask was *ca.* 2/3 full of liquid. The cooling bath was removed and the colorless solution was allowed to stir at room temperature overnight. The solvent was removed with a water aspirator to yield a yellow liquid. The liquid was dissolved in 10 mL double distilled water, and the aqueous solution was washed with seven 75 mL portions of ether until the smell of thiophenol was gone. The

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aqueous solution was reduced in volume on a rotary evaporator fitted to an oil pump to a volume of *ca.* 5 mL, and the colorless solution was applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). The column was eluted with 200 mL of double distilled water, and elution was monitored with a UV detector connected to a chart recorder. The product was collected in a single 100 mL fraction. The solvent was removed on a rotary evaporator at oil pump pressure to yield 677.6 mg (quantitative) of **20** as the dihydrate of the sodium salt which was used in the next step without further purification.

^{1}H NMR (D_2O) d 7.77 (d, J=7.6 Hz, 2, aryl), 7.45 (s, 1, H_6), 7.24-7.12 (d, 3, aryl, H_{6a}), 6.13-5.92 (m, 2, $H_{1'}$,$_{1a'}$), 4.68-4.55 (m, 1.2, DHO, $H_{3'}$), 4.41-4.30 (m, 1, $H_{3a'}$), 4.08-3.50 (m, 8, CH_2, $H_{5'}$,$_{5'}$,$_{5a'}$,$_{5a''}$,$_{4'}$,$_{4a'}$), 2.79 (t, J=6.1 Hz, 2, CH_2), 2.30-1.70 (m, 4, $H_{2'}$,$_{2''}$,$_{2a'}$,$_{2a''}$), 1.77 (s, 2, imp), 1.64 (s, 3, CH_3), 1.41 (s, 3, CH_3).

5'-Phosphorylthymidyl-(3',5')-Thymidine Trisodium Salt (22)

627 mg (0.74 mmol) of 5'-(4-nitrophenethyl)-phosphoryl-thymidyl-(3',5')-thymidine disodium salt (**20**) were dissolved in a solution of 47.5 mL acetonitrile, 2.5 mL water, and 3.8 g DBU. The pale yellow solution was heated to reflux temperature for 2 h after which time HPLC of the crude reaction mixture with an elution mixture of 25% buffer A in B showed only the slightest trace of remaining starting material. The yellow solution containing considerable solid material was cooled to room temperature and reduced in volume on a water aspirator to *ca.* 10 mL. 20 mL of double distilled water was added to the yellow solution, and the mixture was transferred to a separatory funnel. The aqueous phase was washed with ten 30 mL portions of methylene chloride to yield a colorless solution. The aqueous layer was reduced in volume to 2 mL on a rotary evaporator at oil pump pressure, and the residue was applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). The column was eluted with 200 mL of double distilled water, and elution was monitored with a UV detector connected to a chart recorder. The product was collected in a single 150 mL fraction. The pH of the solution was adjusted to 6.8 with 0.1 M HCl, and the solvent was removed on a rotary evaporator at oil pump pressure. The colorless residue was dissolved in 5 mL of double distilled water and applied to a 1.5 x 20 cm column of octadecyl silica gel. The column was eluted with 250 mL of double distilled water followed by elution with *ca.* 500 mL of a 6% solution of methanol in water. Elution was monitored with a UV detector and 100 mL fractions were collected and analyzed by HPLC. Fractions containing product in excess of 98% purity as measured by the integrated peak areas were combined, and the solvent was removed on a rotary evaporator to yield 530 mg (98%) of the product as an amorphous white solid. The solid was dissolved in 20 mL

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of refluxing 95% ethanol and allowed to cool slowly to room temperature for 6 h, then cooled to -10 °C overnight. The resulting white precipitate was collected on a sintered glass filter funnel and rinsed with three 1 mL portions of 95% ethanol to yield 377.0 mg (70%) of the product as white, highly crystalline plates. The plates were dissolved in 20 mL of double distilled water, and the solvent was removed on a rotary evaporator. The process was repeated twice to remove alcohols, and the residue was freeze-dried to yield **22** as a white powder.

^{1}H NMR (D_2O) d 7.63 (s, 1, H_6), 7.52 (s, 1, H_{6a}), 6.18-6.08 (m, 2, $H_{1'}$,$_{1a'}$), 4.75-4.62 (m, 1, $H_{3'}$), 4.64 (s, D_2O), 4.51-4.39 (m, 1, $H_{3a'}$), 4.21-4.12 (m, 1, $H_{4'}$), 4.05-3.85 (m, 3, $H_{5'}$,$_{5'}$,$_{4a'}$), 3.85-3.72 (m, 2, $H_{5a'}$,$_{5a''}$), 2.40-2.14 (m, 4, $H_{2'}$,$_{2''}$,$_{2a'}$,$_{2a''}$), 1.74 (s, 3, CH_3), 1.72 (s, 3, CH_3).

^{31}P NMR ($D_2$0, HEPES, EDTA): -0.418, 3.117

THE SYNTHESIS OF d(TpTp) (27)

3'-Hydroxy-fully protected thymidyl-(3',5')-thymidine (11)

3.74 g (4.14 mmol) of fully protected thymidyl-(3',5')-thymidine (**9**) were dissolved in 100 mL of a 0.15 M solution of freshly distilled t-butylamine in methanol at -78 °C. The colorless slurry was allowed to warm to -20 °C and swirled to yield a colorless solution containing the slightest trace of insoluble starting material. The solution was stored at minus 20 °C for 12 h. TLC analysis indicated the reaction was *ca.* 50% complete, and the solution was allowed to warm to room temperature for 5 h, after which analysis by TLC indicated the complete consumption of starting material. The solvent was removed from the resulting colorless solution on a rotary evaporator at oil pump pressure to yield a colorless glass. The glass was dissolved in 10 mL of methylene chloride and purified on a 4.5 x 30 cm silica gel column using a step gradient of 0-8% methanol in 1% steps to a solvent volume of 2 L. 100 mL fractions were collected and analyzed by TLC. Product fractions were combined, and the solvent was removed on a rotary evaporator to yield 2.636 g (74%) of **11** as an off-white foam.

^{1}H NMR ($CDCl_3$) d 10.5-9.5(br s, 2, CONH), 7.55 (s, 1, $H_{6,}$), 7.45-7.15 (m, 10, aryl, H_{6a}), 6.82 (d, J=8.6 Hz, 4, aryl), 6.45-6.21 (m, 2, $H_{1'}$,$_{1a'}$), 5.20-5.12 (m, 1, $H_{3'}$), 4.55-4.35 (m, 1, $H_{3a'}$),4.40-4.10 (m, $H_{5'}$,$_{5'}$,$_{5a'}$,$_{5a''}$), 3.77 (2s, 6, OCH_3), 3.74 (2d, J=11.4 Hz, 3, $POCH_3$), 3.65-3.25 (m, 2, $H_{4'}$,$_{4a'}$), 2.77-2.05 (m, 4, $H_{2'}$,$_{2''}$,$_{2a'}$,$_{2a''}$), 1.87 (s, 3, CH_3), 1.40 (2s, 1.5, CH_3).

^{31}P NMR ($CDCl_3$) 0.049, -0.713.

Fully Protected 3'-Phosphorylthymidyl-(3',5')-Thymidine (18)

2.64 g (3.06 mmol) of 3'-hydroxy-fully protected thymidyl-(3',5')-thymidine (**11**) and 1.50 g (4.5 mmol, 1.5 eq) of p-nitrophenethyl-

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diisopropylammoniumethoxyphosphine[13] were dried in a 250 mL round-bottomed flask by coevaporation with 50 mL of continuously distilled acetonitrile on a rotary evaporator at oil pump pressure. The reaction vessel was filled with argon, stoppered with a rubber septum, and 20 mL of additional acetonitrile were added by syringe. The mixture was swirled 5 min to yield a colorless solution, and 856 mg (12.2 mmol, 4.0 eq) of tetrazole (Aldrich gold label) was added. The flask was quickly restoppered and swirled to yield a colorless solution which began to precipitate a colorless granular material in a few minutes. The reaction mixture was allowed to stand overnight at room temperature. The resulting mixture was oxidized by titration with a 1 M solution of I_2 dissolved in 2,6-lutidine, THF, water (2:2:1) to a persistent pale orange color, and after 2 min 5 mL of a saturated solution of sodium thiosulfate were added. The colorless emulsion was reduced in volume on a rotary evaporator at oil pump pressure to *ca.* 10 mL to remove acetonitrile. The residue was dissolved in 75 mL of methylene chloride, and the organic layer was washed with a single 50 mL portion of a saturated solution of sodium bicarbonate followed by a single 50 mL portion of saturated sodium chloride solution. Solvent removal from the organic layer on a rotary evaporator yielded a colorless gum which smelled strongly of lutidine. The gum was coevaporated at oil pump pressure on a rotary evaporator with three 20 mL portions of acetonitrile to yield a colorless oil which smelled faintly of lutidine. The oil was dissolved in 10 mL of methylene chloride, and the product was purified by flash chromatography on a 3 x 30 cm silica gel column with a 0%-6% gradient of methanol in methylene chloride in 1% steps to a total solvent volume of 2 L. Fractions were collected and analyzed by TLC. The fractions containing pure product were combined, and the solvent was removed on a rotary evaporator to yield 1.98 g (60%) of **18** as a brittle white foam.

^1H NMR ($CDCl_3$) d 10.0-9.82 (m 2, CONH), 8.22-8.10 (m, 2, aryl), 7.60-7.20 (m, 13, aryl, $H_{6,6a}$), 6.83 (d, J=8.8 Hz, 4, aryl), 6.50-6.38 (m, 1, $H_{1'}$,), 6.38-6.22 (m, 1, $H_{1a'}$), 5.20-5.12 (m, 1, $H_{3'}$), 5.25-4.90 (m, 1, $H_{3a'}$), 4.45-4.10 (m, 8, CH_2, $H_{5',5',5a',5a'',4',4a'}$), 3.76 (2s, 6, OCH_3), 3.74 (2d, J=11.4 Hz, 6, $POCH_3$), 3.13 (t, J=6.6 Hz, 2, CH_2), 2.72-2.10 (m, 4, $H_{2',2'',2a',2a''}$), 1.89 (s, 1.5, CH_3), 1.40 (s, 1.5, CH_3).

^{31}P NMR ($CDCl_3$): -0.192, -0.230, -0.275, -0.283, -0.302, -0.313, -0.471, -0.513. There was also an unknown impurity at 9.612 which was not present in **25**, the subsequent synthetic intermediate.

5'-Hydroxy Fully Protected 3'-Phosphoryl-Thymidyl-(3',5')-Thymidine (25)

1.65 g (1.52 mmol) of fully protected 3'-phosphorylthymidyl-(3',5')-thymidine (**18**) were dissolved in 20 mL of 80% acetic acid in a 100 mL round-

bottomed flask. The orange solution was stirred at room temperature for 3 h, and the solvent was removed on a rotary evaporator at oil pump pressure to yield an orange gum. The gum was dissolved in 20 mL of water, and the solvent was removed on a rotary evaporator at oil pump pressure to yield an orange gum which smelled strongly of acetic acid. 10 mL of a saturated solution of sodium bicarbonate were added, and the solvent was removed on a rotary evaporator to give a yellow gum. The gum was dissolved in 10 mL of methylene chloride and purified by flash chromatography on a 3 x 30 cm silica gel column using a 0%-12% step gradient of methanol in methylene chloride in 1% steps to a total solvent volume of *ca.* 2.5 L. 125 mL fractions were collected and analyzed by TLC. The product fractions were combined, and the solvent was removed on a rotary evaporator to yield 851 mg (60%) of **25** as a brittle white foam.

^{1}H NMR ($CDCl_3$) d 9.65-9.15 (m 2, CONH), 8.25-8.12 (m, 2, aryl), 7.55-7.20 (m, 4, aryl, $H_{6,6a}$), 6.30-6.12 (m, 2, $H_{1',1a'}$), 5.25-4.92 (m, 2, $H_{3',3a'}$), 4.40-4.12 (m, 6, CH_2, $H_{5',5'',5a',5a''}$), 3.90-3.69 (m, 8, $POCH_3$, $H_{4',4a'}$), 3.13 (t, J=6.6 Hz, 2, CH_2), 2.60-2.15 (m, 4, $H_{2',2'',2a',2a''}$), 1.98-1.80 (m, 3, CH_3).

^{31}P NMR ($CDCl_3$) -0.034, -0.079, -0.279, -0.298, -0.317. -0.419, -0.445

3'-(4-Nitrophenethyl)phosphoryl-Thymidyl-(3',5')-Thymidine Disodium Salt (26)

In a magnetically stirred, 100 mL round-bottomed flask, 851 mg (1.08 mmol) of 5'-hydroxy-fully protected 3'-phosphorylthymidyl-(3',5')-thymidine (**25**) were dissolved in 20 mL of a solution of thiophenol, triethylamine, and dioxane (1:2:2 v/v). The yellow solution was stirred for 15 min at room temperature. 6 mL of dry methanol were added to the two phase mixture, and the resulting yellow, single phase solution was stored at -10 ^{o}C overnight. The solvent was removed with a water aspirator to yield a yellow liquid. The liquid was dissolved in 10 mL double distilled water, and the aqueous solution was washed with ten 50 mL portions of ether until the smell of thiophenol was gone. The aqueous solution was reduced in volume on a rotary evaporator fitted to an oil pump to a volume of *ca.* 5 mL, and the colorless solution was applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). The column was eluted with 250 mL of double distilled water, and elution was monitored with a UV detector connected to a chart recorder. The product was collected in a single 100 mL fraction. The pH was adjusted to 6.8 with 0.1 M HCl, and the solvent was removed on a rotary evaporator at oil pump pressure to yield a yellow gum. The gum was dissolved in 5 mL of double distilled water and applied to a 1.5 x 20 cm column of octadecyl silica gel. The column was eluted with 250 mL of double distilled water to remove short retention time impurities, followed by elution with *ca.* 200 mL of a 40% solution of methanol in water.

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Elution was monitored with a UV detector and 100 mL fractions were collected and analyzed by HPLC. Fractions containing product in excess of 98% purity as measured by the integrated peak areas were combined and the solvent was removed on a rotary evaporator to yield 844 mg (quantitative) of **26** as an amorphous white solid.

^{1}H NMR (D_2O) d 7.85 (d, J=8.7 Hz, 2, aryl), 7.40 (s, 1, $H_{6,}$), 7.35-7.24 (m, 3, aryl, H_{6a}), 5.95 (t, J=6.2 Hz, $H_{1'}$), 5.84 (t, J=6.7 Hz, $H_{1a'}$), 4.65 (s, D_2O), 4.60-4.40 (m, 2, $H_{3'}$,$_{3a'}$), 4.10-3.50 (m, 8, CH_2, $H_{5'}$,$_{5''}$,$_{5a'}$,$_{5a''}$, $H_{4'}$,$_{4a'2a'}$,$_{2a''}$), 2.83 (t, J=6.6 Hz, 2, CH_2), 2.32-1.90 (m, 2, $H_{2'}$,$_{2''}$,), 1.63 (2s, 6, CH_3).

^{31}P NMR (D_2O, HEPES): -0.065, -0.396.

3'-Phosphorylthymidyl-(3',5')-Thymidine Trisodium Salt (27)

840 mg (1.00 mmol) of 3'-(4-nitrophenethyl)-phosphorylthymidyl-(3',5')-thymidine disodium salt (**26**) were slurried in a solution of 50 mL acetonitrile, 5 mL water, and 10 g DBU. The pale yellow mixture was heated to reflux temperature for 2 h after which time HPLC of the crude reaction mixture with an elution mixture of 25% buffer A in B showed only the slightest trace of remaining starting material. The yellow slurry was cooled to room temperature, and the solvent was removed on a water aspirator to yield *ca.* 15 mL of a yellow solution. 20 mL of double distilled water were added, and the aqueous phase was washed with ten 30 mL portions of methylene chloride. The colorless aqueous layer was reduced in volume to 2 mL on a rotary evaporator at oil pump pressure and applied to a 2 x 25 cm ion exchange column packed with Dowex 50 (sodium form). The column was eluted with 200 mL of double distilled water, and elution was monitored with a UV detector connected to a chart recorder. The product was collected in a single 150 mL fraction. The pH of the solution was adjusted to 6.8 with 0.1 M HCl, and the solvent was removed on a rotary evaporator at oil pump pressure. The colorless residue was dissolved in 5 mL of double distilled water and applied to a 1.5 x 20 cm column of octadecyl silica gel. The column was eluted with 250 mL of double distilled water followed by elution with *ca.* 500 mL of a 6% solution of methanol in water. Elution was monitored with a UV detector, and 100 mL fractions were collected and analyzed by HPLC. Fractions containing product in excess of 98% purity as measured by the integrated peak areas were combined, and the solvent was removed on a rotary evaporator to yield 710 mg (quantitative) of the product as an amorphous white solid. The solid was dissolved in 20 mL of a methanol, and ethanol was added to give the slightest turbidity at reflux temperature. Methanol was removed slowly in a 90 ^{o}C water bath on a slowly turning rotary evaporator at atmospheric pressure over 2 h to yield a fine white precipitate. The precipitate was collected on a sintered glass filter funnel and rinsed with three 1 mL portions of absolute ethanol to yield a fine white

powder. The powder was dissolved in 20 mL of double distilled water, and the solvent was removed on a rotary evaporator. The process was repeated twice to remove alcohols, and the product was freeze dried to yield 451 mg of white powder.

^{1}H NMR (D_2O) d 7.52 (s, 1, $H_{6,}$), 7.45 (s, 1, H_{6a}), 6.12 (t, J=6.7 Hz, $H_{1'}$), 5.98 (t, J=6.2 Hz, $H_{1a'}$), 4.70- 4.50 (m, 2, $H_{3',3a'}$), 4.15-4.03 (m, 1, $H_{4'}$), 4.03-3.85 (m, 3, $H_{5',5",4a'}$), 3.70-3.50 (m, 2, $H_{5a',5a"}$), 2.45-2.00 (m, 4, $H_{2',2"2a',2a"}$), 1.68 (2s, 6, CH_3).

^{31}P NMR (D_2O, HEPES, EDTA) -0.305, 3.020.

Further Characterization of the Phosphorylated Dimers

The four dimeric nucleotides used in the previous study were characterized by hydrolysis with alkaline phosphatase. The 5'-phosphates, d(pApA) and d(pTpT) eluted in one sharp peak when coinjected with authentic standards (Sigma) on reverse phase HPLC. The two synthetic dimers were also treated for 30 min with a solution of alkaline phosphatase, and the hydrolysates were coinjected with authentic samples of d(ApA) and d(TpT) (Sigma) respectively. In both cases the HPLC traces of each of the coinjected mixtures gave a single sharp peak. The 3'-phosphate dimers d(ApAp) and TpTp were treated with a solution of alkaline phosphatase, and the hydrolysates were coinjected with authentic standards of ApA and TpT respectively (Sigma). In both cases HPLC analysis of the coinjected mixtures gave a single peak.

Acknowledgements: This research was supported by grant CHE-9000187 from the National Science Foundation and a NASA Graduate Student Researchers Award to JRP. Partial funding for the purchase of the Varian 200 MHz and 500 MHz NMRs was provided to the Chemistry Department by NIH and NSF. Hearty thanks is given to Mr. T. Keane and Dr. H. Schwartz for the ^{31}P NMR spectra and to Dr. G. Ertem and Dr. K. J. Prabahar for some of the HPLC data.

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Received 8/31/93
Accepted 1/11/94

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