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T. Sudhakar Rao^a, Michael E. Hogan^b & Ganapathi R. Revankar^a ^a Triplex Pharmaceutical Corporation, 9391 Grogans Mill Road, The Woodlands, TX, 77380

^b Center for Biotechnology, Baylor College of Medicine, 4000 Research Forest Drive, The Woodlands, TX, 77381 Published online: 23 Sep 2006.

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SYNTHESIS OF TRIPLE HELIX FORMING OLIGONUCLEOTIDES CONTAINING 2'-DEOXYFORMYCIN A¹

T. Sudhakar Rao,⁺ Michael E. Hogan,[#] and Ganapathi R. Revankar^{+*}

+Triplex Pharmaceutical Corporation,9391 Grogans Mill Road, The Woodlands, TX 77380

and

[#]Center for Biotechnology, Baylor College of Medicine, 4000 Research Forest Drive, The Woodlands, TX 77381

ABSTRACT: N⁷-Benzoyl-2'-deoxyformycin A ($\underline{5}$) was prepared from formycin A and incorporated into the triple helix forming oligonucleotide PRE2ap at CG inversion sites. The modified oligonucleotide containing three substitutions of 2'-deoxyformycin A displayed a 10-fold increase in binding affinity as compared to its unmodified counterpart. This provided a method to accommodate CG inversion sites within target sites for antiparallel triple helix formation.

The existence of triple helical DNA is an old observation² and has now been documented for synthetic oligomers,³ for the binding of short oligonucleotides to cloned DNA fragments,⁴ and as the result of internal disproportion of tandemly repeated polypurine rich duplex DNA.⁵⁻¹⁰ It has recently been shown that the formation of sequence-specific triple helices can inhibit DNA replication,^{11,12} and block transcription initiation,¹³ thus resulting in the specific inhibition of the synthesis of disease associated proteins. Thus, the therapeutic significance of such triple helix forming oligonucleotides (TFOs) is imminent, e.g. by blocking the transcription of viral proteins, viral infections can be inhibited. Similarly, by inhibiting the

This publication is dedicated to the everlasting memory of Professor Roland K. Robins, 1926-1992

over expression of a particular gene, uncontrolled growth of cancer can be reduced. Immunological diseases associated with aberrant gene expression may also be treatable through therapeutic application of TFOs.

Overwhelming evidence has also been presented to show that in the presence of divalent cations certain G-rich oligonucleotides can bind to specific sites in duplex DNA to form triple helices¹³⁻¹⁵ at physiological pH.

However, the primary goal of TFO design is to develop molecules which can bind to any duplex DNA sequence, without regard for purine content or other symmetry consideration. *H*-bonding of the Hoogsteen or reverse Hoogsteen type normally occurs with purine bases in the major groove of an underlying duplex.¹⁶ Consequently, a polypurine/polypyrimidine duplex target presents an orderly array of bond formers positioned upon one side of the major helix groove. At sites of CG inversion, the corresponding purine target base is placed upon the opposing half of the major groove and can be reached for the purposes of *H*-bonding only; (a) upon extension of the TFO backbone, (b) by distension of the duplex, or (c) both.

Available data suggests that duplex DNA assumes the **A** form upon triplex formation.¹⁷ In the **A** form, the major groove is deep and narrow, with dimensions well suited to the size of a bound third strand. Therefore, at sites of CG or TA inversion within a polypurine/polypyrimidine domain, at the most 3-5 Å of transverse distortion is required to accommodate *H*bonding at the "other side" of the major groove. That much lateral distortion can be partially accommodated by the conformational freedom available to the deoxyribose backbone of a duplex. Preliminary modelling studies¹⁸ suggests that, in order to form a standard TAT or GGC Hoogsteen or reverse Hoogsteen triplex at such sites, distortion of the duplex binding site might also be required.

If triplet formation could be made stable at CG inversion sites, TFOs could be designed against any duplex site, rather than just at purine rich targets. One solution to the binding problem at CG inversion sites would be to alter the position of the two hydrogen bond donors of guanosine, relative to the site of glycosylation (*Figure 1*). We now report that by substituting 2'-deoxyformycin A (F) for dG, the third strand backbone may become less distended at sites of CG inversion.

Formycin A is a naturally occurring nucleoside antibiotic, with structural similarity to adenosine¹⁹. However, due to its unusual C-C glycosidic bond, the aglycon moiety becomes more aromatic (λ_{max} shifts to



Figure 1

310 nm) and its site of protonation is altered relative to adenosine. At neutral pH, the formycin ring has a proton²⁰ at sites N¹/N². As such, the ring is capable of donating two *H*-bonds to an accepter and is therefore homologous to guanosine, with respect to triple helix formation. Based upon this feasibility, we have now converted formycin A (1) to N⁷-benzoyl-2'-deoxyformycin A (5), and incorporated it into a TFO using the solid-support, phosphoramidite chemistry.

Chemistry. Three reports have appeared in the literature for the preparation of 2'-deoxyformycin A. The first two syntheses of 2'-deoxyformycin A, as described by Moffatt and co-workers,²¹ and Robins et al.²² involved the reaction of formycin A (1) with acetoxyisobutyryl bromide/chloride, followed by ammonolysis and reductive dehalogenation. This approach produced a mixture of 2'-deoxyformycin A and the 3'-deoxy isomer, with the latter predominating by a ratio of more than 2:1. The third method involved a four-step deoxygenation procedure²³⁻²⁵ employing phenoxythiocarbonylation of the 2'-hydroxyl group of the corresponding 3',5'-protected formycin A.²⁶ Since a considerable amount of N⁷-protected-2'-deoxyformycin A was required for our studies, we elected to use the deoxygenation procedure.

The protection of the 3',5'-hydroxyl functions of thoroughly dried formycin A (by co-evaporation with dry pyridine) was accomplished by the treatment of 1 with 1.2 equiv of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane



reagent)²⁷ in anhydrous pyridine for 2.5 h at room (Markiewicz's temperature. Work-up of the reaction mixture and purification of the crude product by silica gel column chromatography using 0-5% methanol in dichloromethane gave a 74.2% yield of 2a. It is very crucial to dry formycin A by co-evaporating with pyridine which improves the yield of 2a considerably. Acylation of the 2'-hydroxyl group of the cyclic disiloxy derivative <u>2a</u> using 1.2 equiv of phenyl chlorothionoformate in the presence of 4-dimethylaminopyridine in acetonitrile, gave an 87% yield of 7-amino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-(phenoxythiocarbonyl)-β-Dribofuranosyl]pyrazolo[4,3-d]pyrimidine (2b). Reductive cleavage of the phenoxythiocarbonyl group of 2b with tributyltin hydride in the presence of 2,2'-azobis(2-methylpropionitrile) gave the viable intermediate 7-amino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-β-D-erythro-pentofuranosyl]pyrazolo[4,3-d]pyrimidine $(2c)^{26}$ in a 77% yield. Benzovlation of 2cwith benzoyl chloride in anhydrous pyridine at room temperature gave a 71% yield of the dibenzoylated derivative identified as 7-N.N-dibenzoylamino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-B-D-erythropentofuranosyl]pyrazolo[4,3-d]pyrimidine (6) on the basis of ¹H NMR studies and elemental analysis (see experimental). In addition, a 14.5% yield of the monobenzoylated product $(\underline{3})$ was also isolated. The fact that the compound

<u>6</u> is indeed a N^7 -dibenzoylated derivative and not N^{1} , N^7 -dibenzoylated product was established by the treatment of <u><u>6</u> with tetrabutylammonium fluoride solution in THF at room temperature for 2 h. Under these conditions deprotection of the 3',5'-hydroxyl groups occurred with concomitant mono-debenzoylation to afford 7-N-benzoylamino-3-(2-deoxy- β -D-erythropentofuranosyl)pyrazolo[4,3-d]pyrimidine (<u>5</u>). Since, *N*,*N*-dibenzoylamino group is more labile to basic conditions than mono-*N*-benzoyl derivative (in analogy to purine ring²⁸), it is assumed that compound <u><u>6</u> has the structure that is proposed. A similar treatment of <u>3</u> with fluoride ion (tetrabutyl-ammonium fluoride) also gave <u>5</u>, which was found to be identical with <u>5</u> derived from <u>6</u>.</u></u>

The 5'-hydroxyl group of $\underline{5}$ was protected as the 4,4'-dimethoxytrityl ether by the treatment of $\underline{5}$ with 1.2 molar equiv of 4,4'-dimethoxytrityl chloride in anhydrous pyridine. The crude product thus obtained was purified on a silica gel column to yield the corresponding 5'-O-dimethoxytrityl derivative ($\underline{4a}$). However, the isolated yield of $\underline{4a}$ was only 35.5%, presumably due to the formation of a ditritylated derivative (which was not isolated) and some unreacted starting material, which was recovered. The 3'-hydroxyl group of $\underline{4a}$ was converted into the corresponding phosphoramidite ($\underline{4b}$) by reaction with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in the presence of *N*,*N*-diisopropyl-ethylamine in dry dichloromethane. The pure product was isolated in 86% yield after chromatography over silica gel using dichloromethane:ethyl acetate:triethylamine ($\underline{45:45:10}$, v/v) as the eluent.

Synthesis, Purification and Characterization of Modified TFO.

An oligonucleotide containing 2'-deoxyformycin A (F) residues was prepared with a stepwise coupling efficiency of > 98% by coupling **4b** to the solid support and increasing the reaction time for an additional 90 seconds. Synthesis was carried out on a 0.2 µmole scale on a ABI 380B automated DNA synthesizer. All base-labile protecting groups on the oligodeoxyribonucleotides were removed by the treatment with concentrated NH₄OH at 55 °C for 20 h and the TFO was purified by ionexchange HPLC using a Q-Sepharose (Pharmacia) column.²⁹ The purified product was desalted by passing through C₁₈ sep-pack (Waters) cartridge and analyzed on a 20% denaturing polyacrylamide gel after labeling with ³²P-ATP using polynucleotide kinase.³⁰ Unmodified oligodeoxyribonucleotide was used as Figure 2 36BP site,³¹ 30/36 = 83% purine, 58% GC, 3 sites of F substitution

the standard for comparison of mobility and purity. The modified TFO was found to be > 96% pure and with the expected length. Base composition analysis was performed by Waters reversed-phase HPLC using photodiode array (PDA) detector (Model 996).

2'-Deoxyformycin A (F) was introduced into the 36 base pair TFO PRE2ap.³¹ The TFO specific for this site is presented in the preferred antiparallel orientation (*Figure 2*). As an unmodified TFO, the progesterone response element PRE2ap binds selectively to the purine rich target site.

The binding of PRE2ap and its 2'-deoxyformycin A homologue were monitored at 37 °C by the band shift method,¹³ in the standard binding buffer: 10 mM Tris/HCl, 10 mM MgCl²⁺, pH 7.4. In this experiment, the ³²P 5'-end radiolabelled duplex target fragment of *Figure 2* was titrated with increasing concentration of unlabelled TFO. Binding was then detected by the characteristic electrophoretic mobility difference between a duplex and a triplex on a native 10% acrylamide gel.¹³ The triplex DNA migrates more slowly than duplex DNA, affording easy estimation of the percentage of triple helix formation.

In this assay the 2'-deoxyformycin A containing TFO displayed approximately a **10-fold increase** in affinity as compared to its unmodified counterpart (*Figure 3*). There is an apparent titration midpoint decrease from 10⁻⁷ M to 10⁻⁸ M in TFO equivalents. This significant enhancement of binding affinity resulting from 2'-deoxyformycin A substitution provides a method to accommodate CG inversion sites within target sites for antiparallel mode of triple helix formation.

EXPERIMENTAL

Melting points (uncorrected) were determined in a Thomas-Hoover capillary melting-point apparatus. Elemental analyses were performed by



Figure 3

Quantitative Technologies Inc., Whitehouse, NJ. The presence of solvent as indicated by elemental analysis was verified by ¹H NMR spectroscopy. Thin layer chromatography (TLC) was performed on plates of silica gel 60F-254 (EM Reagents). Silica gel (E. Merck; 230-400 mesh) was used for flash column chromatography. All solvents used were reagent grade. Detection of nucleoside components on TLC was by uv light, and with 10% sulfuric acid in methanol spray followed by heating. Evaporations were conducted under diminished pressure with the bath temperature below 30 °C. Infrared (IR) spectra were recorded in potassium bromide with a Perkin-Elmer 1420 spectrophotometer and ultraviolet spectra (UV) were recorded on a Hewlett Packard 8452A Diode Array spectrophotometer. Nuclear magnetic resonance (¹H NMR) spectra were recorded at 200 MHz with an Bruker 200 spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as the internal standard (key: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad.)

7-Amino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)- β -D-ribofuranosyl]pyrazolo[4,3-d]pyrimidine (<u>2a</u>). Formycin A monohydrate (<u>1</u>, 2.0 g, 7.0 mmol) was dried by co-evaporation with anhydrous pyridine (3 x 25 mL) under a high vacuum. The residue was redissolved in dry pyridine (20 mL) and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.68 mL, 8.39 mmol) was added in one portion. After stirring under anhydrous conditions for 2.5 h, the reaction mixture was diluted with dichloromethane (150 mL) and washed with water (2 x 20 mL). The dried (Na₂SO₄) organic layer was evaporated to dryness. The residue was co-evaporated with toluene (3 x 30 mL) to remove traces of pyridine and purified on a silica gel column (2 x 35 cm) using 0-5% methanol in dichloromethane as the eluent. The appropriate homogeneous fractions were pooled, evaporated and the residue was crystallized from acetonitrile to give 2.65 g (74.2%) of **2a**; mp 130-132 °C [Lit.²⁶ mp 123-126 °C]; ¹H NMR (CDCl₃): δ 1.0 (m, 28 H, 4 isopropyls), 4.06 (m, 3 H, C₄·H and C₅·H₂), 4.68 (m, 2 H, C₂·H and C₃·H), 5.37 (d, 1 H, J = 3.04 Hz, C₁·H), 6.45 (br s, 2 H, NH₂) and 8.23 (s, 1 H, C₅·H).

7-Amino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-(phenoxythiocarbonyl)- β -D-ribofuranosyl]pyrazolo[4,3-d]pyrimidine (2b). To a solution of 2a (2.64 g, 5.18 mmol) and 4-dimethylaminopyridine (1.83 g, 14.98 mmole) in dry acetonitrile (100 mL) was added phenyl chlorothionoformate (0.82 mL, 6.0 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature for 8 h, diluted with dichloromethane (150 mL) and washed successively with cold 0.1 N hydrochloric acid (2 x 75 mL), water (50 mL), and saturated aqueous sodium hydrogen carbonate solution (50 mL). The dried (Na_2SO_4) organic layer was evaporated to dryness. The residue was purified on a silica gel column (2 x 30 cm) using 0-5% methanol in dichloromethane as the eluent. The homogeneous fractions were combined, evaporated to dryness, and the residue was crystallized from acetonitrile to give 2.7 g (80.7%) of 2b; mp 138-140 °C [Lit.26 mp 142-145 °C]; 1H NMR (CDCl₃): § 1.05 (m, 28 H, 4 isopropyls), 4.15 (m, 4 H, C₃·H, C₄·H and C_5 : H_2), 5.1 (m, 1 H, C_2 :H), 5.73 (d, 1 H, J = 3.25 Hz, C_1 :H), 6.2 (br s, 2 H, NH₂), 7.05-7.45 (m, 5 H, Ph) and 8.25 (s, 1 H, C₅H).

7-Amino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-β-D-erythro-pentofuranosyl]pyrazolo[4,3-d]pyrimidine (2c). To a solution of 2b (2.7 g, 4.1 mmol) and 2,2'-azobis(2-methylpropionitrile) (0.25 g) in anhydrous toluene (100 mL) was added tributyltin hydride (3.28 mL, 12.18 mmol) under an argon atmosphere. The reaction mixture was stirred at 80 °C for 2.5 h and evaporated to dryness. The residue was purified on a silica gel column (2 x 30 cm) using 0-5% methanol in dichloromethane as the eluent. The homogeneous fractions were combined, evaporated to dryness to yield 1.6 g (77%) of <u>2c</u>; mp 168-170 °C; ¹H NMR (CDCl₃ + DMSO- d_6): δ 1.1 (m, 28 H, 4 isopropyls), 2.40 (m, 1 H, C₂·H), 2.85 (m, 1 H, C₂·H), 3.80-4.10 (m, 3 H, C₄·H and C₅·H₂), 4.83 (m, 1 H, C₃·H), 5.56 (t, 1 H, J = 7.24 Hz, C₁·H), 6.40 (br s, 2 H, NH₂), 8.34 (s, 1 H, C₅H) and 12.3 (br s, 1 H, N₁H).

7-N-Benzoylamino- and 7-N,N-Dibenzoylamino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-β-D-erythro-pentofuranosyl]pyrazolo-[4,3-d]pyrimidine (3 and 6). To a cooled (0-5 °C) solution of 2c (1.5 g, 2.92 mmol) in anhydrous pyridine (25 mL) was added benzoyl chloride (1.36 mL, 11.7 mmol). The reaction mixture was stirred at room temperature for 2.5 h and then it was partitioned between dichloromethane (150 mL) and water (50 mL). The organic layer was separated and the aqueous phase was reextracted with dichloromethane (2 x 50 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was azeotroped with toluene (4 x 50 mL) to remove traces of pyridine. The crude material was purified on a silica gel column (2.5 x 25 cm) using 0-1% methanol in dichloromethane as the eluent. The following two nucleosides were isolated in the order listed. 7-N,N-Dibenzoylamino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-divl)-2-deoxy- β -D-erythro-pentofuranosyl]pyrazolo[4,3-d]pyrimidine (6): crystall-ized from acetonitrile, 1.45 g (70.8%); mp 74-75 °C; IR (KBr): v_{max} 1720 (C=O), 3240 (NH) cm⁻¹; UV (MeOH): λ_{max} 230 nm (ϵ 24,000), 318 nm (ε 14,500); ¹H NMR (DMSO-d₆): δ 0.98 (br s, 28 H, 4 isopropyls), 2.40-2.90 (m, 2 H, C₂'H and C_{2"}H), 3.7-4.0 (m, 3 H, C₄'H and C₅'H₂), 4.90 (q, 1 H, C₃'H), 5.50 (dd, 1 H, C₁'H), 7.30-8.15 (m, 10 H, 2 COC₆H₅), 9.07 (s, 1 H, C₅H) and 12.0 (br s, 1 H, NH). Anal. Calcd. for C₃₆H₄₇N₅O₆Si₂: C, 61.59; H, 6.75; N, 9.97. Found: C, 61.68; H, 6.81; N, 9.86.

7-N-Benzoylamino-3-[3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-β-*D-erythro*-**pentofuranosyl]pyrazolo[4,3-d]pyrimidine** (<u>3</u>): crystallized from acetonitrile, 0.25g (14.5%); mp 120-122 °C; IR (KBr): v_{max} 1710 (C=O), 3260 (NH) cm⁻¹; UV (MeOH): λ_{max} 228 nm (ε 23,500), 318 nm (ε 14,500); ¹H NMR (DMSO-*d*₆): δ 1.0 (m, 28 H, 4 isopropyls), 2.40 (m, 1 H, C₂·H), 2.70 (m, 1 H, C₂·H), 3.80 (m, 3 H, C₄·H and C₅·H₂), 4.85 (m, 1 H, C₃·H), 5.4 (dd, 1 H, C₁·H), 7.4-8.0 (m, 5 H, COC₆H₅) and 8.39 (s, 1 H, C₅H). *Anal*. Calcd. for C₂₉H₄₃N₅O₅Si₂: C, 58.26; H, 7.25; N, 11.71. Found: C, 58.42; H, 7.51; N, 11.93. 104

7-N-Benzoylamino-3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrazolo-[4,3-d] pyrimidine (5). To a cooled (0-5 °C) solution of either 3 (1.17 g, 1.96 mmol) or 6 (1.37 g, 1.96 mmol) in anhydrous tetrahydrofuran (30 mL) was added tetrabutylammonium fluoride (8 mL, 1M solution in THF). After stirring the reaction mixture at room temperature for 2 h, the solvent was removed and the residue was purified by chromatography over a silica gel column using 0-5% methanol in dichloromethane as the eluent. The homogeneous fractions were pooled and evaporated to give 0.46 g (68%) of pure <u>5</u>; mp 250-251 °C; IR (KBr): v_{max} 1690 (C=O), 3100-3500 (OH, NH) cm⁻¹; UV (MeOH): λ_{max} 248 nm (ϵ 14,500), 320 nm (ϵ 13,700); ¹H NMR (DMSO- d_6): δ 2.00-2.20 (m, 1 H, C₂·H), 2.60-2.80 (m, 1 H, C₂·H), 3.40-3.70 (m, 2 H, C₅·H₂), 3.90 (m, 1 H, C₄'H), 4.40 (d, 1 H, C₃'H), 5.5 (br s, 2 H, 3'OH and 5'OH), 5.50 (dd, 1 H, C₁·H), 7.50-8.15 (m, 5 H, COC₆H₅), 8.75 (s, 1 H, C₅H), 11.80 (br s, 1 H, N₁H) and 13.00 (br s, 1 H, N7H). Anal. Calcd. for C17H17N5O4•1/4 H2O : C, 56.74; H, 4.90; N, 19.46. Found: C, 56.93; H, 4.96; N, 19.35.

7-N-Benzoylamino-3-[5-O-(4,4'-dimethoxytrityl)-2-deoxy-β-D-erythropentofuranosyl]pyrazolo[4,3-d]pyrimidine (4a). To a solution of 5 (0.40 g, 1.12 mmol) in anhydrous pyridine (10 mL) was added 4,4'-dimethoxytrityl chloride (0.46 g, 1.35 mmol). After stirring at room temperature for 3 h, the reaction mixture was partitioned between dichloromethane and water (50 mL of each). The organic layer was separated, dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was azeotroped with toluene (4 x 50 mL) to remove traces of pyridine, and then purified by chromatography over a silica gel column using 0-2% methanol in dichloromethane as the eluent. The homogeneous fractions were pooled and evaporated to give a solid, which was dissolved in a minimum amount of dichloromethane and precipitated from hexanes to yield 0.26 g (35.5%) of 4a; mp 107-108 °C; IR (KBr): υ_{max} 1690 (C=O), 3200-3400 (OH, NH) cm⁻¹; UV (MeOH): λ_{max} 238 nm (ε 31,700), 320 nm (ε 18,000); ¹H NMR (DMSO-d₆): δ 2.05-2.30 and 2.75-2.95 (2m, 2 H, C₂'H and C₂"H), 3.70 (s, 3 H, OCH₃), 3.71 (s, 3 H, OCH₃), 3.98 (m, 2 H, C₅·H₂₎, 4.40 (m, 2 H, C₄·H), 5.20 (m, 1 H, C₃·H), 5.55 (dd, 1 H, C₁·H), 6.70-8.30 (m, 18 H, COC_6H_5 and DMT), 8.65 (s, 1 H, C_5H), 11.85 (br s, 1 H, N_1H) and 13.10 (br s, 1 H, N7H). Anal. Calcd. for C38H35N5O6+3/4 hexane: C, 70.66; H, 6.35; N, 9.69. Found: C, 70.32; H, 6.40; N, 9.70.

7-N-Benzoylamino-3-[3-O-(P-β-cyanoethoxy-N,N-diisopropylaminophosphinyl)-5-O-(4,4'-dimethoxytrityl)-2-deoxy-\beta-D-erythro-pentofuranosyl]pyrazolo[4,3-d]pyrimidine (4b). To a solution of 4a (0.16 g, 0.2 mmol) and N,N-diisopropylethylamine (0.14 mL, 0.83 mmol) in anhydrous dichloromethane (3 mL) was added with stirring 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (56 µL, 0.25 mmol) under an argon atmosphere.

After 30 min, an additional 45 µL of the phosphitylating reagent was added. After stirring for an additional 15 min, the reaction mixture was diluted with 10% triethylamine in ethyl acetate solution (50 mL) and washed with cold aqueous saturated sodium hydrogen carbonate solution (10 mL). The aqueous phase was separated and reextracted with ethyl acetate (50 mL). The combined organic layers was dried (Na₂SO₄) and evaporated to dryness. A solution of the residue in 10% triethylamine/ethyl acetate solution (2 mL) was passed through a silica gel column packed in dichloromethane:ethyl acetate:triethylamine (45:45:10, v/v) and the column was eluted with the same solvent system. The appropriate, homogeneous fractions were collected and evaporated to dryness. The residue was dissolved in dichloromethane (3 mL) and the product was precipitated by addition to dry hexanes. The pure product 4b was collected by filtration and dried under high vacuum to yield 0.18 g (86.2%). ¹H NMR (CDCl₃): δ1.20 [m, 12 H, N(CHMe₂)₂], 2.40-2.70 (m, 2 H, C₂·H and C₂·H), 2.80-4.0 (m, 6 H, OCH₂CH₂CN and C₅·H₂), 3.75 (s, 6 H, 2OCH₃), 4.35 (m, 1 H, C₄'H), 4.75 (m, 1 H, C₃'H), 5.75 (q, 1 H, C₁'H), 6.50-8.20 (m, 18 H, COC₆H₅ and DMT), 8.64 and 8.65 (2 s, 1 H, C₅H of the isomers), and 12.20 (br s, 1 H, N₇H); ³¹P NMR (CDCl₃): δ 148.36. Anal. Calcd. for C₄₇H₅₃N₇O₇P•1/2H₂O: C, 65.11; H, 6.28; N, 11.31; P, 3.57. Found: C, 65.16, H, 6.32; N, 10.87; P, 3.70.

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- 29. HPLC Conditions: Elution with buffer A (0.5 M NaCl in 10 mM NaOH): buffer B (1.5 M NaCl in 10 mM NaOH); flow rate 2.5 mL/min. Initial conditions, A:B = 90:10 (5 min), then A:B = 40:60 (45 min), finally A:B = 0:100 (60 min).
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