Chiral Chemical Synthesis of DNA Containing (*S*)-9-(1,3-Dihydroxy-2-propoxymethyl)guanine (DHPG) and Effects on Thermal Stability, Duplex Structure, and Thermodynamics of Duplex Formation†

Stephen J. Marshalko,‡ Barry I. Schweitzer,§,|| and G. Peter Beardsley*,‡,§

Departments of Pharmacology and Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT: The antiviral compound 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, Ganciclovir) is used clinically to treat cytomegaloviral infections in immunocompromised hosts and more recently is being investigated as a chemotherapeutic agent to be used in combination with retroviral gene therapy. Structurally, DHPG, an analog of guanosine, lacks the 2′-deoxyribose carbon atom and is acyclic. It is therefore prochiral at the 4′-deoxyribose carbon, having both *pro-R* and *pro-S* isomers. This stereochemistry is critical during biochemical conversions. DHPG retains the equivalent of 3′-hydroxyl and 5′-hydroxyl moieties. These can potentially support not only initial misincorporation of DHPG into DNA but also subsequent nucleotide addition. The mechanism of DHPG antiviral action may thus not strictly be through chain terminations. To investigate the structural and biochemical consequences of incorporation of DHPG into DNA, with particular attention to the relative contributions of the deoxyribose sugar to the overall structure and stability of DNA, we have developed a methodology for the chiral chemical synthesis of DNA oligomers containing DHPG. The stereochemistry of the DHPG phosphoramidite was established by a stereoselective acetyl transfer reaction catalyzed by porcine pancreatic lipase. The DNA resisted enzymatic digestion at DHPG sites. Circular dichroism and copper phenanthroline cleavage studies indicated that the incorporation of DHPG into DNA does not significantly perturb the global B-conformation structure. Detailed thermodynamic investigations into DNA containing DHPG revealed reduced thermal stability, as evidenced by a decrease in melting temperature, with significant alteration of the enthalpy, entropy, and free energy of duplex formation. These data demonstrate that an intact deoxyribose ring significantly contributes to the stability of a DNA duplex.

The antiviral compound 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, Ganciclovir) (Martin et al., 1983; Ogilvie & Gillen, 1980) is known to have potent activity against a variety of herpes viruses, including herpes simplex virus (Smith et al., 1982; Cheng et al., 1983a; Smee et al., 1983), varicella-zoster virus (Smith et al., 1982), Epstein-Barr virus (Chent et al., 1983a), and most notably cytomegalovirus (CMV) (Smee et al., 1983; Cheng et al., 1983a). Clinically, DHPG is used extensively to treat infections with CMV, an important pathogen in immunocompromised hosts which often leads to life-threatening disease in bone marrow/organ transplant patients as well as in patients with the acquired immune deficiency syndrome (AIDS) (Erice et al., 1987; Zaia, 1993; Buckner & Pomeroy, 1993; Nevins & Dunn, 1992; Faulds & Heel, 1990; Emanuel, 1990). As a nucleoside analog, DHPG has been shown to inhibit DNA polymerases upon its conversion to the biologically active triphosphate form (Frank et al., 1984). Selectivity against virally infected cells is established at the level of the initial phosphorylation of DHPG to DHPG monophosphate (DHPGMP), which is catalyzed by a virally encoded kinase (Littler et al., 1992; Sullivan et al., 1992), while subsequent conversions to the di- and triphosphate forms are catalyzed by host cellular kinases (Cheng et al., 1983b). Most recently, DHPG has been revisited not as an antiviral drug but as a chemotherapeutic agent to be used in combination with gene therapy. Tumor cells can be retrovirally transfected with the HSV thymidine kinase gene (Culver et al., 1992, 1994; Smythe et al., 1994; Chen et al., 1994), which confers susceptibility to DHPG.

From a structural standpoint, DHPG (Figure 1), an analog of guanosine, has no chemical modification to its base, but instead lacks the 2′-carbon atom of the deoxyribose moiety, and the "sugar" is therefore acyclic. An important stereochemical feature of DHPG is that it is prochiral at what would normally be the 4′-carbon. The *pro-R* and *pro-S* isomers are distinguishable in a chiral environment such as the active site of an enzyme. This stereochemistry is extremely critical during the biochemical conversion to the mono-, di-, and triphosphate forms, where there are known stereochemical requirements. The phosphorylation of DHPG by HSV-TK has been shown to be stereospecific for the *pro-S* hydroxyl (Karkas et al., 1987), as evidenced by the rapid conversion of (*S*)-DHPGMP by GMP kinase to the diphosphate, while only half of a chemically synthesized (±)-DHPGMP, a racemic mixture, is converted to the diphosphate under similar conditions (Tolman, 1989).

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* Address correspondence to this author at the Department of Pediatrics, Yale University School of Medicine, P.O. Box 208064, New Haven, CT 06520-8064.

‡ Department of Pharmacology.

§ Department of Pediatrics.

|| Present address: Walt Disney Memorial Cancer Institute, Orlando, FL 32826.

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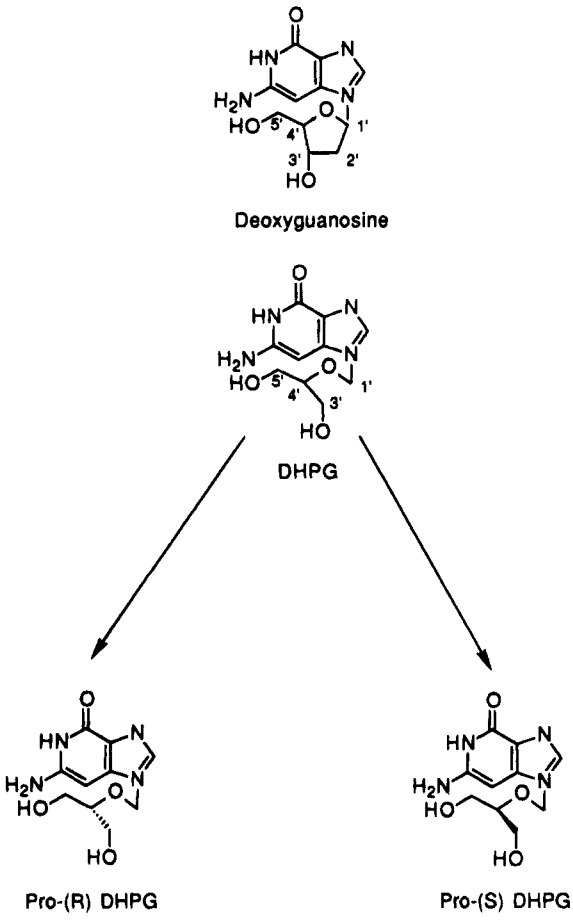

FIGURE 1: Structure comparison of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and deoxyguanosine. The acyclic deoxyribose of DHPG is missing the 2′-carbon atom and is therefore prochiral at its 4′-position, giving rise to both a *pro-R* and a *pro-S* enantiomer.

Although lacking a 2′-carbon, DHPG retains the equivalent of both 3′-hydroxyl and 5′-hydroxyl moieties of natural deoxynucleotides. These are potentially capable of supporting not only misincorporation of DHPG into DNA but subsequent nucleotide addition and elongation of the growing DNA strand. As such, DHPG is not restricted to a role as an obligate chain terminator. Evidence from several investigators (Frank et al., 1984; Cheng et al., 1983b; Reid et al., 1988; Ilsey et al., 1995) indeed indicates that DHPG is incorporated into growing DNA where it appears both at 3′-terminal and at internucleotide positions. The mechanism of its antiviral action may not strictly be through chain terminations.

Because DHPG retains an unmodified guanine base, it is capable of participating in hydrogen-bonding interactions with dC in a standard Watson−Crick base-pair. Therefore, in addition to its potential as an antiviral and chemotherapeutic agent, DHPG may be a unique tool with which to probe the relative contribution of the deoxyribose sugar to the overall stability of DNA. Additionally, DHPG can be used as a functional probe to investigate the contributions of the deoxyribose ring to interactions with polymerases.

To investigate the structural, physicochemical, and biochemical consequences of DHPG incorporation into DNA, we have developed methodology for the chemical synthesis of DNA oligomers containing DHPG. This required the chiral chemical synthesis of a DHPG phosphoramidite, which permits synthesis of DNA containing enantiomerically pure (*S*)-DHPG. This report details this synthetic method, characterizes oligodeoxynucleotides containing DHPG, and describes thermodynamic investigations of these oligodeoxynucleotides, with particular attention to the contributions of the deoxyribose portion to the overall stability, and the effects on the thermodynamic parameters including enthalpy, entropy, and free energy of duplex formation.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. DHPG was a gift from Dr. Julian Verheyden, Syntex Inc. HSV-TK was kindly provided by Dr. William Summers, Yale University. 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite and other chemicals and solvents were purchased from Aldrich Chemical Co., J.T. Baker, Bio-Rad, or American Bioanalytical and were of the highest purity available. Guanylate kinase (GMP kinase), phosphoenolpyruvate, porcine pancreatic lipase, Type I−crude, and phosphodiesterase I were from Sigma. Lactate dehydrogenase and pyruvate kinase were from Boehringer-Mannheim. (*S*)-(+)-α-Methoxy-α-trifluoromethylphenylacetic acid chloride [(*S*)-(+)-MTPA chloride, Mosher's acid chloride] was from Fluka. "Reverse" 5′-cyanoethylphosphoramidites were from Glen Research Corporation.

Synthesis of Chiral DHPG Phosphoramidite (Figure 2). Preparation of Triisobutyryl-DHPG (**2**). Essentially, the method of isobutyryl protection of the guanine base as initially described (Buchi & Khorana, 1972) was employed with the following modifications. To 3 g (11.76 mmol) DHPG (**1**) was added 45 mL of anhydrous pyridine. This slurry was cooled to 4 °C on ice. Four equivalents (4.93 mL, 47.06 mmol) of isobutyryl chloride was slowly added via syringe. Upon complete addition of isobutyryl chloride, the ice bath was removed and the non yellow-white suspension was allowed to stir at room temperature for 4 h. TLC on silica with a mobile phase of 1:9 methanol/chloroform demonstrated complete conversion to a higher R_f, UV-active spot ($R_f = 0.53$). The solution was poured over ice to quench any unreacted isobutyryl chloride and left at room temperature overnight. The resulting clear solution was concentrated using a rotary evaporator. Following the removal of approximately 10−20 mL of liquid, a yellow-white precipitate formed. The resulting slurry was filtered and dried under vacuum. The purity of this material was assessed using two TLC systems (R_f = 0.53, 1:9 methanol/chloroform; R_f = 0.60, 3:3.5:2.5:1 chloroform/ethyl acetate/acetone/triethylamine) and found to be pure.

Preparation of N-Isobutyryl-DHPG (**3**). A solution of 10 mL of methanol and 40 mL of dry pyridine was cooled to 4 °C on ice. To this cooled stirring mixture was added 4 g (8.6 mmol) of the triisobutyryl-DHPG (**2**) reaction product from above. At this point, the solution was pH 5. Sodium hydroxide (2 N) was slowly added dropwise until the pH was 13.5. The reaction mixture was then allowed to stir at 4 °C on ice. The reaction was followed by TLC on silica with 1:9 methanol/chloroform and after 15 minutes demonstrated two major products. An intermediate (diisobutyryl) *N,O*-isobutyryl-DHPG ($R_f = 0.24$) from incomplete hydrolysis and the desired *N*-isobutyryl DHPG ($R_f = 0.07$). After 25−35 min, TLC demonstrated greater than 95% conversion to the lower UV-active spot. The pH was then adjusted to 8.0 with 2 N hydrochloric acid. DOWEX (strongly acidic cation exchange) resin was suspended in 20% pyridine in

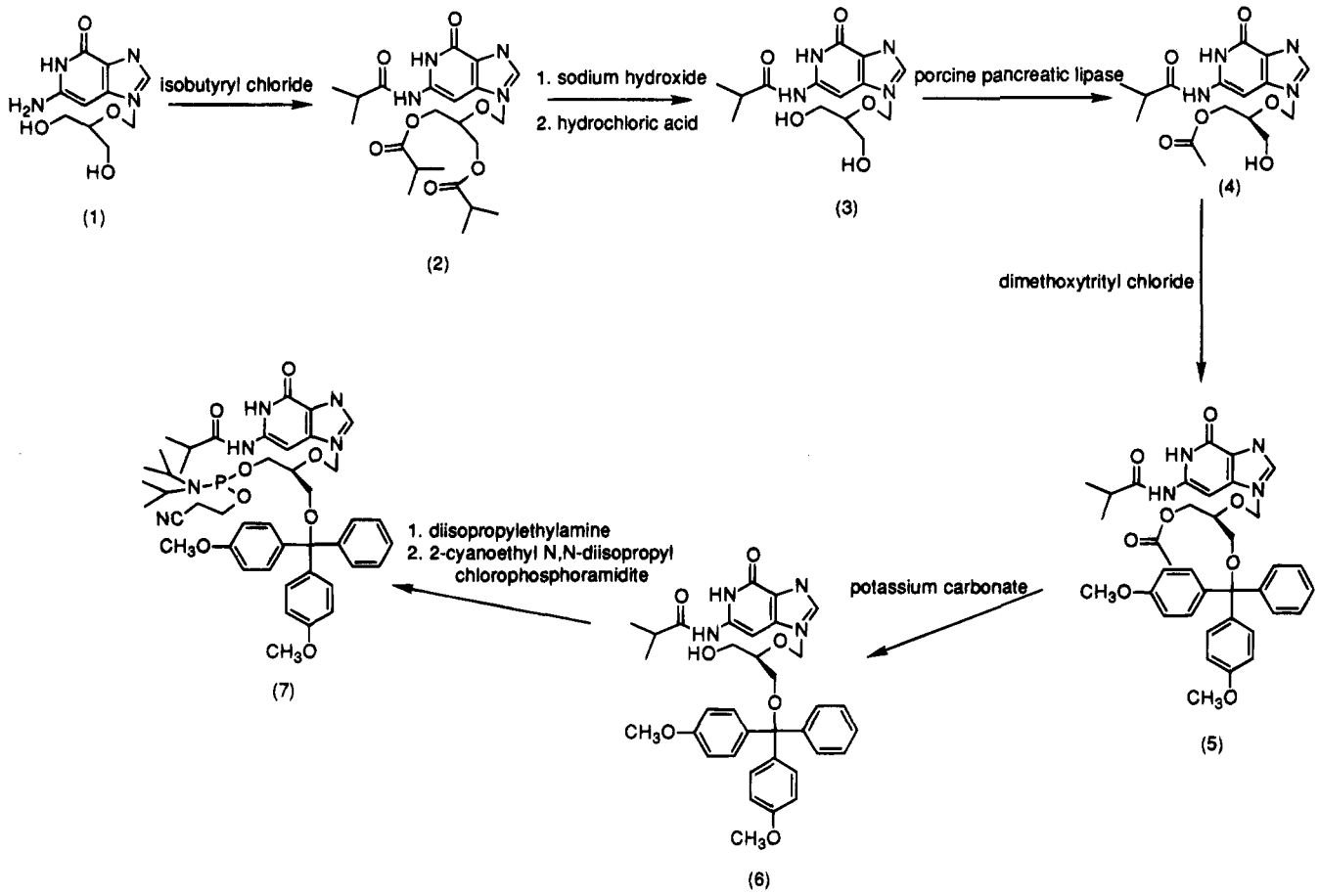

FIGURE 2: Reaction scheme for the chiral chemical synthesis of DHPG phosphoramidite. Following chemical protection of the guanine ring with an isobutyryl group, a stereoselective acetyl transfer reaction catalyzed by porcine pancreatic lipase established the illustrated stereochemistry. Subsequent protection of the remaining hydroxyl with a dimethoxytrityl group, removal of the acetyl group, and ultimately addition of the phosphoramidite moiety to this site yields the "reverse-type" 5′-DHPG phosphoramidite. Further details of this reaction pathway are given in Experimental Procedures.

water. After 20 min, this suspension was poured into a Buchner funnel to create a DOWEX pad, through which the reaction mixture was poured and collected. The DOWEX was then washed with methanol, and the wash was combined with the flow-through and concentrated on a rotary evaporator at 40 °C. The residue was further dried in vacuo. The crude solid was then dissolved in 10% methanol in chloroform, chromatographed on silica gel, and eluted with a gradient of 10−40% methanol in chloroform. Fractions containing *N*-isobutyryl-DHPG (**3**) were combined and concentrated in vacuo to yield 2.54 g (7.8 mmol) of a white solid. The experimental yield was 90%, and the material was judged to be pure by two TLC systems (R_f = 0.07, 1:9 methanol/chloroform; R_f = 0.09, 3:3.5:2.5:1 chloroform/ethyl acetate/acetone/triethylamine).

Preparation of (S)-O-Acetyl-N-isobutyryl-DHPG (**4**). *N*-Isobutyryl-DHPG (**3**), 2.54 g (7.8 mmoles), was dissolved in a mixture of 400 mL of pyridine and 200 mL benzene, and 60 mL of vinyl acetate and 60 g of porcine pancreatic lipase were added (Ramos Tombo et al., 1986). This suspension was allowed to stir at room temperature. After 24 h, TLC on silica developed with 10% methanol in chloroform indicated that approximately 50% of the starting material had been converted to the *O*-acetyl-*N*-isobutyryl-DHPG (R_f = 0.09). An additional 20 g of porcine pancreatic lipase was added, and the suspension was allowed to stir for an additional 24 h. At this point, TLC demonstrated 98% monoacetylated product. The crude reaction mixture was filtered and concentrated in vacuo prior to chromatography on silica gel with a gradient of 10−30% methanol in chloroform. Fractions containing the desired product were combined and concentrated in vacuo to afford 1.90 g (5.2 mmol) of light brown thick oil. The experimental yield was 66%, and the material was determined to be pure using two TLC systems (R_f = 0.09, 1:9 methanol/chloroform; R_f = 0.11, 3:3.5:2.5:1 chloroform/ethyl acetate/acetone/triethylamine). ^{1}H NMR assignments (ppm) 1.1 (*N*-isobutyryl methyl protons), 1.85 (*O*-acetyl methyl protons), 2.6 (*N*-isobutyryl proton), 3.9−4.1 (H5′, H5″, H3′, and H3″ protons), 4.9 (H4′ proton), 5.6 (H1′ proton), 8.1 (H8 proton), 11.8 (H1 proton), 12.1 (NH proton).

Preparation of (S)-DMT-O-acetyl-N-isobutyryl-DHPG (**5**). (*S*)-*O*-Acetyl-*N*-isobutyryl-DHPG (**4**), 1 g (0.0027 mol), was dissolved in 20 mL of dry pyridine and stirred for 10 min at room temperature. Dimethoxytrityl chloride (DMTCl) (1.007 g, 2.9 mmol) (Beardsley et al., 1988; Smith et al., 1962) was then added. The reaction was followed by TLC on silica with 5% ethanol in chloroform. After approximately 3−4 h at room temperature, the reaction was judged to be complete as evidenced by essentially complete conversion to more mobile material (R_f = 0.33). The reaction mixture was then poured into 30 mL of an ice−water mixture. This mixture was extracted three times with 25 mL of chloroform. The combined organic layers were dried over sodium sulfate, filtered, and concentrated in vacuo. The crude residue was then dissolved in chloroform, chromatographed on silica, and eluted with a gradient of 0−5% ethanol in chloroform. Fractions containing the desired product were combined and

concentrated in vacuo to afford 1.617 g (2.4 mmol) of (*S*)-DMT-*O*-acetyl-*N*-isobutyryl-DHPG (**5**). The experimental yield was 89%, and the product was determined to be pure by two TLC systems (R_f = 0.33, 1:19 ethanol/chloroform; and R_f = 0.94, 50:48:2 ethanol/water/ammonium hydroxide). ^{1}H NMR assignments (ppm) 1.1 (*N*-2-isobutyryl methyl protons), 1.85 (*O*-acetyl methyl protons), 2.6 (*N*-isobutyryl proton), 3.6−4.1 (H5′, H5″, H3′, and H3″ protons), 3.7 DMT $(OMe)_2$ protons), 5.6 (H1′ proton), 6.9−7.3 (DMT aromatic protons), 8.1 (H8 proton).

Preparation of (S)-DMT-N-isobutyryl-DHPG (**6**). (*S*)-DMT-*O*-acetyl-*N*-isobutyryl-DHPG (**5**), 1.617 g (2.4 mmol), was dissolved in 30 mL of 5% potassium carbonate in aqueous methanol, which was prepared by the addition of 5 g of potassium carbonate to a solution of 60 mL of methanol and 40 mL of distilled, deionized water. The reaction mixture turned a light pink color, and the reaction was followed by TLC on silica with 1:9 ethanol/chloroform. After 10 min, the reaction was complete as judged by near complete conversion to a lower R_f, UV-active spot (R_f = 0.35), and the solution was added to a separatory funnel containing 50 mL of chloroform. The organic layer was collected, and the remaining aqueous layer was extracted three times with chloroform. The organic layers were combined, dried over sodium sulfate, and concentrated in vacuo to yield a white-yellow solid. This solid was dissolved in 10% ethanol in chloroform, chromatographed on silica, and eluted with 10% ethanol in chloroform. The fractions containing pure product, as judged by two TLC systems (R_f = 0.35, 1:19 ethanol/chloroform; R_f = 0.08, 3:3.5:2.5:1 chloroform/ethyl acetate/acetone/triethylamine), were combined and concentrated in vacuo to yield 1.025 g (1.6 mmol) of (*S*)-DMT-*N*-isobutyryl-DHPG (**6**). The experimental yield was 70%. ^{1}H NMR assignments (ppm) 1.1 (*N*-isobutyryl methyl protons), 2.6 (*N*-isobutyryl proton), 3.7 (DMT $(OMe)_2$ protons), 3.9−4.1 (H3″, H3′, H5″ and H5′ protons), 5.6 (H1 protons), 6.9−7.3 (DMT aromatic protons), 8.1 (H8 proton).

Preparation of (S)-DMT-N-isobutyryl-DHPG-cyanoethyl Phosphoramidite (**7**). (*S*)-DMT-*N*-isobutyryl-DHPG (**6**), 140 mg (0.22 mmol), was added to 1 mL of dry, distilled tetrahydrofuran (Sinha et al., 1983) in a preflushed, flamed 10-mL round bottom fitted with a nitrogen balloon. Four equivalents (150 μL) of diisopropylethylamine (McBride & Caruthers, 1983; Beaucage & Caruthers, 1981) was added under nitrogen, and the reaction mixture was cooled to 4 °C in an ice bath. Slowly and dropwise, 2 equiv (174 μL) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (Sinha et al., 1983) was added over several minutes. Upon complete addition of the chlorophosphoramidite to the reaction mixture, a white precipitate of the ammonium salt formed. TLC on silica with a mixture of 30% chloroform/35% ethyl acetate/25% acetone/10% triethylamine was used to follow the progress of the reaction. After 5 min, TLC demonstrated near complete conversion of starting material to a higher R_f, UV-active spot (R_f = 0.38). The reaction mixture was filtered through a 0.45 μm PTFE filter to remove the precipitated salt. The filter was washed twice with 10 mL of ethyl acetate with 1% triethylamine. The combined filtrate and washes were then washed twice with saturated aqueous sodium chloride, and the aqueous washes were back-extracted with ethyl acetate. The combined organic phases were dried over sodium sulfate and concentrated on a rotary evaporator. Silica chromatography with 99:1 ethyl acetate/triethylamine was utilized to further purify the DHPG-phosphoramidite. Fractions containing desired product were combined and concentrated to yield 166 mg (0.22 mmol) of (*S*)-DMT-*N*-isobutyryl-DHPG cyanoethyl phosphoramidite (**7**) for an experimental yield of 92%. Mass spectrum−fast atom bombardment (FAB$^+$) *m*/*z* 828. ^{1}H NMR assignments (ppm) 0.8−0.9 (isopropyl protons), 1.0 (*N*-isobutyryl methyl protons), 2.5−2.7 (cyanoethyl methylene protons), 2.6 (*N*-isobutyryl proton), 2.8−3.9 (H3′, H3″, H4′, H4″ and H5′ protons), 3.6 (DMT $(OMe)_2$ protons) 5.5 (H1′ protons), 6.5−7.3 (DMT aromatic protons), 8.1 (H8 proton), 11.8 (H1 proton), 11.9 (NH proton). ^{31}P NMR assignments (ppm) 148.8 (phosphoramidite P) (referenced to phosphoric acid).

Preparation of Oligodeoxynucleotides Containing DHPG. (*S*)-DMT-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite (**7**), 166 mg (0.20 mmol), was dissolved in 2 mL of synthesizer grade acetonitrile to give a 0.1 M solution, which was filtered through a PTFE filter prior to use in the synthesis of oligodeoxynucleotides. A decamer of sequence 5′-CTG XAT CCA G-3′ (where X denotes DHPG incorporation) (Nilges et al., 1987) was synthesized at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Conventional, solid-phase oligodeoxynucleotide synthesis routinely utilizes 5′-DMT−3′-phosphoramidites in its 3′ to 5′ chemical synthesis. The above (*S*)-DMT-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite, however, is the equivalent of a 3′-DMT−5′-phosphoramidite, and as such, oligodeoxynucleotides synthesized to contain DHPG required the use of "reverse" 3′-DMT−5′-phosphoramidites (Glen Research Corporation) and were synthesized in a 5′ to 3′ direction. Oligodeoxynucleotides were deprotected for at least 16 h at 55 °C in concentrated ammonium hydroxide. Self-complementary oligodeoxynucleotides containing DHPG were purified prior to trityl removal on reverse-phase HPLC as follows. The oligodeoxynucleotides were diluted to 1 mL in water, and 500 μL was injected onto a Hamilton PRP-1 reverse-phase column (305 × 7 mm) and eluted using an increasing gradient of acetonitrile in aqueous 0.1 M triethylammonium acetate, pH 7.0. Fractions containing tritylated oligodeoxynucleotides were combined, lyophilized to dryness, and resuspended in 100 μL of water. Four hundred microliters of 80% acetic acid was added, and the solution was heated for 30 min at 37 °C prior to reinjection and subsequent collection of the detritylated product. DNA concentrations were determined by UV absorbance using an extinction coefficient of 12.00 nmol/A_{260} or 37.3 μg/A_{260}.

Preparation of NMR Chiral Shift Adducts. To determine whether the (*S*)-*O*-acetyl-*N*-isobutyryl-DHPG (**4**) produced in the reaction employing porcine pancreatic lipase was indeed enantiomericly pure, an NMR chiral shift adduct (Figure 3a) was prepared (Dale & Mosher, 1973). Pure (*S*)-*O*-acetyl-*N*-isobutyryl-DHPG (**4**), 20 mg, was dissolved in 2 mL of dry pyridine, and 20 μL of (*S*)-(+)-MTPA chloride was added. The reaction mixture was stirred at room temperature, and the reaction was followed by TLC on silica with 5% methanol/94% chloroform/1% triethylamine. After 1 h, the reaction was judged to be complete as evidenced by the nearly complete conversion to a higher UV active material (R_f = 0.18), and the reaction mixture was diluted with 8 mL of chloroform, transferred to a separatory funnel, and washed with 10 mL of water. The aqueous layer was back-extracted with 10 mL of chloroform. The combined organic layers were dried over sodium sulfate and concentrated in vacuo. This crude material was chromatographed on silica with 5% methanol/94% chloroform/1% triethyl-

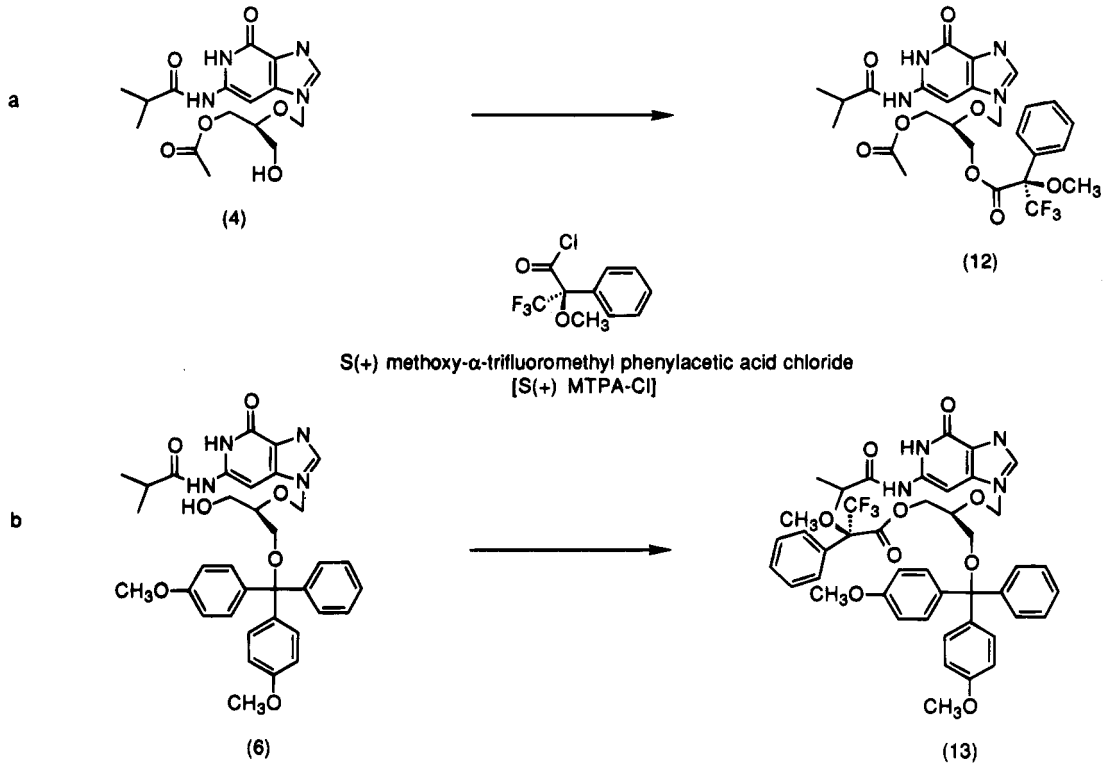

FIGURE 3: Preparation of NMR chiral shift adducts. To illustrate the stereoselectivity of the lipase-catalyzed acetyl transfer reaction, (*S*)-(+)-MTPA chloride was employed to create chiral shift adducts, with the demonstrated stereochemistry, of (*S*)-*O*-acetyl-*N*-isobutyryl-DHPG (a). To confirm that no racemization or acetyl group migration had occurred during subsequent chemical steps, the NMR chiral shift adduct of the penultimate (*S*)-DMT-*N*-isobutyryl-DHPG (b) was prepared. Further details are given in Experimental Procedures.

amine prior to the start of NMR experiments.

To guarantee that no racemization nor acetyl protecting group migration occurred during any of the subsequent chemical manipulations, an NMR chiral shift adduct of (*S*)-DMT-*N*-isobutyryl-DHPG (**6**), the precursor to the phosphoramidite, was prepared in a similar manner (Figure 3b).

Preparation of (S)-DHPG Monophosphate (**8**) *(Figure 5a).* To a stirred solution of 50 mg (0.08 mmol) of (*S*)-DMT-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite (**7**) in 1.25 mL of dry acetonitrile was added 45 μL of β-cyanoethanol (3-hydroxypropionitrile) and 0.5 mmol of tetrazole in 1 mL of acetonitrile to prepare the intermediate dicyanoethyl phosphoramidite (Westerduin et al., 1986). The reaction mixture was stirred at room temperature for 30 min. To oxidize the trivalent phosphate to its desired pentavalent state, an oxidation solution consisting of 0.1 M iodine (0.254 g of iodine in 5 mL of tetrahydrofuran/2.5 mL of lutidine/2.5 mL of water) was prepared and added dropwise to the above dicyanoethyl phosphoramidite until the brown color persisted (Shimidzu et al., 1980). The reaction mixture was allowed to stir at room temperature for an additional 5 min. The remaining iodine was quenched by the addition of freshly prepared 5% hydrogen sulfite, and the entire reaction mixture was concentrated on a rotary evaporator to yield a white solid. To remove the *N*-isobutyryl protection moiety completely, concentrated ammonium hydroxide was added, and the solution was heated to 55 °C for 12 h, and concentrated in a rotary evaporator. To liberate the desired DHPG monophosphate as the free acid, as well as to remove the DMT moiety, the deprotected DHPG was treated with acetic acid for 10 min. Ultimately, (*S*)-DHPG monophosphate was purified by HPLC on a SAX column with a gradient from 10 to 500 mM potassium phosphate, pH 3.0.

Preparation of (R)-DHPG Monophosphate (**11**) *(Figure 5b).* The (*R*)-DHPG monophosphate was prepared in a manner similar to that for the (*S*)-DHPG monophosphate as described above. Briefly, 25 mg (0.068 mmol) of (*S*)-*O*-acetyl-*N*-isobutyryl-DHPG (**4**) was initially converted to the *O*-acetyl-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in a solution of diisopropylethylamine in tetrahydrofuran. The addition of this phosphoramidite moiety occurs on the opposite (*pro-R*) hydroxyl group, as opposed to the *pro-S* hydroxyl as was previously described. The resulting (*R*)-*O*-acetyl-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite (**9**) was concentrated in vacuo but not purified by chromatography. The crude (*R*)-*O*-acetyl-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite was dissolved in 1.25 mL of dry acetonitrile, converted to the intermediate dicyanoethyl phosphoramidite (**10**) as previously described, subsequently oxidized with iodine, deprotected, and purified by HPLC on a SAX column to afford the (*R*)-DHPG monophosphate (**11**).

Preparation of Enzymatically Derived (S)-DHPG Monophosphate. An authentic sample of DHPG monophosphate was prepared, employing HSV-TK to convert DHPG to its biochemically relevant (*S*)-isomer. To 0.5 mM DHPG in TK buffer (74 mM sodium phosphate, pH 6.0; 18.5 mM sodium fluoride; 7.4 mM magnesium chloride; 44 mg/mL BSA; 7.4 mM ATP; 2 mM DTT; 15 mM phosphocreatine; and 0.1 unit/μL creatinine phosphokinase) was added 10−50 μL of HSK-TK, and the reaction mixture was heated at 37 °C. At specific time intervals, samples were withdrawn and analyzed by HPLC. Within 2−3 h, the reaction was judged to be complete, and the (*S*)-DHPG monophosphate was purified by HPLC as above.

Guanylate (GMP) Kinase Assay. Stock GMP kinase assay buffer (0.1 M Tris−acetate, pH 7.6; 0.1 M potassium chloride; 10 mM magnesium chloride; 4 mM ATP; 1.5 mM phosphoenol pyruvate; 0.1 mg/mL BSA; 11 units/mL lactate dehydrogenase; and 5 units/mL pyruvate kinase) was prepared. To 1 mL of HPLC-purified DHPG monophosphate, either the (*R*) or (*S*) chemically prepared isomer or the material prepared enzymatically using HSV thymidine kinase, was added 0.5 mL of stock GMP kinase assay buffer. Four microliters of 1.5 mM NADH was added, and the mixture was allowed to preincubate at 34 °C for 5 min. To this was added 2 μL (0.0288 unit) of guanylate kinase (GMP kinase), and the reaction was allowed to proceed at 34 °C. At specific time intervals, a 200-μL aliquot was removed, diluted with 0.5 mL of 10 mM potassium phosphate, pH 3.0, filtered through a 0.45 μm filter, and analyzed by HPLC as described below for the presence of DHPG diphosphate. The conversion of each of the individual DHPG monophosphate isomers to their corresponding DHPG diphosphate is shown in Figure 6.

HPLC Chromatography of Phosphorylated DHPG. All HPLC purifications and analyses of various phosphorylated species of DHPG were conducted using a Varian 5000 liquid chromatograph fitted with a 10-μm Partisil SAX (strong anion exchange) column (250 × 4.6 mm, Alltech Associates) and a buffer system consisting of 10 mM potassium phosphate, pH 3.0 (buffer A), and 1 M potassium phosphate, pH 3.0 (buffer B). Gradients from 0 to 50% buffer B were typically employed and found to afford excellent resolution of all important species.

Phosphodiesterase I Enzymatic Digestion. Approximately 1 μM annealed 5′-^{32}P-labeled duplex DNA was dissolved in 100 mM Tris-HCl, pH 8.4, and 0.5 μL of phosphodiesterase I was added to initiate the digestion. The reaction mixtures were incubated at room temperature, and the reactions were stopped at various time points by adding gel loading buffer and heating to 95 °C for 5 min prior to analysis on a denaturing polyacrylamide gel.

Melting Temperature Studies. The absorbance versus temperature profile for the DHPG-containing, self-complementary duplex and control duplex was performed on a Perkin-Elmer Lambda 2 UV/vis spectrometer equipped with water-jacketed cuvette holders. Temperature ramping was controlled with a Perkin-Elmer Peltier system. Approximately 0.3 OD_{260nm} unit was annealed in 100 mM NaCl, and the absorbance at 260 nm was measured over a temperature range from 20 to 70 °C. The temperature was increased linearly at a rate of 2 °C/min. The melting temperatures (T_m) were determined from a derivative plot of the absorbance versus temperature profiles.

Circular Dichroism Spectropolarimetry. All circular dichroism investigations were conducted at the Yale University Chemical Instrumentation Center and utilized an AVIV 60 DS circular dichroism spectropolarimeter. Approximately 1 OD_{260nm} unit of each decamer was dissolved in 100 mM NaCl, heated to 95 °C, and annealed by slowly cooling to room temperature. The circular dichroism spectrum were obtained at 23 °C by varying the wavelength over a range from 400 to 210 nm. A graph of the data was obtained using Kaleidograph.

Copper Phenanthroline Chemical Cleavage. A fresh 10× stock solution of the cleavage reagent containing 500 mM TrishHCl, pH 7.8, 1.7 mM 1,10-phenanthroline, 400 μM $CuSO_4$, and 48 mM 3-mercaptopropionic acid was prepared and added to approximately 1 μM annealed 5′-^{32}P-labeled duplex DNA. The reaction mixtures were incubated at room temperature and quenched by the addition of 2,9-dimethylphenanthroline to 2 mM at various time points. These reaction mixtures were then denatured at 95 °C for 5 min prior to analysis on a denaturing polyacrylamide gel.

NMR Spectroscopy. All NMR experiments were carried out on a Bruker AM-500 spectrometer (500.13 MHz for protons and 202.45 MHz for phosphorus). The sample temperature was regulated at 303 K for all experiments.

RESULTS

Chiral Chemical Synthesis of the DHPG DNA Precursor (Figure 2). To investigate the antiviral compound 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, Ganciclovir), we have synthesized DNA containing the biologically active isomer of DHPG. To protect the reactive primary amine moiety of the guanine in anticipation of the automated DNA synthesis process, DHPG was first converted to an *N*-isobutyryl-protected compound (**3**), via a completely protected triisobutyryl intermediate (**2**). Differential alkaline lability between *N*-isobutyryl (amide)- and *O*-isobutyryl (ester)-protected functional groups allowed selective removal of the two *O*-isobuyryl groups, affording *N*-isobutyryl DHPG (**3**).

Introduction of chirality was achieved through a highly stereoselective acetyl transfer reaction catalyzed by porcine pancreatic lipase. This reaction distinguished between the two chemically equivalent hydroxyl moieties and introduced the chemically manipulatable acetyl group. The enantiomeric purity of the *O*-acetyl-*N*-isobutyryl-DHPG (**4**) was assessed through the formation of a NMR chiral shift adduct employing (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride [(*S*)-(+)-MTPA chloride] (Figure 3a). As a chiral moiety, the introduction of (*S*)-(+)-MTPA into any racemic mixture must result in the formation of a pair of diastereomers which are, by definition, not chemically equivalent, and each would be expected to generate significantly distinct NMR spectra. (*S*)-(+)-MTPA chloride was first reacted with a *racemic* mixture of (*R,S*)-*O*-acetyl-*N*-isobutyryl-DHPG prepared by direct chemical acetylation. As expected, the ^{1}H NMR spectra of the product (Figure 4a) demonstrated a doublet peak assignable to diastereometric *O*-acetyl methyl protons as well as a characteristic pattern of paired resonances between 3.9 and 5.6 ppm assignalbe to the diastereometric protons of the acyclic sugar moiety. (*S*)-(+)-MTPA chloride was then reacted with the lipase-derived *O*-acetyl-*N*-isobutyryl-DHPG (**4**) in pyridine, and the product was purified. Comparison to the initial racemic ^{1}H NMR spectrum (Figure 4b) now demonstrates a singlet peak, rather than a doublet, assignable to the *O*-acetyl methyl resonance at approximately 1.8 ppm as well as only *one* set of characteristic resonances between 3.9 and 5.6 ppm, demonstrating the presence of a single diastereomer. No evidence to suggest the presence of a racemic mixture in the sample containing lipase-derived *O*-acetyl-*N*-isobutyryl-DHPG was found. The NMR chiral shift investigations indicate that the lipase-catalyzed acetyl transfer reaction was completely stereoselective, and only one enantiomer was produced.

The chemically derived (*S*)-*O*-acetyl-*N*-isobutyryl-DHPG (**4**) was converted to the dimethoxytrityl (DMT)-protected species (**5**), the *O*-acetyl protecting group was removed to yield (**6**), and the compound was ultimately elaborated to

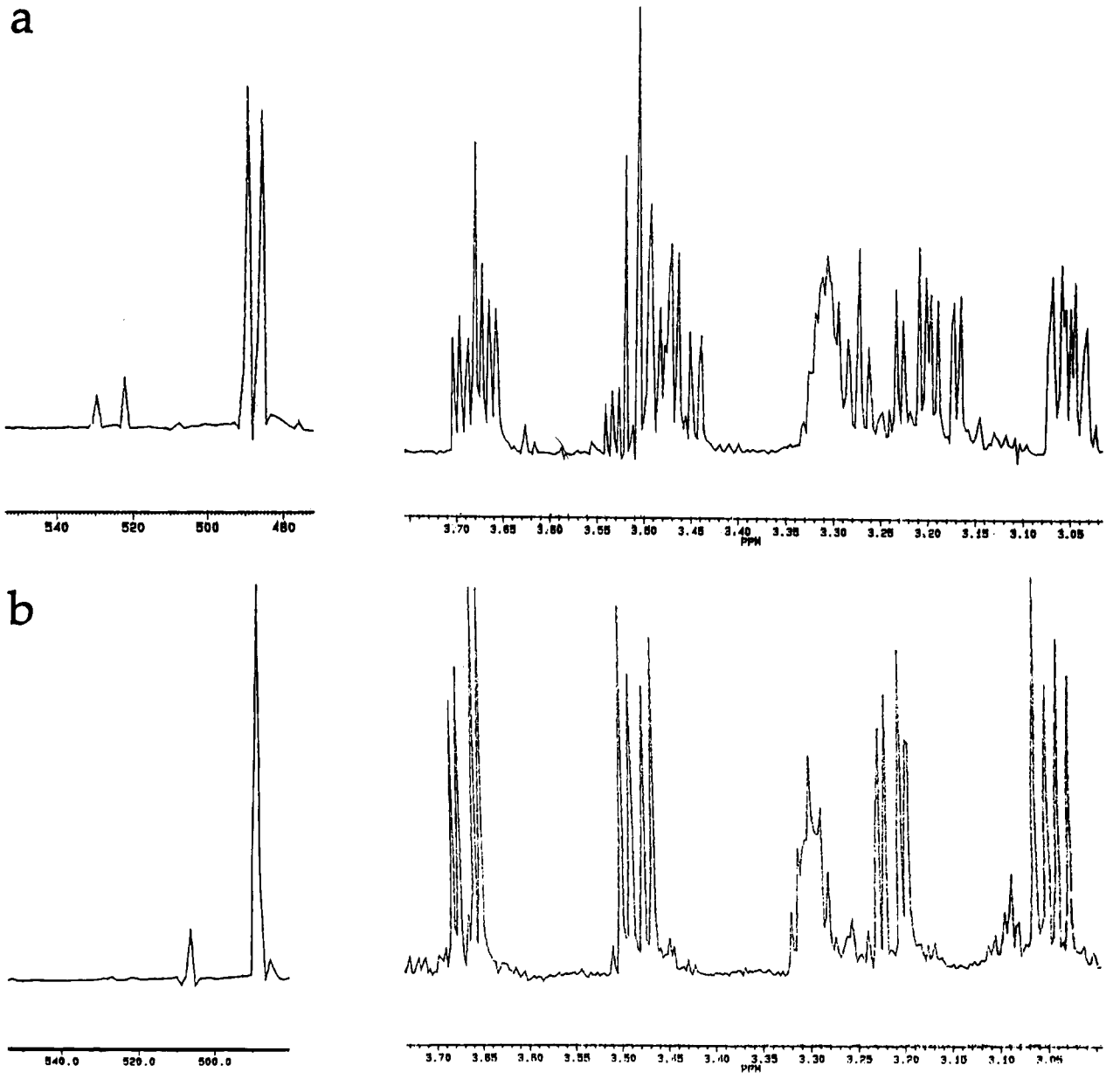

FIGURE 4: ^{1}H NMR spectra of chiral shift adducts. The spectra of enzymatically (lipase)-prepared (*S*)-(+)-MTPA adduct (b) demonstrate a singlet peak, assignable to the *O*-acetyl methyl resonance at approximately 1.8 ppm, as well as only *one* distinct set of characteristic resonances between 3.9 and 5.6 ppm. The spectra of chemically (racemic)-prepared (*S*)-(+)-MTPA adduct (a) demonstrate a *doublet* peak, assignable to the diastereometric *O*-acetyl methyl resonance, as well as a characteristic pattern of paired resonances between 3.9 and 5.6 ppm that are assignable to the diastereometric protons of the acyclic deoxyribose moiety. No evidence to suggest the presence of a racemic mixture in the sample containing lipase-derived *O*-acetyl-*N*-isobutyryl-DHPG was found. The NMR chiral shift investigations indicate that the lipase-catalyzed acetyl transfer reaction was completely stereoselective, and only one enantiomer was produced.

the protected phosphoramidite (**7**). To guarantee that no racemization through acetyl group migration had occurred prior to the synthesis of the final phosphoramidite, ^{1}H NMR chiral shift studies employing the (*S*)-(+)-MTPA adduct of the penultimate DMT-*N*-isobutyryl-DHPG were conducted and demonstrated the presence of only one characteristic set of proton resonances, indicating that the (*S*)-DMT-*N*-isobutyryl-DHPG (**6**) remained enantiomerically pure (data not shown).

Chiral Chemical Synthesis of the DHPG DNA Precursor: Stereochemical Assignment. To determine specifically which isomer, (*R*) or (*S*), was the product of the lipase-catalyzed reaction, we chemically synthesized both possible 5′-monophosphate isomers and compared each of these with DHPG monophosphate prepared using recombinant HSV-TK to catalyze the conversion of DHPG to (*S*)-DHPG monophosphate. The ability of each form of DHPG monophosphate to act as a substrate in the enzymatic conversion to the diphosphate by guanylate kinase was determined. GMP kinase has been demonstrated to have strict stereochemical substrate requirements, and preferentially converts the (*S*)-DHPG monophosphate to its corresponding diphosphate much faster than the (*R*)-DHPG monophosphate.

To prepare chemically what was eventually shown to be the (*S*)-DHPG monophosphate, (*S*)-DMT-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite (**7**) was reacted with β-cyanoethanol (3-hydroxypropionitrile) and tetrazole in acetonitrile, in a reaction analogous to the coupling reaction employed during the automated solid-phase synthesis of oligonucleotide, yielding the intermediate dicyanoethyl phosphoramidite (Figure 5a). This trivalent phosphorus compound was easily oxidized to its pentavalent form with iodine. Subsequent

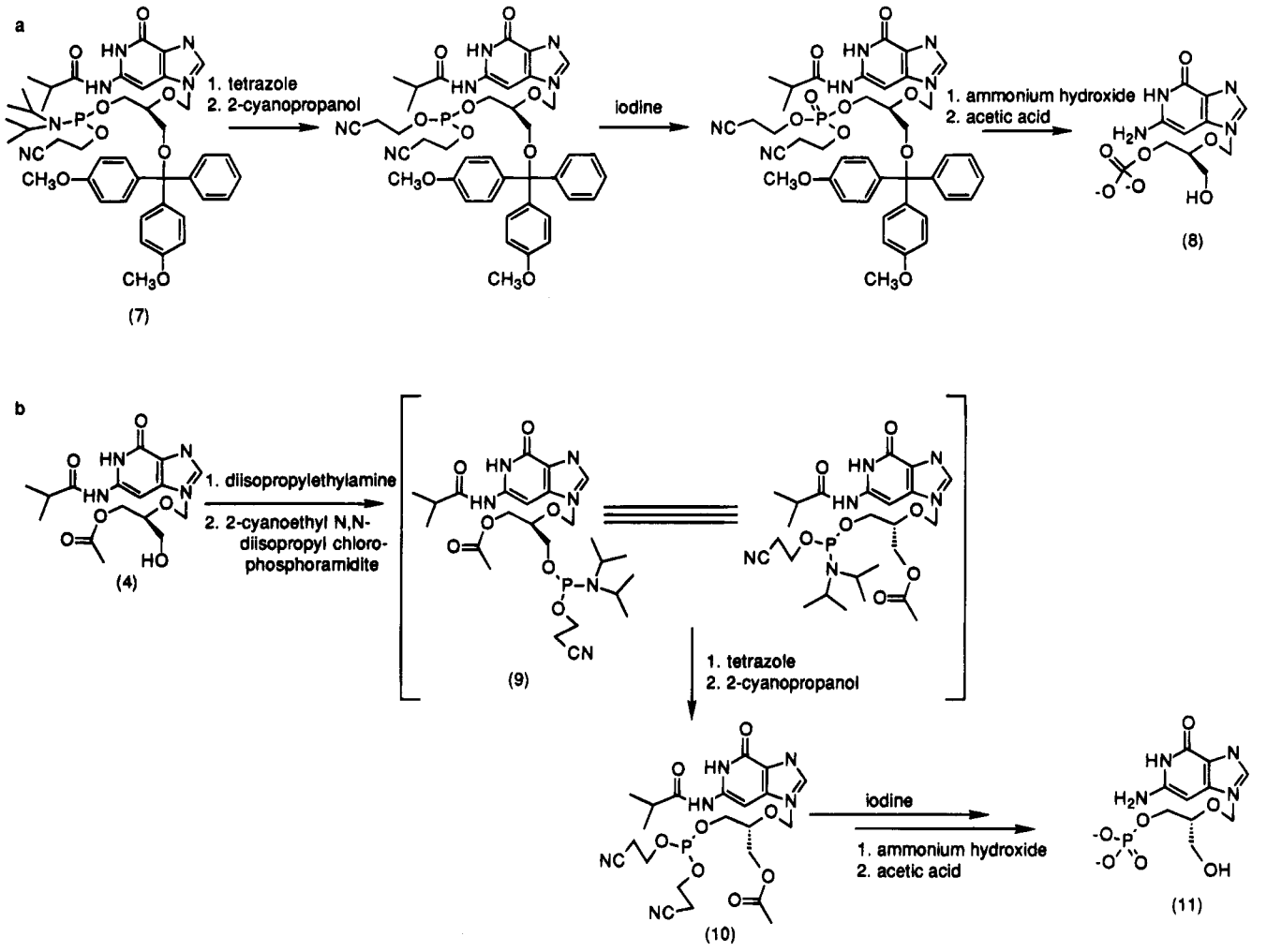

FIGURE 5: Preparation of DHPG monophosphate isomers. The (*S*)-DHPG monophosphate (a) was prepared from the 5′-DHPG phosphoramidite in reactions analogous to the coupling, oxidation, and deprotection reactions employed during solid-phase synthesis of DNA. The (*R*)-DHPG monophosphate (b) was prepared from the 3′-phosphoramidite in a similar manner. Further details are provided in Experimental Procedures.

deprotection afforded the (*S*)-DHPG monophosphate (**8**).

In a similar fashion, the (*R*)-DHPG monophosphate (**11**) was prepared (Figure 5b) by first reacting (*S*)-*O*-acetyl-*N*-isobutyryl-DHPG (**4**) with 2-cyanoethyl *N*,*N*-diisopropyl-chlorophosphoramidite. In contrast to the previously synthesized (*S*)-DHPG monophosphate, the addition of the phosphoramidite moiety here is to the opposite, *pro-R* hydroxyl group, resulting in the formation of the (*R*)-*O*-acetyl-*N*-isobutyryl-DHPG-cyanoethyl phosphoramidite (**9**). Conversion to the intermediate dicyanoethyl phosphoramidite and subsequent oxidation and deprotection afforded what was ultimately shown to be (*R*)-DHPG monophosphate (**11**).

Three compounds, (i) chemically synthesized (*R*)-DHPG monophosphate, (ii) chemically synthesized (*S*)-DHPG monophosphate, and (iii) enzymatically synthesized (*S*)-DHPG monophosphate, were all compared as substrates for their conversion to the diphosphate by guanylate (GMP) kinase (Figure 6). The rate of conversion to the diphosphate of the chemically synthesized (*S*)-DHPG monophosphate was identical to that of the enzymatically synthesized (*S*)-DHPG monophosphate, indicating the equivalency of these two compounds. Under these reaction conditions, both enzymatically and chemically derived (*S*)-DHPG monophosphate were quantitatively converted to the diphosphate in approximately 2 h. The time course for the conversion to the diphosphate of the chemically synthesized (*R*)-DHPG monophosphate was dramatically slower, with no observable conversion to the diphosphate at 2 h and only minimal conversion even at 24−36 h. This investigation completely assigns the absolute stereochemistry introduced by the lipase-catalyzed reaction and demonstrates that the chemically synthesized (*S*)-DHPG monophosphate is indistinguishable from the enzymatically derived (*S*)-DHPG monophosphate but is unquestionably different from the chemically derived (*R*) isomer under conditions of the GMP kinase assay.

Chemical Synthesis of DHPG-Containing DNA Oligodeoxynucleotides. In the scheme utilized to prepare the DHPG-DNA precursor above, the hydroxyl group protected with DMT is stereochemically analogous to the 3′-OH group of a normal deoxyribose ring, and the phosphoramidite is at the position analogous to the 5′-OH group. Most conventional phosphoramidites for use in automated 3′ to 5′ DNA synthesis are 5′-DMT−3′-phosphoramidites. The placement of the DMT group on the 3′-OH and the subsequent placement of the phosphoramidite moiety on the 5′-OH results in a DHPG phosphoramidite that is of "reverse" orientation with respect to standard 3′-phosphoramidites. This issue was addressed by conducting the DNA oligomer synthesis in the reverse sense through the use of commercially available 5′-phosphoramidites. This allows DHPG placement at the 3′-termini or at any internucleotide position.

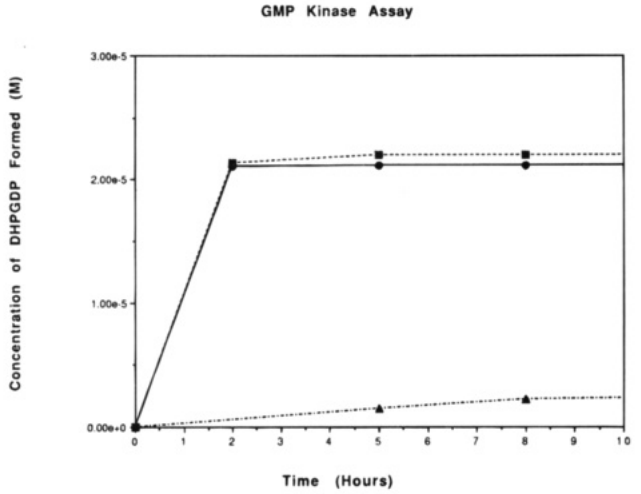

FIGURE 6: GMP kinase assay. The ability of the following monophosphate isomers to act as substrates for the enzymatic conversion to their respective diphosphates was examined. The chemically derived (*S*)-DHPG monophosphate (■) and the HSV-TK-derived (*S*)-DHPG monophosphate (●) were essentially completely converted to the diphosphate in approximately 1 h, whereas the chemically derived (*R*)-DHPG monophosphate (▲) was only minimally converted to the diphosphate even after 10 h. The chemically derived (*S*)-DHPG monophosphate and the HSV-TK-derived (*S*)-DHPG monophosphate were indistinguishable in this assay. The conditions for the GMP kinase assay are described in Experimental Procedures.

A self-complementary decamer of sequence 5′-CTG XAT CCA G-3′ (where X denotes DHPG) was synthesized and purified by reverse-phase HPLC. Electrophoresis of this material (Figure 7, lanes 1 and 5) showed a homogeneous band with nearly the same mobility as an identical sequence decamer containing dG in place of DHPG. Electrospray negative ion mass spectrum analysis demonstrated a 12 atom mass unit difference between the control and DHPG decamers, which was consistent with the loss of a single carbon atom and DHPG incorporation. These decamers were utilized for all subsequent structural characterizations and thermodynamic studies.

Enzymatic Stability. To demonstrate that DHPG had indeed been incorporated into an internucleotide position in the decamer, we investigated its stability to enzymatic hydrolysis. Previous work in our laboratory (unpublished results) and others (Augustyns et al., 1991; Ogilvie & Gillen, 1980; Ogilvie et al., 1984; Usman et al., 1988; Hakimelahi et al., 1987) had suggested that acyclic nucleotides were resistant to various endo- as well as exonucleases. Phosphodiesterase I from bovine intestinal mucosa, a 5′-exonuclease which sequentially liberates 5′-nucleotides from 3′-terminal ends, was chosen for our investigation. Figure 7 illustrates the results of this enzymatic hydrolysis. In contrast to the control decamer, which is fully degraded into all possible products, the DHPG-containing decamer is only partially degraded. Phosphodiesterase I readily hydrolyzes the DHPG duplex until it encounters DHPG at position 4. This DHPG-terminated tetramer accumulates and is wholly resistant to continued hydrolysis, as no further digestion is observed.

Melting Temperature Stability. To determine to what extent the presence of DHPG altered the thermodynamic stability and melting temperature (T_m) of a DNA duplex, UV absorbance versus temperature studies were undertaken. Self-complementary DHPG-containing decamers and sequence-matched controls were each dissolved in 100 mM sodium

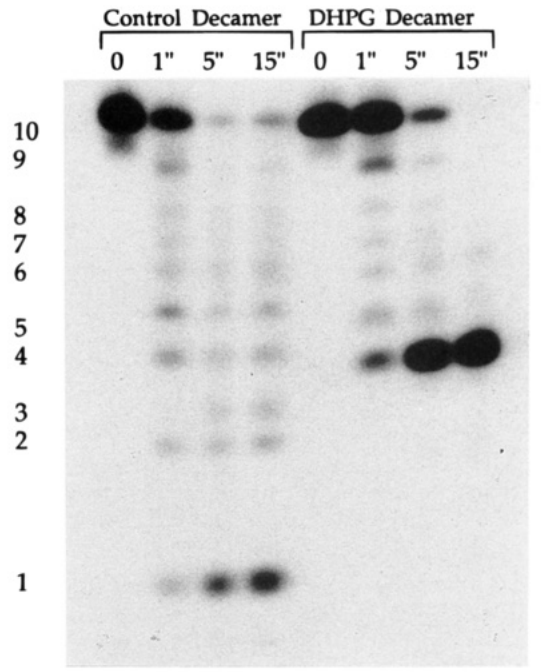

FIGURE 7: Phosphodiesterase I digestion. This panel shows the time-dependent enzymatic hydrolyses of both control and DHPG-containing decamers as described in Experimental Procedures. Aliquots were withdrawn, denatured, and fractionated on denaturing polyacrylamide gels. Incubation of the control decamer with phosphodiesterase I (lames 2−4) demonstrates the complete and sequential degradation of the starting decamer (lane 1) to all nine possible products. Phosphodiesterase I incubation of DHPG-DNA, with DHPG at position 4 (lane 5), clearly shows that the digestion fails to proceed past DHPG and results in an accumulation of DHPG-terminated tetramer (lanes 6−8). Incorporation of DHPG into DNA renders the DNA resistant to phosphodiesterase I digestion.

chloride. This salt concentration has been demonstrated to minimize hairpin formation and promote true duplex formation (Marky et al., 1983). The concentrations of oligodeoxynucleotides were approximately 2.5−5 μM. First derivatives of these absorbance versus temperature curves were calculated using the Perkin-Elmer PECSS software and used to identify the melting temperature. As shown in Figure 8, the $T_{m_{average}}$ for self-complementary control oligodeoxynucleotide was determined to be 43.9 °C, while the $T_{m_{average}}$ for the DHPG-containing duplex was 31.1 °C. There is thus a 13 °C decrease in melting temperature attributable to the presence of two DHPG sites, or a 6.5 °C decrease per DHPG site. The shapes of both the control and the DHPG-containing oligodeoxynucleotide melting curves were monophasic, indicating that the observed melt was that of a true DNA duplex with no component of hairpin loop formation. The shapes of the control and the DHPG melting curves are superimposable, demonstrating that the presence of DHPG does not alter the melting cooperativity.

Helix Conformation of DHPG DNA. To be certain that the introduction of DHPG into DNA, with its associated decrease in thermal stability, did not result in global changes to the overall helix conformation, we investigated the circular dichroism (CD) of these oligodeoxynucleotides as well as their susceptibility to chemical cleavage by the nucleic acid cleavage reagent copper phenanthroline [$Cu(OP)_2$].

Circular dichroism (CD) spectroscopy can be used effectively to study the conformations of nucleic acids in solution and to distinguish between the B-, A-, and Z-form conformations of DNA (Gray et al., 1992; Tunis-Schneider

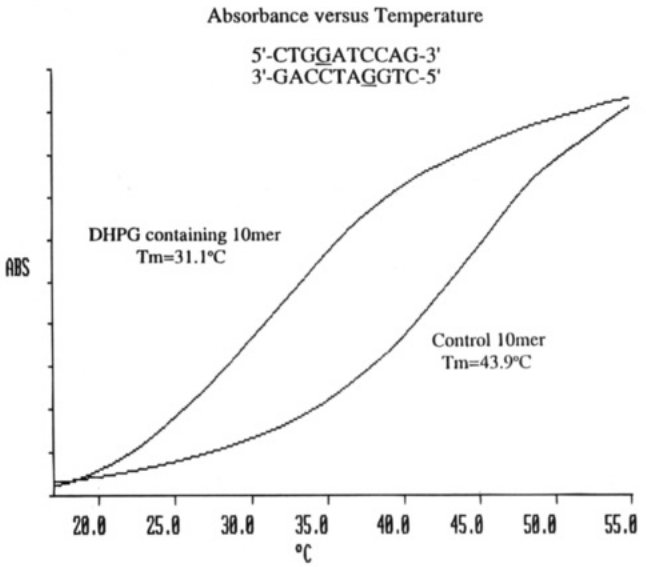

FIGURE 8: The sequence and UV melting curves of the DHPG decamer (T_m = 31.1 °C) and the control decamer (T_m = 43.9 °C). Sample (0.3−0.5 OD unit) was suspended in 100 mM NaCl, and absorbance at 260 nm was measured over a temperature range of 20−60 °C.

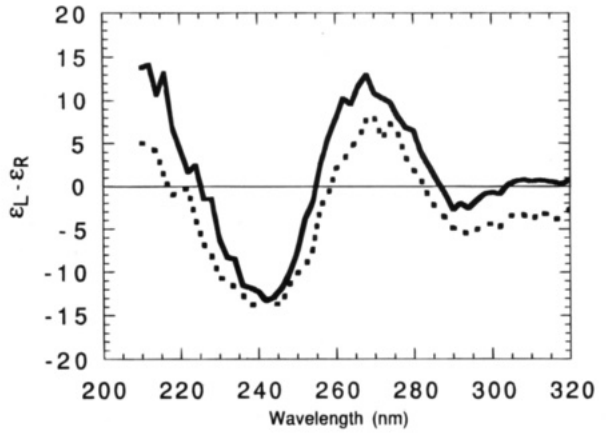

FIGURE 9: Circular dichroism spectra obtained for the DHPG-containing decamer (- - -) and the control decamer (—). The spectra are strikingly similar and display maximum positive bands at 275 nm, crossovers from positive to negative at approximately 257 nm, and additional maximum positive bands at approximately 220 nm. These findings are characteristic of B-form DNA.

& Maestre, 1970; Pohl & Jovin, 1972). Figure 9 illustrates the CD spectra obtained for both the DHPG-containing decamer and the sequence-matched control decamer. The spectra are strikingly similar and display maximum positive bands at 275 nm, crossovers from positive to negative at approximately 257 nm, and additional maximum positive bands at approximately 220 nm. These findings are characteristic of B-form DNA. Features consistent with A-form DNA, including shifts in the maximum positive band from 275 to 262 nm and in the crossover from 257 to 243 nm, as well as the disappearance of the maximum positive band at 220 nm and the appearance of a new negative minimum band at 210 nm, were not found. Preliminary NMR investigations have also confirmed that the introduction of DHPG into DNA fails to alter the helix conformation and that the DHPG-containing decamer remains B-form.

Chemical cleavage by $Cu(OP)_2$ exhibits different specificity for various secondary structures of DNA because it requires a DNA orientation such that the copper ion is accessible to the C1′ hydrogen of the deoxyribose moiety in

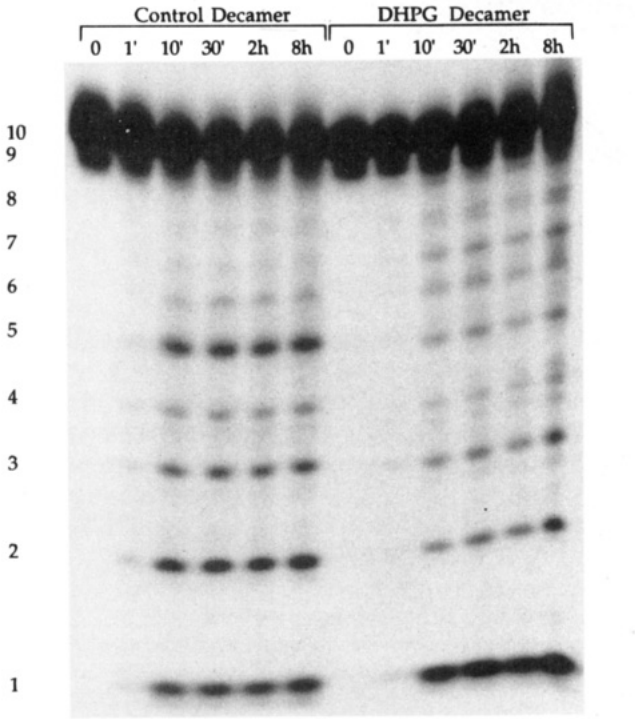

FIGURE 10: Products of the $Cu(OP)_2$ cleavage reaction for both control (lanes 2−6) and DHPG-containing decamers (lanes 8−12). Both strands of the duplex were labeled with ^{32}P at their 5′-ends and were incubated for various time intervals as described in Experimental Procedures. Aliquots were withdrawn, denatured, and fractionated on denaturing polyacrylamide gels. As shown, both the control and the DHPG-containing decamer are equally susceptible to $Cu(OP)_2$ cleavage, suggesting that both duplexes are of the same helix conformation, namely, B-form, and that the presence of DHPG fails to perturb the overall global structure of the helix. Minor differences in the banding pattern centered around the DHPG site may reflect local structural or chemical perturbations introduced by DHPG (see Discussion).

the minor groove (Sigman et al., 1993). The B-form of DNA is more susceptible to $Cu(OP)_2$ cleavage by a factor of 6−7 over the A-form of DNA, and by even a greater factor over the Z-form (Marshall et al., 1981; Pope & Sigman, 1984). $Cu(OP)_2$ can therefore probe global duplex structural issues. Figure 10 illustrates the results of the time-dependent $Cu(OP)_2$ cleavage of DHPG-DNA and its control. The rates and pattern of degradation are very similar, indicating that the DHPG-containing decamer and the control decamer are equally susceptible to the cleaving reagent, implying that both decamers are B-form, reconfirming the CD and NMR observations.

Thermodynamic Parameters. To investigate further the thermodynamic consequences of the presence of DHPG in DNA, ΔH and ΔS were determined using the concentration dependence of the melting temperature and the van't Hoff equation, $1/T_m = R/\Delta H \ln C_T + \Delta S/\Delta H$, where C_T is the oligomer concentration is single stands, T_m is the melting temperature in degrees K, and ΔH and ΔS are respectively the enthalpy and entropy for duplex formation (Petruska et al., 1988). From a linear least-squares fit (Figure 11), ΔH and ΔS were obtained for both the DHPG-containing oligomers and the control oligomers. These experiments reveal that the introduction of *two* DHPG sites in a single self-complementary duplex decamer reduced the enthalpy of duplex formation ($\Delta\Delta H$) by 19.5 kcal/mol as well as reduced the entropy of duplex formation ($\Delta\Delta S$) by 54.7 cal/mol deg. Additionally, ΔG for duplex formation was calculated using $\Delta G = \Delta H - T\Delta S$ at 298 K. The free

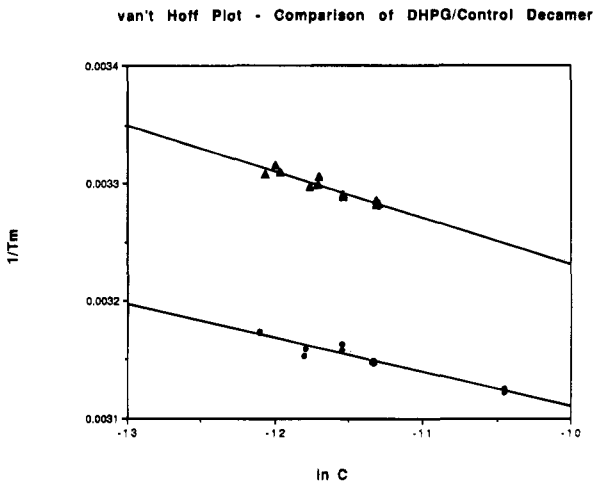

FIGURE 11: Concentration dependence of melting temperature of DHPG decamer (▲) and control decamer (●). The van't Hoff relationship was employed to estimate the ΔH and ΔS of duplex formation from a linear least−squares fit of $1/T_m$ versus ln C.

Table 1: Thermodynamic Values for Duplex Formation[a]

	ΔH (kcal/mol)	ΔS (cal/mol deg)	ΔG (kcal/mol)
control decamer	−69.5	−196.7	−10.9
DHPG decamer	−50.0	−142.0	−7.6

[a] Thermodynamic values for duplex formation shown in this table were derived from the concentration−dependent melting behavior using a linear least−squares analysis of the van't Hoff equation: $1/T_m = R/(\Delta H \ln C_T) + \Delta S/\Delta H$. ΔH and ΔS were then used to determine ΔG at 25 °C: $\Delta G = \Delta H - T\Delta S$.

energy calculated at 25 °C for the DHPG decamer was −7.6 kcal/mol, which respresents a reduction in the free energy of duplex formation ($\Delta\Delta G$) equal to 3.3 kcal/mol. Values for each of these thermodynamic parameters are summarized in Table 1.

DISCUSSION

The therapeutic antiviral drug 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, Ganciclovir) is an intriguing compound from both a structural and a functional standpoint. To investigate and characterize DNA containing DHPG, we have designed a chiral chemical synthesis of the phosphoramidite of DHPG, thus enabling the automated chemical synthesis of DNA containing the biologically relevant isomer of DHPG. The key chemical step employs an enzyme, porcine pancreatic lipase, to catalyze a stereospecific acetyl transfer reaction. The product of this reaction is a single enantiomer, as demonstrated by NMR chiral shift investigations. The absolute stereochemistry has been rigorously determined by comparison of the rates of utilization by GMP kinase of each of the two enantiomers of DHPG monophosphate with that produced enzymatically by phosphorylation with HSV-TK.

NMR chiral shift studies, in addition to demonstrating complete stereochemical selectivity in the lipase-catalyzed reaction, also demonstrate that no racemization had occurred in any of the synthetic steps prior to the final conversion to the phosphoramidite. Given the stereochemistry that is defined during the lipase-catalyzed acetylation, the most direct route to the synthesis of the phosphoramidite proved to be protection of the remaining hydroxyl group with a dimethoxytrityl protecting group and selective replacement of the acetyl group with the phosphoramidite. This results in the synthesis of a 5′-phosphoramidite. To create a standard 3′-DHPG phosphoramidite would involve several additional chemical steps and potentially result in racemization or nonselective loss of one of the protecting groups. We therefore employed the more direct synthetic pathway and adapted the automated chemical synthesis of DNA to utilize our 5′ (reverse)-DHPG phosphoramidite.

5′-Phosphoramidites were developed primarily to accommodate less conventional situations including the construction of parallel-stranded oligodeoxynucleotide hairpins and nuclease-resistant linkages for antisense compounds. We have utilized these 5′-phosphoramidites for use in the automated synthesis of DNA containing DHPG. The synthesis of DNA using these precursors is analogous to the standard system and employs identical chemistry; however, the synthesis proceeds in a 5′ to 3′ direction. Using this method also provides the opportunity of directly adding DHPG to the 3′-terminus of a single-stranded oligodeoxynucleotide without the necessity of preparing a separate precursor. Oligomers containing DHPG at 3′-termini are necessary to study the functional consequences of this lesion at the 3′ primer terminus of a template/primer duplex.

To investigate the structural, thermodynamic, and functional consequences of the presence of DHPG in DNA, we synthesized a self-complementary decamer of sequence 5′-CTG XAT CCA G-3′ (where X denotes DHPG) and an identical control sequence with DHPG replaced by dG. Mass spectral analysis was consistent with the incorporation of DHPG into this DNA.

Enzymatic hydrolysis of this decamer by phosphodiesterase I was undertaken as a further demonstration that DHPG had indeed been incorporated at internucleotide positions in this decamer. We found that the incorporation of DHPG into DNA renders it resistant to exonuclease degradation, which is consistent with previous investigations (Augustyns et al., 1991; Ogilvie & Gillen, 1980; Ogilvie et al., 1984; Usman et al., 1988; Hakimelahi et al., 1987). This increased resistance to digestion has been suggested to be of potential advantage for antisense oligodeoxynucleotide constructs, where a major requirement is an increased stability to enzymatic degradation (Stein & Cheng, 1993).

Because DHPG lacks the 2′-carbon atom of the deoxyribose moiety, it is acrylic, and therefore this nucleotide analog presents an interesting opportunity to explore the contributions of the deoxyribose sugar ring to the structure and thermodynamic stability of duplex DNA. DHPG is unmodified at the guanine base and is therefore potentially capable of forming standard Watson−Crick base pairs with cytosine, preserving the hydrogen-bonding as well as the base pair stacking interactions that contribute to duplex stability. However, because the base is not tethered to the phosphodiester backbone through a restrained cyclic deoxyribose sugar, one might expect changes in duplex structure and thermodynamic stability.

UV absorbance versus temperature studies demonstrated a decrease of 13 °C in the melting temperature of a self-complementary DNA duplex containing two DHPG sites, as compared to the control. Care was taken to ensure that true DNA duplexes, rather that hairpin loops, are represented by these experiments. This suggests a lowering of the duplex melting temperature by 6.5 °C per DHPG site. This is a significant decrease in thermal stability. Comparable values for a *single* dG/dT mismatch are 12−17 °C (Mikita & Beardsley, 1994; Aboul-ela et al., 1985). The decreased

thermal stability of duplex DNA containing DHPG provides initial validation of the argument that the cyclic deoxyribose ring is an important contributor to the overall thermal stability of DNA. In our investigation, the magnitude of the melting temperature change is significantly less than initially suggested by previous work (Schneider & Benner, 1990), where the introduction of two acyclic deoxythymine nucleotides into a nonamer decreased the melting temperature by at least 25 °C.

Comparison of these results with previous investigations into the impact of abasic and anucleosidic sites on the thermodynamic stability of DNA duplexes is worthwhile. Breslauer and co-workers (Vesnaver et al., 1989) found that a single abasic site lowered the melting temperature by almost 14 °C and caused a decrease in the global stability of the duplex. This degree of destabilization was attributable to both the loss of a favorable Watson−Crick base pair and the loss of the stabilizing base pair stacking interactions at the abasic site. Comparisons of this reduction in melting temperature to that of a completely anucleosidic site, lacking both base and pentose ring, failed to reveal further destabilization. The investigators concluded that the presence or absence of the sugar ring at the modified site had little effect on the stability and the temperature-dependent melting behavior of the resulting duplex, and that the primary thermodynamic impact resulted from the absence of the heterocyclic base.

In our investigations, the introduction of DHPG, whose base is tethered by a flexible straight chain to the phosphodiester backbone, results in significantly decreased stability as evidenced by a decrease in the temperature-dependent melting behavior. The additional structural flexibility introduced by a noncyclic "deoxyribose" might fail to properly orient the base or allow a much wider sampling of conformational space, leading to decrease stability.

The combination of decreased thermal stability coupled with the resistance to enzymatic digestion makes DHPG an attractive candidate in the design of ribozymes. DHPG, with its potential for Watson−Crick hydrogen bonding, may prove to be useful in maintaining specificity and decreasing thermal stability while conferring resistance to enzymatic digestion.

To evaluate to what extent the decrease in thermal stability observed upon the introduction of DHPG into DNA might be attributable to perturbations of the overall DNA helix conformation, circular dichroism (Figure 9) and copper phenanthroline [$Cu(OP)_2$] (Figure 10) were used to identify the helix conformation of the duplex decamers. These investigations as well as initial NMR spectroscopy have confirmed that the introduction of DHPG maintains the overall B-conformation of the helix.

CD spectroscopy can also distinguish subtle changes in secondary structure within B-conformations. Comparison of the CD spectra of the DHPG decamer and its control (Figure 9) reveals a slight height decrease in the long-wavelength positive band at 275 nm with a small shift in the 258-nm crossover to a longer wavelength upon incorporation of DHPG. Such changes in the CD spectrum have been observed previously upon mild dehydration of DNA (Gray et al., 1992). Though the exact structural changes that give rise to these observed CD changes are not clearly established, they may result from a cancellation of two bands, a positive band from light absorbed parallel to the helix axis and a negative band, induced by dehydration, from light absorbed perpendicular to the helix axis (Chung & Holzwarth, 1975). Though it is clear that the DNA remains B-form, these observed changes to the CD spectrum of the DHPG decamer suggest that there may be local disruptions to the duplex. The altered intensities of the banding pattern at positions 3−5 in the $Cu(OP)_2$ cleavage (Figure 10) may also suggest local structural perturbations arising from the introduction of DHPG. Alternatively, these changes may be chemical in nature, as DHPG lacks the 2′-carbon atom of the deoxyribose moiety and cannot undergo the β-elimination that is thought to follow the oxidation of the 1′-carbon atom by $Cu(OP)_2$ (Sigman et al., 1993; Goyne & Sigman, 1987). Though these investigations suggest that there may be local structural or chemical perturbations as a result of the introduction of DHPG, there do not appear to be any gross changes in the overall helix structure which would explain the observed changes in the thermal stability of duplex formation.

To extend the investigation further to include thermodynamic parameters, the concentration dependence of the melting temperature, the van't Hoff relationship, and the Gibbs relationship were used to estimate the entropy, enthalpy, and free energy. The presence of *two* DHPG sites in a single self-complementary duplex decamer reduced the entropy of duplex formation ($\Delta\Delta S$) by 54.7 cal/mol deg, but also significantly reduced the enthalpy of duplex formation ($\Delta\Delta H$) by 19.5 kcal/mol. For comparison, the $\Delta\Delta S$ for a single abasic site was found to be 69.2 cal/mol deg, while the $\Delta\Delta H$ for a single abasic site was found to be 27.2 kcal/mol. The free energy of duplex formation ($\Delta\Delta G$) at 25 °C was reduced by 3.3 kcal/mol.

In contrast to our results, Breslauer and co-workers found that the subsequent removal of the deoxyribose moiety after the initial removal of the base failed to produce further significant reductions in either ΔS, ΔH, or ΔG and described the removal of a sugar as a "thermodynamically neutral structural alteration". The unique nature of DHPG allows us to address the contributions of the deoxyribose ring to overall DNA stability from a different perspective. The guanine base of DHPG is still tethered through a "natural" linker to the phosphate backbone but is no longer restrained by a cyclic sugar. DHPG affords the opportunity to investigate the relative contributions of the cyclic deoxyribose moiety to overall DNA stability and structure in a situation where the potential for base-pairing and base-stacking interactions is preserved.

Although it is impossible to investigate the absolute contribution of the deoxyribose sugar to the overall stability of DNA by completely untethering the base from the phosphodiester backbone, the acyclic but tethered DHPG provides a nearly perfect alternative. Our melting temperature studies suggest that the presence of a tethered base is not sufficient to preserve the thermal stability of a DNA duplex, and therefore an intact cyclic deoxyribose ring, which serves as the connection to the phosphodiester backbone, necessarily contributes to stability. The magnitude of this melting temperature decrease, as well as the decrease in both the entropy and the enthalpy for DHPG-DNA duplex formation, is consistent given the potential preservation of both hydrogen-bonding and base pair stacking interactions in the DHPG duplex. The reductions in entropy and enthalpy are significantly greater than those observed when the sugar is sequentially removed from an already abasic site.

The reduction in the entropy term observed in the presence of DHPG is not surprising given the understanding that an

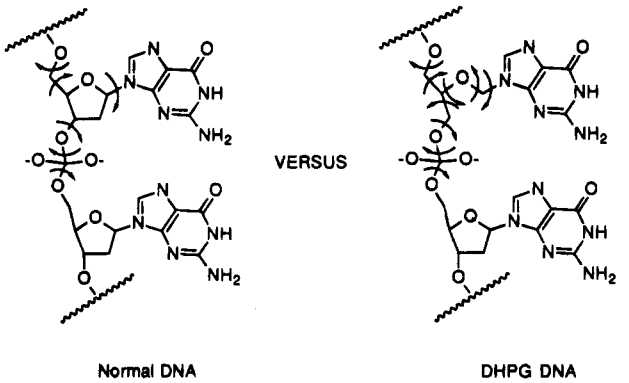

FIGURE 12: Restricted rotor analysis of DHPG in DNA. The placement of DHPG, with its acyclic "deoxyribose" moiety, into DNA introduces three additional rotors, bringing the total number to nine. These extra degrees of freedom are apparent in the entropy for duplex formation.

acyclic sugar should have increased flexibility and be far less constrained than a normal deoxyribose ring, allowing the base to sample greater conformational volume. In a recent examination of the thermodynamic stability of nucleic acids (Searle & Williams, 1993), entropic factors are characterized through a set of six rotors whose rotations become more restricted within the deoxyribose−phosphate backbone during coil−helix transitions. Such an examination in the case of DHPG is enlightening (Figure 12). The loss of the 2′-carbon atom is generally expected to increase the degrees of freedom, but specifically, under this analysis, the loss of the 2′-carbon atom introduces an additional three rotors to the existing set of six. The entropy of coil−helix and helix−duplex transitions for DNA containing DHPG must be reduced to reflect these additional rotational freedoms.

However, the explanation for the reduction in enthalpy during duplex formation may be less apparent. Initially, we expected that the destabilization of the duplex, as evidenced by the decrease in melting temperature, would be primarily due to entropic contributions, given the preserved potential for hydrogen-bonding and base stacking interactions within DHPG. However, significant reductions in enthalpy were also observed, indicating that the stabilizing contributions of the deoxyribose ring through proper base-stacking and base-pairing interactions may be more significant than was concluded from abasic and aglycosidic site data. Our data suggests that the cyclic deoxyribose is critically important to the preservation of the stability of a GC base pair. An investigation employing acyclic deoxythymine nucleotides (Schneider & Benner, 1990), although it did not address detailed thermodynamic considerations and focused only on thermal stability, found decreases in the melting temperature of at least 25 °C upon the introduction of two acyclic deoxythymine moieties. These studies suggest that the cyclic sugar may be even more important in an AT base pair. The loss of the cyclic constraint, in addition to increasing the possible degrees of freedom through facilitated rotation, may also fail to secure the base in the proper orientation to achieve optimal hydrogen-bonding or base stacking interactions. Perturbation of these interactions could account for the reduction in the overall enthalpic contributions.

The existence of enthalpy/entropy compensation has been described for thermodynamic measurements (Searle & Williams, 1992) and addressed in the context of nucleic acid stability (Searle & Williams, 1993) and melting thermodynamics (Petruska et al., 1988; Petruska & Goodman, 1995). The biological role of DNA necessitates its ability to form single- and double-stranded structures *reversibly*. Though duplex DNA is favored over single-stranded DNA, as evidenced by the net negative free energy of duplex formation, the absolute magnitude of this difference must be modest to main reversibility. A recent investigation of this compensation as observed in the melting thermodynamics of DNA doublets (Petruska & Goodman, 1995) revealed that ΔS varies with ΔH as a rectangular hyperbola, thus affirming the existence of a significant enthalpy/entropy compensation. Though the exact molecular basis of this compensation is unclear, Petruska and Goodman suggest that complex solvent interactions with DNA may provide insights not only into the basis of this compensation but also into the basis of DNA polymerase fidelity during replication.

The structure of DNA may have evolved to allow tolerance of local structural anomalies while still permitting overall favorable thermodynamics for reversible duplex formation. As described by Searle and Williams (Searle & Williams, 1993), entropy and enthalpy are the essence of this compensatory phenomenon. Our investigations concerning DHPG highlight this natural plasticity of DNA to tolerate structural perturbations by sacrificing and manipulating entropic and enthalpic contributions to maintain this modest net free energy free difference. The thermodynamic changes resulting from the introduction of DHPG into DNA are primarily driven by entropic contributions stemming from tremendous increases in the degrees of freedom, which are balanced by compensatory changes in enthalpy to preserve the free energy of the entire system.

We have seen that the introduction of DHPG into DNA, in addition to rendering this DNA resistant to enzymatic degradation, also decreases the thermal stability of duplex formation, demonstrating that the presence of a tethered base is insufficient to maintain the thermal stability of a DNA duplex and that an intact deoxyribose ring is essential. Changes in global helix conformation, as evidenced by circular dichroism and copper phenanthroline cleavage, were not found, and thus do not explain this decreased thermal stability. However, these experiments do raise the question of potential local perturbations to the DNA structure at the DHPG site and highlight the importance of fully investigating the thermodynamic parameters of enthalpy, entropy, and free energy in addition to melting temperature stability as a means of fully evaluating the impact of structural changes. Although the detailed effects of DHPG on DNA structure remain to be determined, it is clear that the perturbations observed upon the introduction of DHPG into DNA are made manifest by changes in the thermodynamic characteristics of the entire system. To evaluate the effects of DHPG on the base-pairing and base-stacking interactions further, and to provide structural correlations to these thermodynamic investigations, we are currently examining oligodeoxynucleotides containing DHPG through multidimensional NMR. To assess the functional consequences of these structural issues, detailed investigations into the steady-state and pre-steady-state kinetics of DHPG incorporation and extension by DNA polymerases, including CMV DNA polymerase, are underway.

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