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Synthesis of the Analogue Nucleoside 3-Deaza-2'-deoxycytidine and its Template Activity with DNA Polymerase

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A new synthesis of the nucleoside analogue 3-deaza-2'-deoxycytidine is described. The N^3-nitrogen of dC is involved in the central hydrogen bond of a dG-dC base pair. The relative importance of hydrogen bonding as a controlling factor in the activity of DNA polymerase is examined by studying both duplex stability and template effects for this dc^3C nucleoside. Duplexes containing dG-dc^3C base pairs were strongly destabilized while templates containing the analogue nucleoside were found to incorporate only the complementary dG triphosphate, similar to observations with templates containing the native 2'-deoxycytidine.

Keywords: Nucleic acid analogues, polymerization, pyridones, hydrogen bonding

The 3-deazapyrimidine nucleosides have been shown to have a wide scope of biological, biochemical, and medicinal applications. They have functioned effectively as antivirals, tumor growth inhibitors, anti-bacterial agents[1] and as RNA viral suppressors[2]. While the chemical, biochemical and pharmacological properties of 3-deazauridine have been widely evaluated[3,4], we find startlingly little recent studies of 3-deazacytidine. Neither derivative appears to have been incorporated into DNA sequences for further studies.

The earliest method for synthesizing 3-deazapyrimidines by Currie and coworkers was a many-stepped task and produced the 2'-deoxynucleosides in low yield[5]. This method was improved upon by Cook, Day and Robins[6] and the latter synthetic route has been used exclusively as the method for the synthesis of 3-deaza-2'-deoxycytidine (dc^3C). Unfortunately this process requires the use of the dangerous alkyne starting material 1-methoxy-1-butene-3-yne that is no longer readily available and represents an unattractive synthetic target. We now wish to report a novel method for synthesizing the dc^3C analogue that involves the construction of the heterocycle in relatively few steps followed by its glycosylation. Suitable protection results in a derivative suitable for DNA synthesis.

The N^3-nitrogen is present on the Watson-Crick face of the nucleobase, thus this analogue should be incapable of forming a Watson-Crick base pair with dG. In this respect, when present in a DNA template, this residue should interfere with DNA elongation as catalyzed by DNA polymerase I. However recent studies with isosteric "shape" mimics of the common nucleosides has suggested that such hydrogen bonding interactions may be unnecessary so long and the shape of the nucleobase remains common[7]. In light of these reports, we have also examined the template effects of dc3C and we report on the ability of DNA polymerase I to recognize this analogue and correctly incorporate a complementary dGTP at this site.

Results and Discussion

Synthesis. The most common synthesis of 3-deaza-2'-deoxycytidine begins with the construction of the

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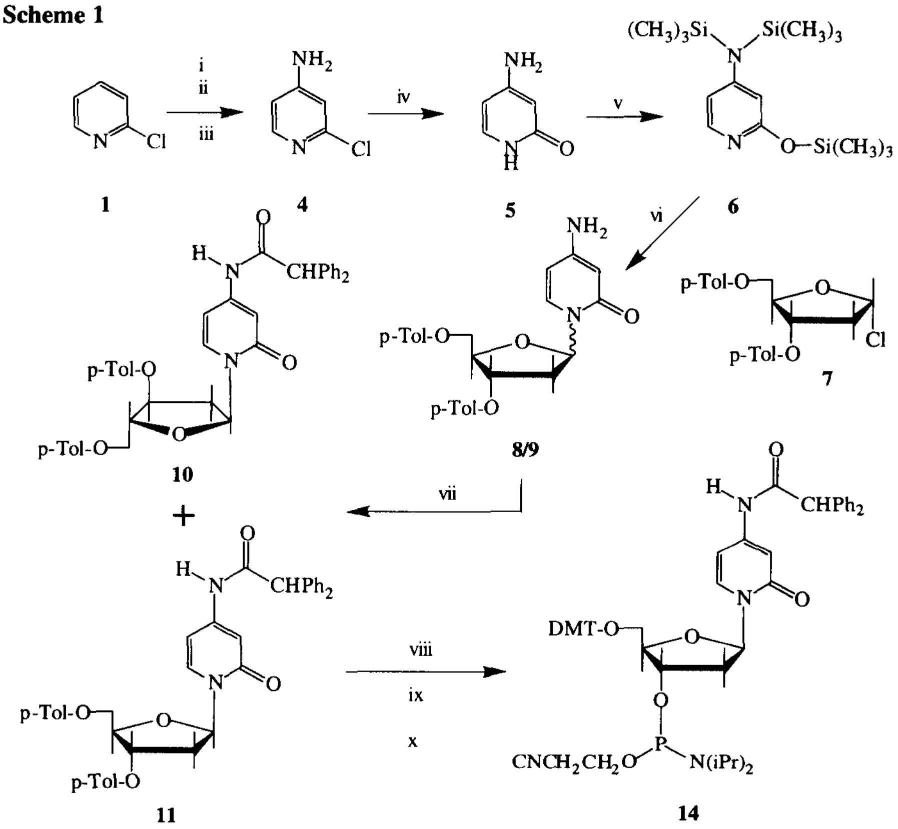
pyrimidine heterocycle using 1-methoxy-1-butene-3-yne, a material that is no longer imported into the United States and represents an unattractive synthetic target owing to its explosive nature. We opted for a new route that begins with the intact pyridine (2-chloropyridine) and relies upon the ability to introduce the requisite exocyclic amino group through nitration/reduction followed by hydrolysis of the chloro substituent to generate the 2-pyridone ring system. Synthesis of the (pyrimidine) nucleoside lacking the N^3-nitrogen began with the preparation of the target 4-amino-2-pyridone heterocycle. This synthesis could be accomplished from 2-chloropyridine in four steps with product isolation and characterization only after steps three and four. The first three reactions were as follows: (i) treatment of 2-chloropyridine **1** (Scheme 1) with hydrogen peroxide and trifloroacetic acid gave 2-chloropyridine-n-oxide (**2**); (ii) refluxing the N-oxide with nitric acid and sulfuric acid yielded 4-nitro-2-chloro-pyridine-N-oxide (**3**) as a yellow solid; and (iii) reduction of **3** with iron powder and concentrated hydrogen chloride gave 4-amino-2-chloropyridine (**4**) in 62% overall yield. Reaction of **4** with sodium hydroxide under pressure with heating provided a nearly quantitative yield of 4-amino-2-pyridone (**5**).

Scheme 1



(i) H_2O_2, (ii) HNO_3/H_2SO_4, (iii) Zn/CH_3COOH, (iv) $NaOH/CH_3OH$, (v) BSA, (vi) $SnCl_4$, (vii) $Ph_2CHCOCl$/pyridine, (viii) $NaOCH_3/CH_3OH$, (ix) DMT-Cl/pyridine, (x) $CNCH_2CH_2OP(Cl)iPr_2$/DIEA

The ^{1}H-NMR spectrum of **5** in DMSO-d_6 agrees with precedent for this heterocycle[6]. The proton resonance peak coresponding to the exo-cyclic amino was exchangeable with D_2O, as expected.

Treatment of **5** with bis(trimethylsilyl)acetamide (BSA) converts the heterocycle to **6**, a derivative that is soluble in 1,2-dichloroethane (the more polar **5** slowly dissolves into 1,2-dichloroethane as the silylation takes place). Glycosylation of the heterocycle was accomplished using 1-α-D-chloro-3,5-di-O-p-toluyl-2-deoxy-*erythro*-pentofuranose[8] (**7**) catalyzed by tin (IV) chloride. Condensation of **6** with **7** resulted in a mixture of α and β anomers (**8** and **9**) that could not be resolved through conventional chromatography. A variety of derivatives of the anomeric nucleosides were prepared in order to effect separation. These included removal of the two *p*-toluyl esters and/or conversion to the exocyclic amino group to the acetamide, benzamide or amidine derivative. Only through introducing increased steric bulk on the heterocycle; by transformation of the nucleoside to the diphenylacetamide derivative (**10**, **11**) could the desired chromatographic separation be achieved and result in purified **11**. The diphenylacetyl protecting group, to our knowledge, has not been previously described for the protection of nucleosides used for DNA synthesis, but the use of the related phenylacetyl group has been reported[10-12]. The β isomer (**11**) was assigned on the basis of NOESY NMR analysis that identified a proton-proton interaction between H_1' and H_4' (data not shown). The corresponding α isomer (**10**) was similarly assigned on the basis of NOE interactions between H_1' and H_3' as well as H_1' and H_5' (data not shown).

The remaining steps in the synthesis (Scheme 1) involved removal of the *p*-toluyl esters (→**12**), protection of the 5'-hydroxyl as the DMT derivative (**13**) and conversion of this product to the corresponding phosphoramidite (**14**). The nucleoside analogue produced in this fashion could be incorporated into oligonucleotides using conventional solid-phase DNA synthesis. Deprotection of the synthesized oligonucleotides was performed in concentrated aqueous ammonia, but complete cleavage of the diphenyl acetyl required at least a 12 h reaction at 50 °C. Digestion of a small quantity of the analogue-containing sequence with snake venom phosphodiesterase and alkaline phosphatase confirmed the presence of the deprotected dc^{3}C residue in the prepared sequences.

DNA Base Pairing. We examined two different DNA duplexes containing the dc^{3}C residue. Initially we prepared the self-complementary Dickerson dodecamer, 5'-[CGCGAATT(c^{3}C)GCG]$_2$ such that two dG-dc^{3}C base pairs were present. We were unable to observe a cooperative thermally induced helix to coil transition for this duplex. We then prepared a 15-mer duplex containing a single dG-dc^{3}C base pair: 5'-[TTTCTTTTCT(c^{3}C)TCTT]·5-[AAGAGAGAAAAGAAA]. This duplex exhibited a Tm value of 23 °C, reduced some 17 °C from the native duplex. Both of these experiments suggest that the presence of dG-dc^{3}C base pairs are disruptive and this effect can probably be explained best in that replacement of the N^3-nitrogen by a C-H introduces a hydrogen atom opposed to the N^1-H of the dG residue and interferes with the formation of Watson-Crick hydrogen bonds (Figure 1).

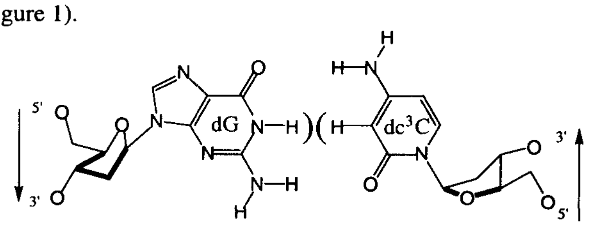


Figure 1. Expected base pair disruption for a dG-dc^{3}C base pair

(a)

5'-CGATACCA$_{OH}$
3'-GCTATGGTGAXTGGTATTCG

(b)

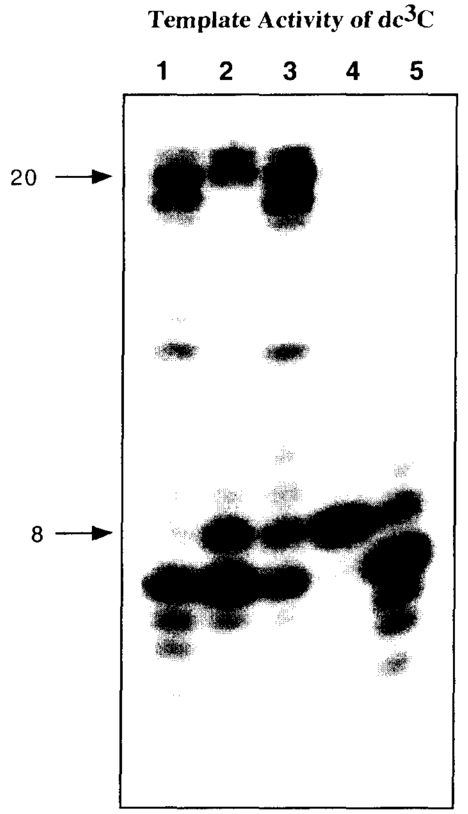


Figure 2. (a) Primer template complex with X = dC or dc^{3}C. (b) Radioactive image of a denaturing polyacrylamide gel illustrating template elongation with Lane 1, X = dC; Lane 2, X = dc^{3}C; Lane 3, X = equivalent amounts of the dC and dc^{3}C-containing templates; Lane 4, control experiment containing all materials except the enzyme; Lane 5, control experiment containing all materials except the template strand.

DNA Polymerase Template Activity. DNA polymerase activity has evolved to the extent that the enzyme can effectively select the correct complementary triphosphate from a background of non-cognate triphosphates. The conventional wisdom has suggested that effective base pairing between the template residue and the incoming triphosphate is critical to the selection process[13-20]. Errors (in the absence of proofreading) occur at roughly 1 in every 10^3 to 10^5 insertions reflecting a selection criterion of about 4 kcal/mole[21].

In order to examine this selectivity, we inserted the analogue nucleoside into a 20-mer DNA template and complexed this template to an 8-mer DNA primer (Figure 2a). We had expected to observe that the elongation of the 8-mer primer by *E.coli* DNA polymerase I (Klenow fragment) would terminate at the site of the dc^{3}C analogue owing to the poor base-base interactions present. Early studies[22] had indicated that in the presence of a mismatch the exonuclease activity of the enzyme removes the mismatch before further elongation (polymerization past the mismatch can be enhanced using the α-thio dNTPs that inhibit the exonuclease activity[23]). However, upon incubation of the polymerase with primer and the dc^{3}C-containing template, primer elongation occurred and extended beyond the site of the analogue such that the majority of the product was fully elongated (Figure 2b). A comparison of this result with that obtained with the dC-containing template, or with that obtained when a mixture of the dC- and dc^{3}C-containing templates indicated no substantial differences.

In a second experiment, we placed either dC or the analogue dc^{3}C residue in the first template position after the primer (Figure 3a). In each experiment a single dNTP was added to a reaction mixture containing primer, template and polymerase. In both cases only dGTP was effective as the nucleotide substrate for elongation of the primer by a single residue (Figure 3b).

These template effects at first glance may seems surprising since DNA polymerase I has difficulty in bypassing any of the common Watson-Crick mismatches. In fact primer-template complexes containing terminal mismatches (and non-cleavable phosphorothioate diester) cannot be effectively elongated by DNA polymerase I. One could expect that the dG-dc^{3}C base pair might function as a mismatch since the duplex stability studies suggest that the base pair is very destabilizing.

(a)

5'-CTATACCACT$_{OH}$
3'-GCTATGGTGAXTGGTATTCG

(b)

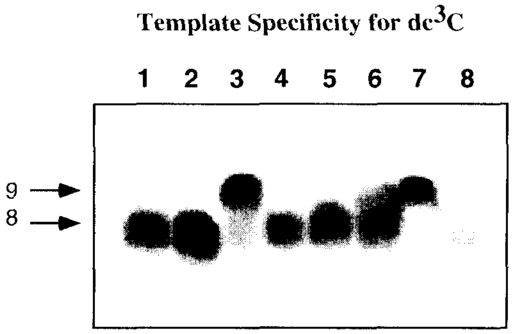


Figure 3 Radioactive image of a denaturing polyacrylamide gel illustrating single-nucleotide insertions with X = dC (Lanes 1 - 4) or dc^{3}C (Lanes 5 - 8) in the template strand. In each case a single triphosphate was added to the reaction: Lanes 1 and 5, dATP; Lanes 2 and 6, dCTP; Lanes 3 and 7, dGTP; Lanes 4 and 8, TTP.

On the other hand, the results reported here are very similar to those reported by the Kool laboratory for selected nucleoside analogues that mimic the shape and not the hydrogen bonding characteristics of the common nucleobases. That work has suggested that efficient hydrogen bonding between template base and incoming nucleotide is not an absolute requirement for nucleoside incorporation into an elongating primer[7,24-27]. Non-polar size and shape mimics of dA and T have been observed to form very unstable DNA duplexes, yet can be incorporated efficiently by the polymerase so long as the corresponding template residue has the complementary shape. For example, the triphosphate of the analogue dZ, a shape mimic of dA is incorporated when difluorotoluene (dF) appears in the template strand - although the putative base pairing between the template residue and the incoming triphosphate would suggest significant steric interference between two opposing hydrogen atoms (Figure 4). Similar results are observed when the dZ residue is present in the template, although in this latter case in addition to observing substrate activity with dFTP, dTTP is also moderately active. In both cases opposing steric interactions should be present.

The current observations with dc3C in the template strand could also be explained in terms of "shape" mimics in the absence of effective interbase hydrogen bonding. The dc^{3}C analogue resulting from the substitution of a C-H for the N^3-nitrogen would be expected to retain much of the geometric shape of dC.

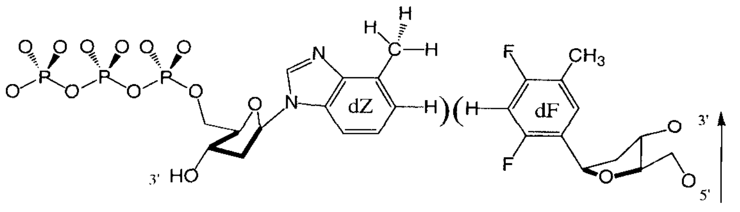


Figure 4. Watson-Crick positioning of dZTP and a dF template residue (see reference 7)

An alternative explanation is that the presence of the C3-H and the subsequent steric effects forces a transient tautomeric shift in the N1-H of the dG residue (Figure 5). Such a tautomeric shift might permit a closer approach of the dG to the dc^{3}C residues such that two of the "normal" Watson Crick bonds could form. This explanation would permit dNTP selection on the basis of the generally held belief that both base-base hydrogen bonding and geometric effects play important roles in the fidelity of DNA replication[13-20].

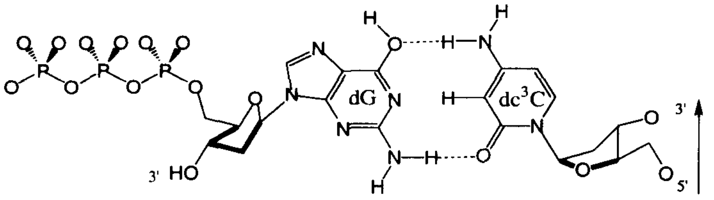


Figure 5. Possible base pair for the rare enol tautomer of dGTP and a dc^{3}C template analog residue

Experimental

^{1}H-NMR spectra were determined on Varian spectrophotometers (300, 400 & 500 MHz). NMR samples contained tetramethylsilane as internal standard unless otherwise specified. Rotary evaporations under reduced pressure were accomplished using Buchi systems. Thin layer chromatography (TLC) was performed using Silica Gel 60 F_{254} pre-coated on aluminum sheets (EM Separations Technology) using methanol:CH_2Cl_2 (1:19) unless otherwise specified. Flash column chromatography was run on Silica Gel 60, particle size 0.040-0.063 (EM Separations Technology), using a methanol gradient in CH_2Cl_2 unless otherwise specified. Glass chromatography plates were Uniplate (TM) 20x20 cm and 1000 µm precoated on glass (Analtech, Inc). Dichloroethane was dried by heating under reflux over calcium hydride for several hours followed by distillation and stored over Linde No. 4 molecular sieves. Other dry solvents were purchased from Aldrich Chemical Co. and used without further purification. UV scans were performed on a Perkins-Elmer 8452A Diode Array Spectrophotometer scanning from 180-820 nm.

2'-Deoxynucleotide phosphoramidites and 3' terminal nucleoside controlled pore glass (CPG) supports were purchased from Glenn Research (Sterling, VA). Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA Synthesizer. High performance liquid chromatography (HPLC) was performed on a Beckman HPLC system using C-18 reversed-phase columns (ODS-Hypersil, 5 µm particle size, 120Å pore) detecting at 260 nm. UV analysis of oligodeoxynucleotides were performed on a Perkins-Elmer Lambda 3B UV/Vis Spectrophotometer.

T4 polynucleotide kinase and large fragment DNA polymerase (Klenow) were purchased from New England BioLabs (Beverly, MA). Denaturing polyacrylamide gels (20%; 8M urea) were run at 24 x 16 x 0.1 cm. Radioactivity was visualized using a Molecular Dynamics 425 phosphorimager.

Methods

2-Chloropyridine-1-oxide (2). To 15.0 g (132.2 mmol) of 2-chloropyridine was added 200 g (135 ml) trifluoroacetic acid and 13 ml 30% hydrogen peroxide. The mixture was heated on a steam bath for 4 h. The solution was evaporated to dryness *in-vacuo* and 300 ml dichloromethane was added to the residue. The organic phase was dried over sodium sulfate and evaporated to dryness. TLC indicated nearly complete conversion to **2**, resulting in a yield of 13.6 g (91%).

R_f(methanol/dichloromethane, 1/19): 0.25.

This crude product was used directly in the following procedure.

2-Chloro-4-nitropyridine-1-oxide (3). To 50 ml 93-98% sulfuric acid was slowly added 25 ml 90% nitric acid (fuming) at 0 °C. To the acid solution was added 13.0 g (0.100 mol) of **2** and the mixture was heated on a steam bath for 2 h. The mixture was cooled and poured onto 100 g crushed ice. The solution was neutralized by slow addition of sodium carbonate while the temperature was kept below 30 °C. 200 mL dichloromethane was added with stirring. The biphasic solution was decanted from the inorganic precipitates. The organic layer was separated and the aqueous filtrate was extracted three times with dichloromethane. The organic solutions were combined, dried and evaporated *in vacuo* to yield 13 g (72%) of a light yellow solid. TLC indicated nearly complete conversion to a new product.
R_f (methanol/dichloromethane, 1/19): 0.54. This crude product was used directly in the following procedure.

4-Amino-2-chloropyridine (4). To 13 g (74.5 mmol) **3** was added 20 g iron powder, 50 ml 80% aqueous ethanol and 5 ml concentrated hydrochloric acid. The mixture was refluxed for 3 h. Sodium carbonate was added to neutralize the residual acid. The resulting mixture was filtered through Celite and was concentrated to dryness. This reaction yielded **4** and some material corresponding to the original starting material **1**. Using flash column chromatography (dichloromethane with a 0-10% gradient of methanol), 6.8 g (52.4 mmol, 95%) of **4** were obtained.
R_f (methanol/dichloromethane, 1/19): 0.29; mp = 80-81 °C.
U.V. (solvent): λ_{max} = 218 nm, 248 nm; λ_{min} = 226 nm.
^{1}H NMR ($CDCl_3$): δ = 6.45-6.47 (dd, 1H, C_5H), 6.55 (d, 1H, C_3H), 7.93-7.94 (d, 1H, C_6H) ppm.
^{13}C NMR ($CDCl_3$): δ = 108.5, 109.9, 149.4, 151.9, 155.2 ppm.
HRMS: calcd for $C_5H_5N_2Cl$ ($M+H^+$), 128.0141, found 128.0145.

4-Amino-2-pyridone (5). To 22.4 g NaOH (s) (0.56 moles) in 120 ml absolute methanol was added 10.0 g (77.8 mmol) of **4**. The mixture was heated 15 hours at 170 °C in a steel bomb. The solution was cooled to 0 °C and neutralized slowly by the dropwise addition of cold concentrated hydrochloric acid. Methanol (100 mL) was added and the solution was filtered through a sintered glass funnel. The product **5** was dried on silica and purified by flash column chromatography (dichloromethane with a 0-20% gradient of methanol). 8.1 g (74.3 mmol, 96%) of **5** were obtained as a yellow solid.
R_f (methanol/dichloromethane, 1/9): 0.05; mp = 64-65 °C.
U.V. (methanol): λ_{max} = 222 ($\varepsilon = 8.80 \times 10^{-3}$), 266 nm ($\varepsilon = 2.38 \times 10^{-3}$); λ_{min} = 238 nm.
^{1}H NMR (DMSO-d_6): δ = 5.17-5.18 (d, 1H, C_3H), 5.63-5.65 (dd, 1H, C_5H), 6.05 (s, 2H, NH_2), 7.00-7.02 (d, 1H, C_6H) ppm.
^{13}C NMR (DMSO-d_6): δ = 94.6, 99.8, 119.4, 136.1, 159.5 ppm.
HRMS: calcd for $C_5H_6N_2O$ ($M+H^+$), 110.0480, found 110.0480.

4-Amino-1-(3',5'-di-O-p-toluyl-2'-deoxy-α/β-D-*erythro*-pentofuanosyl)-2-pyridone (8) and (9). To 2.61 g (23.70 mmol) of residue **5** co-evaporated from anhydrous pyridine (3x) was added 50 mL anhydrous acetonitrile followed by 5.0 ml of bis(trimethylsilyl)acetamide (BSA). As silylation of the heterocycle occurred, the brown immiscible oil slowly dissolved to create a yellow solution (**6**). The solution was stirred under argon for 12 hours at ambient temperature after which time the solvents were removed *in vacuo* and the residue dissolved in 20.0 mL anhydrous dichloroethane followed by the addition of a further 1.0 ml BSA. After 1 h, 4.30 g (6.41 mmol) 1-α-D-chloro-3,5-di-O-p-toluyl-2-deoxy-*erythro*-pentofuranose[8] (7) dissolved in 20 ml anhydrous dichloroethane was added dropwise followed by 0.4 mL (3.47 mmol) of tin (IV) chloride, also dropwise. After 45 minutes the reaction was complete, with one major product and a number of minor side products evident from TLC analysis. The solution was poured slowly into 250 mL of vigorously stirred 5% aqueous sodium hydrogen carbonate and after 15 minutes of vigorous stirring 300 mL of dichloromethane was added. The organic layer was separated, dried over magnesium sulfate and concentrated to a yellow foam. Flash column chromatography (dichloromethane with a 0-10% gradient of methanol) resulted in a mixture of the α and β deoxynucleoside anomers. Yield of the mixture of **8** plus **9** was 1.75 g (3.78 mmol, 59%).

Characterization of the mixture of anomers:

R_f (methanol/dichloromethane, 1/9): 0.39; mp = 82-83 °C.

U.V. (methanol): λ_{max} = 224 nm, 242 nm (sh); λ_{min} = 234 nm.

^{1}H NMR ($CDCl_3$): δ = 2.18-2.26 (m, 1H, H_2'), 2.40-2.43 (d, 6H, CH_3), 2.81-2.98 (m, 1H, H_2'), 4.33 (s, 2H, H_5'β), 4.55-4.57 (quartet, 2H, H_5'α), 4.65-4.70 (triplet, 1H, H_4'β), 4.84-4.86 (triplet, 1H, H_4'), 5.57-5.61 (m, 1H, H_3'), 5.64-5.65 (d, 1H, C_3H), 5.71-5.74 (dd, 1H, C_5H), 6.46-6.48 (dd, 1H, H_1'α), 6.64-6.67 (dd, 1H, H_1'β), 7.18-7.29 (m, 4H, Ar-H.), 7.43-7.45 (d, 1H, C_6Hβ), 7.72-7.74 (d, 1H, C_6Hα), 7.90-7.97 (dd, 4H, Ar-H.) ppm.

^{13}C NMR ($CDCl_3$): δ = 22.8, 40.2, 40.3, 65.4, 65.5, 76.2, 83.6, 86.2, 86.3, 89.2, 96.7, 97.3, 99.7, 100.8, 127.5, 127.8, 130.2, 130.3, 130.4, 130.4, 130.7, 130.8, 130.8, 130.9, 133.2, 133.7, 145.3, 145.5, 156.5, 156.7, 164.2, 166.8, 167.2 ppm.

HRMS: calcd for $C_{26}H_{27}N_2O_6$ (M+H$^+$), 462.186 912, found 463.187 400.

4-Diphenylacylamino-1-(3',5'-di-p-toluyl-2'-deoxy-α/β-D-*erythro*-pentofuanosyl)-2-pyridone (10) and (11). To 1.25 g (2.70 mmol) of **8** plus **9** co-evaporated from anhydrous pyridine (3x) was added 3.0 ml anhydrous pyridine and the solution was cooled to 0 °C. To the cooled solution was added 2.50 g (10.9 mmol, 4 eq) diphenylacetyl chloride. After stirring 1 h at 0 °C, the ice bath was removed and the solution stirred 12 h at ambient temperature. The pyridine was removed *in-vacuo* and the product coevaporated three times from toluene. The residue was dissolved in 30 ml dichloromethane, washed with saturated sodium bicarbonate, dried over sodium sulfate, and evaporated *in-vacuo* to yield a yellow solid. Preparative thin layer silica gel chromatography in a solvent system of 1:50 methanol:dichloromethane resolved two major products. The two bands were removed from the plate and dissolved in methanol/dichloromethane (1:20), washed through a sintered glass funnel, and evaporated *in vacuo*. Flash column chromatography (dichloromethane with a 0-10% gradient of methanol) produced two principal products: The upper band yielded 0.40 g (0.61 mmol, 23%) of **11**, and the lower band yielded 0.50 g (0.76 mmol, 28%) of **10**. NOESY NMR spectra of **11** (data not included) indicated a cross peak between H_1' and H_4'. NOESY NMR spectra of **10** indicated a cross peak between H_1' and H_3', as well as between H_1' and H_5'. These results confirmed that the faster migrating material was the β anomer and the slower material was the α anomer.

Characterization of **10**:

R_f (methanol/dichloromethane, 1/9): 0.455; mp = 171-173 °C.

U.V. (methanol): λ_{max} = 244 nm, 310 nm (sh); λ_{min} = 216 nm.

^{1}H NMR ($CDCl_3$): δ = 2.26 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 2.41-2.46 (d, 1H, $H_{2'}$), 2.74-2.78 (m, 1H, $H_{2'}$), 4.42-4.48 (m, 2H, $H_{5'}$), 4.79 (m, 1H, $H_{4'}$), 5.16 (s, 1H, CHα), 5.48-5.49 (m, 1H, $H_{3'}$), 6.29-6.31 (d, 1H, $H_{1'}$), 6.81(s, 1H, C_3H), 6.93-6.95 (d, 1H, C_5H), 7.06-7.30 (m, 14H, p-Tol Ar-H and DPhAc Ar-H), 7.56-7.58 (d, 1H, C_6H), 7.64-7.93 (dd, 4H, p-Tol Ar-H) ppm.

^{13}C NMR ($CDCl_3$): δ = 22.8, 22.8, 40.1, 60.2, 65.3, 75.8, 86.5, 89.5, 102.1, 105.6, 127.3, 127.7, 128.4, 129.7, 129.9, 130.0, 130.4, 130.5, 130.8, 133.7, 140.1, 140.1, 145.4, 145.5, 150.8, 164.3, 166.8, 167.3, 172.9 ppm.

Characterization of **11**:

R_f (methanol/dichloromethane, 1/9): 0.54; mp = 78-80 °C.

U.V. (methanol): λ_{max} = 238 nm 306 nm (sh); λ_{min} = 218 nm.

^{1}H NMR ($CDCl_3$): δ = 2.15-2.22 (m, 1H, $H_{2'}$), 2.38 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.86-2.91 (m, 1H, $H_{2'}$), 4.54-4.56 (m, 1H, $H_{5'}$), 4.64-4.68 (m, 2H, $H_{4'}$ and $H_{5'}$), 5.06 (s, 1H, CHα), 5.55-5.57 (m, 1H, $H_{3'}$), 6.44-6.46 (d, 1H, C_3H), 6.53-6.56 (quartet, 1H, $H_{1'}$), 6.65-6.68 (d, 1H, C_5H), 7.21-7.36 (m, 14H, p-Tol Ar-H and DPhAc Ar-H), 7.57-7.59 (d, 1H, C_6H), 7.86-7.94 (dd, 4H, p-Tol Ar-H) ppm.

^{13}C NMR ($CDCl_3$): δ = 10.8, 22.9, 22.9, 31.0, 40.5, 61.3, 65.6, 76.5, 84.6, 87.3, 97.4, 102.6, 106.4, 129.5, 130.7, 130.7, 130.8, 130.9, 131.1, 131.2, 131.4, 131.7, 134.1, 140.3, 146.2, 146.4, 150.1, 151.8, 164.7, 168.2, 168.3, 173.0 ppm.

HRMS: calcd for $C_{40}H_{37}N_2O_7$ (M+H$^+$), 657.2601, found 657.2592.

4-Diphenyacetylamino-1-(2'-deoxy-β-D-*erythro*-pentofuanosyl)-2-pyridone (12). A 0.070 M solution of sodium methoxide was created by dissolving 0.16 g Na (s) in 100 ml anhydrous methanol. To 0.20 g (0.29 mmol) of the protected β-nucleoside **11** in 3 ml of methanol and cooled to 0 °C was added 15 ml of the sodium methoxide solution. After 10 minutes the reaction was quenched with 1 mL H_2O and a small quantity of Dowex™ ion-exchange resin was added. The solution was stirred for a subsequent 15 minutes until the pH was between 7 and 8 and then filtered through sintered glass. The solution was evaporated under high vacuum until the methyl ester could no longer be detected by scent. Residual sodium hydroxide was eliminated by dissolving the green tinted product in a small quantity of methanol and decanting away from the insoluble sodium hydroxide. No further purification was required. Yield of **12**: 0.13 g (0.23 mmol, 96%).

R_f (methanol/dichloromethane, 1/9): 0.26; decomposes at >130 °C.

U.V. (methanol): λ_{max} = 224 nm, 258 (sh); λ_{min} = 244 nm.

^{1}H NMR (CD_3OD-d_4): δ = 2.09-2.14 (m, 1H, $H_{2'}$), 2.40-2.43 (m, 1H, $H_{2'}$), 3.36-3.82 (m, 1H, $H_{5'}$), 3.97-4.00 (m, 1H, $H_{4'}$), 4.36-4.39 (m, 1H, $H_{3'}$), 5.21 (s, 1H, CHα), 6.45-6.48 (triplet, 1H, $H_{1'}$), 6.60-6.63 (dd, 1H, C_5H), 7.05-7.06 (d, 1H, C_3H), 7.22-7.32 (m, 10H, DPhAc Ar-H), 7.99-8.01 (d, 1H, C_6H) ppm.

^{13}C NMR (CD_3OD-d_4): δ = 42.1, 59.5, 62.5, 71.7, 86.6, 88.8, 102.4, 104.7, 128.0, 129.3, 129.6, 129.9, 130.2, 134.6, 140.1, 150.5, 164.9, 173.6 ppm.

HRMS: calcd for $C_{24}H_{25}N_2O_5$ (M+H$^+$), 421.1764, found 421.1761.

4-Diphenyacetylamino-1-(5'-4,4'-dimethoxytrityl-2'-deoxy-β-D-*erythro*-pentofuanosyl)-2-pyridone (13). To 0.22 g (0.53 mmol) of **12** co-evaporated three times with anhydrous pyridine and dissolved in 1.5 mL of anhydrous pyridine was added 0.30 g (1.02 mmol) of 4,4'-dimethoxytrityl chloride (DMT-Cl) and a catalytic amount of dimethylaminopyridine. The reaction was stirred under an argon atmosphere for 15 hours at ambient temperature. The reaction was quenched with 1 mL of methanol and dried *in vacuo.* The solution was coevaporated three times with toluene to remove the remaining pyridine. TLC of the product (**13**) showed a characteristic orange spot that charred to black when dipped in a 10% H_2SO_4:H_2O solution and heat dried. The DMT-containing material was isolated using flash column chromatography (dichloromethane with trace quantities of triethylamine and a 0-15% gradient of methanol). Yield of **13** was 0.19 g (0.27 mmol, 72%).
R_f (methanol/dichloromethane, 1/9): 0.37; mp = 86-88 °C.
^{1}H NMR ($CDCl_3$): δ = 2.09-2.12 (m, 1H, H_2'), 2.57-2.61 (m, 1H, H_2'), 3.35-3.44 (m, 1H, H_5'), 3.76 (s, 6H, $-OCH_3$), 4.07-4.08 (m, 1H, H_4'), 4.44-4.45 (m, 1H, H_3'), 5.11 (s, 1H, CHα), 6.39-6.40 (d, 1H, C_3H), 6.45-6.48 (triplet, 1H, H_1'), 6.72-6.75 (dd, 1H, C_5H), 7.21-7.46 (m, 23H, DMT and DPhAc Ar-H), 7.79-7.81 (d, 1H, C_6H) ppm.
^{13}C NMR ($CDCl_3$): δ = 43.1, 47.1, 56.3, 64.1, 72.5, 86.56, 87.0, 101.8, 114.3, 128.7, 131.1, 134.5, 139.6, 159.7, 182.11, 197.8 ppm.
HRMS: calcd for $C_{45}H_{42}N_2O_7$ (M+H$^+$), 722.9591, found 723.3065.

4-Diphenyacetylamino-1-(5'-4,4'-dimethoxytrityl-3'-cyanoethyl-N,N-diisopropyl phosphoramidite-2'-deoxy-β-D-*erythro*-pentofuansyl)-2-pyridone (14). To 190 mg (0.27 mmol) of (**13**) dissolved in 2.0 mL of fresh distilled dichloromethane was added 0.190 mL (10.9 mmol) of N,N-diisopropylethylamine and 0.190 mL (0.86 mmol) cyanoethyl-N,N-diisopropylchlorophosphoramidite. The solution was stirred one hour at ambient temperature then quenched with 1 mL of methanol. The solution was extracted using 100 mL of dichloromethane and 100 mL of a 5% sodium hydrogen carbonate solution. The organic layer was dried through magnesium sulfate and dried *in vacuo.* The crude reaction products were dissolved in 1 mL dichloromethane followed by slow addition of 75 mL of hexane. The solution was swirled slowly and the phosphite product (**14**) allowed to precipitate. The hexane was poured off slowly. This precipitation process was repeated three times and the material was dried *in vacuo.* The yield of **14** was 85 mg (0.094 mmol, 35%) of white solid.
^{32}P NMR ($CDCl_3$): 148 ppm.

***DNA Synthesis*.** Oligonucleotides were synthesized from 1 μmol of bound nucleoside on 500Å CPG silica supports using conventional automated phosphoramidite chemistry[9]. Oligodeoxynucleotides created of sequence: 3'-(GCTATGGTGAXTGGTAGTCG)-5', (3'-(TCACCATAGC)-5', 3'-(ACCATAGC)-5', 3'-GCG(c^{3}C)TTAAGCGC-5', 3'-TTCTXTCTTTTTTTT-5', and 3'-AAAAAAAAGAGAGAA-5', with X either the native dC or the dc^{3}C modified nucleotide. Coupling times for the 3-deaza-2'-cytidine nucleotide were increased from 30 seconds to 4 minutes. Controlled pore glass and protecting groups were removed from the dry oligomers using concentrated ammonium hydroxide at 50 °C. The deprotection of native strands required 4 hours, but the exocyclic amino group of dc^{3}C required 12 hours to fully deprotect.

Purification of the oligonucleotide was accomplished by HPLC (trityl on) using 50 mM triethylammonium acetate (pH 7.0) with a linear gradient of 14-45% acetonitrile (40 minutes) at a flow rate of 3.0 mL/min through a column of diameter of 9.4 x 250 mm. Strands still protected at the base's exocyclic amine by the diphenyacetyl group elute 1 minute after deprotected strands.

After reduction to a small volume, the 5' terminal dimethoxytrityl group was removed with addition at 0°C of an equal volume of 80% acetic acid followed by a 30 minute incubation. The strand was desalted using a Sephadex G-10 size exclusion column eluting with water. The oligomer strands were reduced to a volume of 1 mL and concentration determined by UV spectroscopy (260 nm).

Nucleotide Analysis. Approximately 0.2 A_{260} unit of each purified oligomer was incubated for 4 hours at 37 °C with snake venom phosphodiesterase (1 unit) and alkaline phosphatase (1 unit) in a buffering solution containing Tris-HCl and $MgCl_2$. This digested material was analyzed by HPLC using the KH_2PO_4/methanol system described above and peaks integrated using a Shimadzu CF601 Chromatograph integrator and corrected with established extinction coefficients at pH 5.5. Retention time in minutes for the nucleosides were 7.9 (dC), 9.8 (dc^{3}C), 12.8 (dG), 13.6 (dT), 17.4 (dA).

Tm Studies. To obtain T_m values the DNA duplex was formed by heating a 1:1 mixture of complementary strands at 1 µM to 95 °C and cooling to 4 °C overnight. The buffer conditions used were 20 mM KH_2PO_4 (pH = 7.0), and 0.5 M NaCl (12-mers) or 1.0 M NaCl (15-mers). Measurements were made by heating the samples from 0 °C - 95 °C in 1 degree increments using an AVIV 14DS spectrophotometer (AVIV Associates, Lakewood, NJ). After temperature stabilization absorbance readings were taken. Absorbance vs. temperature plots were made using Igor Pro software (AVIV Associates) and the T_m was determined by first derivative.

Radioisotopic Labeling. The 8-mer and 10-mer oligodeoxynucleotides used as primer strands were labeled using [γ^{32}P]ATP and T4 polynucleotide kinase in a buffer of 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT. The product was purified by polyacrylamide gel electrophoresis. The product band was excised, extracted with 0.3 M sodium acetate, pH 6.0 and desalted with a Waters Sep-pac C18 cartridge.

Primer Extension Reactions. The labeled primer was annealed to the template in a buffer of 0.4 M HEPES (pH 6.6), 0.4 mM Tris HCl (pH 7.0), 0.02 mM EDTA and 0.8 mg ml^{-1} BSA. Polymerase reactions were started by mixing equal volumes of solution A containing the DNA-enzyme complex and solution B containing dNTP substrates. Solution A was made by adding the large-fragment (Klenow) polymerase (New England Biolabs) diluted in annealing buffer to the annealed duplex DNA and incubating 2 min at 37 °C. Solution B contained various concentration of dNTPs in a buffer of 200 mM Tris HCl (pH 8.0), 20 mM $MgCl_2$ and 6 mM mercaptoethanol. The reaction mixture was incubated at 37 °C and terminated by adding one volume of stop buffer (50% glycerol, 20 mM EDTA, 0.025% xylene cyanol and bromophenol blue). For single nucleotide insertions, the final concentrations used were: primer/template 537 µM, Klenow fragment 104 nM and dNTP 60 mM. The reaction was incubated 60 min and the extents of reaction were determined by running quenched reaction samples on a 20% denaturing polacrylamide gel. For multiple nucleotide insertion experiments the final concentrations used were: primer/template 1.0 μM, Klenow fragment 154 nM and dNTP 86 mM, reactions were stopped after 60 minutes and analyzed on a 20% denaturing polyacrylamide gel.

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