Synthesis and Characterization of Oligonucleotides Containing 2′-Deoxyxanthosine Using Phosphoramidite Chemistry

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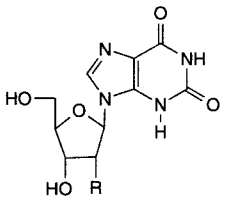
Dedicated to Prof. *Colin B. Reese* on the occasion of his 70th birthday

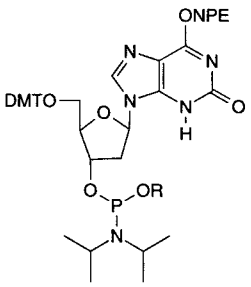
Oligodeoxynucleotides containing 2′-deoxyxanthosine (X_d) were synthesized in good yield from a O^2,O^6-bis[2-(4-nitrophenyl)ethyl](NPE)-protected phosphoramidite of X_d. Attempts to synthesize a O^6-monoNPE-protected phosphoramidite resulted in formation of a major by-product. The NPE protecting groups were removed by treatment with oximate ion after other protecting groups were removed with aqueous NH_4OH solution. The composition of the synthetic oligonucleotides was verified by enzymatic degradation and MALDI-TOF mass spectrometry. The efficacy of this procedure allowed isolation of oligodeoxynucleotides containing multiple X_d residues.

1. Introduction. – Xanthosine (X, **1**) is a naturally occurring nucleoside containing a purine heterocycle that presents a H-bonding pattern to a complementary strand distinct from that presented by standard purines found in encoded oligonucleotides. In the mid-1980s, xanthine was proposed as a 'universal base', a heterocycle that can pair equally well with all four of the naturally occurring bases [1]. Oligonucleotides incorporating 2′-deoxyxanthosine (X_d; **2**) were, therefore, synthesized to examine duplex hybridization and enzymatic incorporation properties. The original interest in X as a nucleoside with a universal base waned because it failed to show the desired base-pairing characteristics. More recently, X_d was used as the purine partner in a base pair with 5-(β-D-ribofuranosyl)pyrimidine-2,4-diamine, a pyrimidine analogue presenting a H-bonding pattern complementary to that of X. This increased the number of possible base pairs in oligonucleotides [2–4].

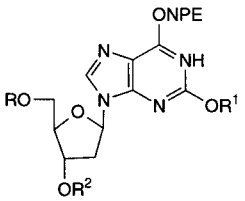
Eschenmoser and co-workers successfully synthesized hexopyranosyl (6′-4′) oligonucleotide analogues containing xanthine using phosphoramidite chemistry with O^2,O^6-di(allyloxy)-protected xanthine [5]. However, published phosphoramidite-based methods to synthesize oligodeoxyribonucleotides containing X_d proved to be low-yielding. *Eritja et al.* used **3**, an O^6-[2-(4-nitrophenyl)ethyl](NPE)-protected phosphoramidite derivative of X_d in the first solid-phase synthesis of an oligodeoxynucleotide containing 2′-deoxyxanthosine [1], but characterized **3** only by TLC analysis. No problems were reported in oligonucleotide synthesis with **3**, even though the building block had an unprotected O=C(2). Later, *Krauch* employed a similar synthetic scheme to make and characterize **3** and the analogous 2-cyanoethyl derivative **4** [6]. During the phosphoramidite-forming reactions in the syntheses of **3** and **4**, however, a major by-product was



1 R = OH
2 R = H



3 R = Me
4 R = $(CH_2)_2CN$



5 R = DMT, $R^1 = R^2$ = P(MeO)($^iPr_2N$)
6 R = DMT, $R^1 = R^2$ = P($NCCH_2CH_2O$)($^iPr_2N$)
7 R = 4-MeOTr, R^1 = NPE, R^2 = P(O)(2–Cl–C_6H_4O)O^-
8 R = DMT, R^1 = NPE, R^2 = P($NCCH_2CH_2O$)($^iPr_2N$)

DMT = 4,4'-$(MeO)_2$Tr
NPE = (4–NO_2–C_6H_4)CH_2CH_2

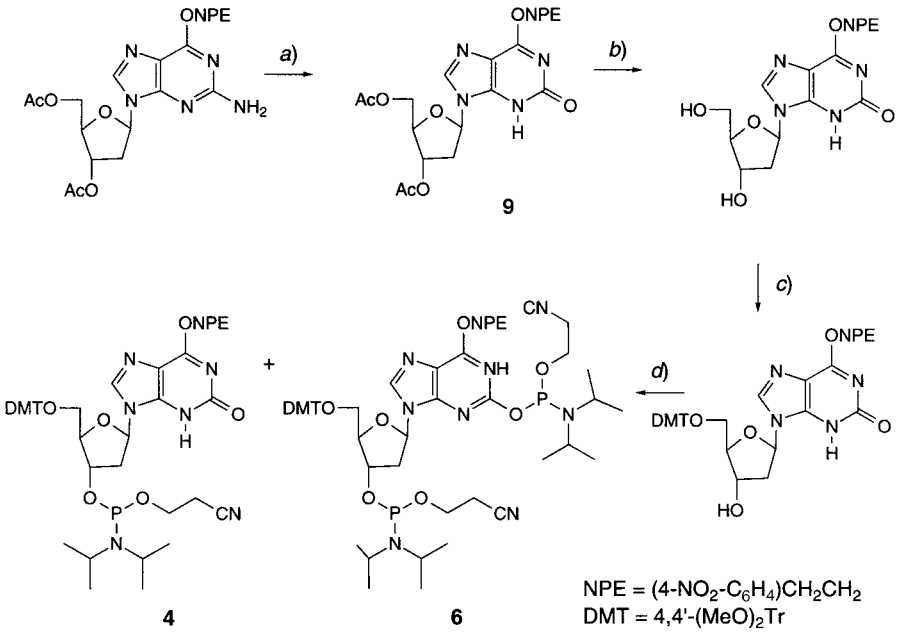
observed and was attributed to the formation of bis-adducts **5** and **6**, respectively. The desired phosphoramidites were purified by silica-gel flash chromatography. Further, solid-phase synthesis with either of the purified phosphoramidites was problematic, with coupling yields (monitored by trityl release) of less than 20%.

Soon after, *Van Aerschot et al.* reported a solid-phase synthesis with protected phosphotriester analog **7** [7]. Like *Krauch*, these workers first attempted to form **7** having an unprotected O=C(2). By-products were observed when subjecting singly NPE-protected X_d to the phosphorylation and condensation conditions required for phosphotriester solid-phase synthesis. *Van Aerschot et al.* finally synthesized doubly NPE-protected phosphotriester **7** to prevent side reactions at position O^2, and then used **7** to synthesize a short oligonucleotide containing X_d.

Finally, post solid-phase synthetic deamination of guanine has been used to introduce X_d into oligonucleotides, although this method is limited to oligonucleotides containing only nucleosides without exocyclic amine functionality, thus severely limiting the range of possible product oligonucleotides [8].

Results and Discussion. – As part of our work to expand the genetic alphabet, we required a suitably protected phosphoramidite of X_d for solid-phase oligonucleotide synthesis. Our first efforts to synthesize oligonucleotides containing X_d followed the work of *Eritja et al.* closely (see synthesis of **4** and by-product **6** in *Scheme 1*) and began

Scheme 1



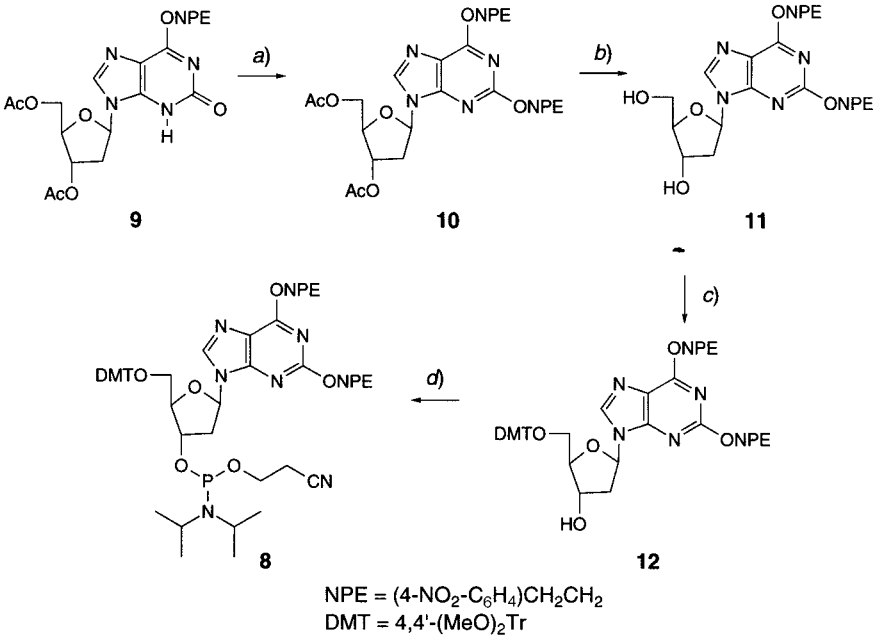
a) AcOH, $NaNO_2$; 57%. *b*) NH_3, H_2O/MeOH; 99%. *c*) $(MeO)_2TrCl$, pyridine; 37%. *d)* 2-Cyanoethyl diisopropylphosphoramidochloridite, $^iPr_2NEt$, CH_2Cl_2; 42% **4**, 7% **6**.

with a phosphoramidite where O=C(2) of X_d was left unprotected, with the purine moiety carrying a single NPE protecting group [1][6]. In this synthesis, 3′,5′-di-*O*-acetyl-2′-deoxyguanosine was NPE-protected at position O^6 and then converted to NPE-protected 3′,5′-di-*O*-acetyl-2′-deoxylxanthosine **9** by treatment with nitric acid. We were, however, unable to form phosphoramidite **3** in good yield by this strategy. As reported by *Krauch*, the unprotected O=C(2) evidently reacted during formation of the 3′-phosphoramidite, resulting in two major products requiring chromatographic separation. Even if only 0.9 equiv. of the phosphoramidite-forming reagent were used, which reduced the yield of **3**, the formation of the contaminant was not completely suppressed (data not shown). Because of these difficulties, this approach was abandoned.

Next, double NPE protection as previously employed in the synthesis of phosphotriester **7** was adapted for the synthesis of a cyanoethyl phosphoramidite of X_d. Starting from 3′,5′-di-*O*-acetyl-2′-deoxy-O^6-[2-(4-nitrophenyl)ethyl]xanthosine (**9**) [9], the desired phosphoramidite **8** was synthesized in four steps (*Scheme 2*). NPE Protection at position O^2 was achieved by treatment of **9** with 2-(4-nitrophenyl)ethyl iodide [10] and Ag_2CO_3 ($\rightarrow$ **10**), by the method of *Pfleiderer* and co-workers [11]. After removal of the acetyl protecting groups ($\rightarrow$ **11**), 5′-*O*-dimethoxytrityl ($\rightarrow$ **12**) and 3′-(cyanoethyl diisopropylphosphoramidite) groups were introduced [12] to give **8** in 55% yield over four steps.

Solid-phase oligonucleotide synthesis with **8** was performed according to standard methods with an extended coupling time for **8** and 1*H*-imidazole-4,5-dicarbonitrile

Scheme 2



a) 2-(4-Nitrophenyl)ethyl iodide, Ag_2CO_3, benzene; 93%. *b*) NH_3, H_2O/MeOH. *c*) $(MeO)_2TrCl$, pyridine; 70% (2 steps). *d*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^iPr_2NEt$, MeCN; 85%.

instead of 1*H*-tetrazole, a less acidic activator, and dichloroacetic acid to detritylate. The last two were precautions to avoid depurination of X_d. Observed coupling yields, monitored by trityl-group release at 470 nm, were quite satisfactory under these synthetic conditions, as demonstrated by the coupling yields for the 1-μmol scale synthesis of the 14-mer oligonucleotide **13** (*Fig.*).

In previous syntheses involving NPE groups at X_d, DBU and/or oximate treatment [13][14] were used to deprotect X_d-containing products [1][6][7][11]. Synthetic oligonucleotides made from **8** were deprotected in two steps. The protected synthetic oligonucleotide was incubated in concentrated aqueous NH_4OH solution at room temperature, followed by incubation in oximate solution at 70°. This deprotection scheme was especially convenient because, during NH_4OH treatment, an otherwise deprotected oligonucleotide containing O^2,O^6-bis-NPE-protected X_d was generated. The progress of the NPE cleavage reaction was then monitored to completion by HPLC. It was particularly useful to monitor the reaction progress when scaling up the deprotection, which often required extended reaction times.

Aqueous NH_4OH solution at 60° for 48 h also removed the NPE protecting groups. Oligonucleotide **13** was subjected to these deprotection conditions and then enzymatically digested with snake-venom phosphodiesterase and alkaline phosphatase. The component nucleosides were separated with reversed-phase HPLC. The standard nucleosides were present in the expected ratio, but only about two thirds of the expected amount of X_d and an unexpected component were found. This unanticipated

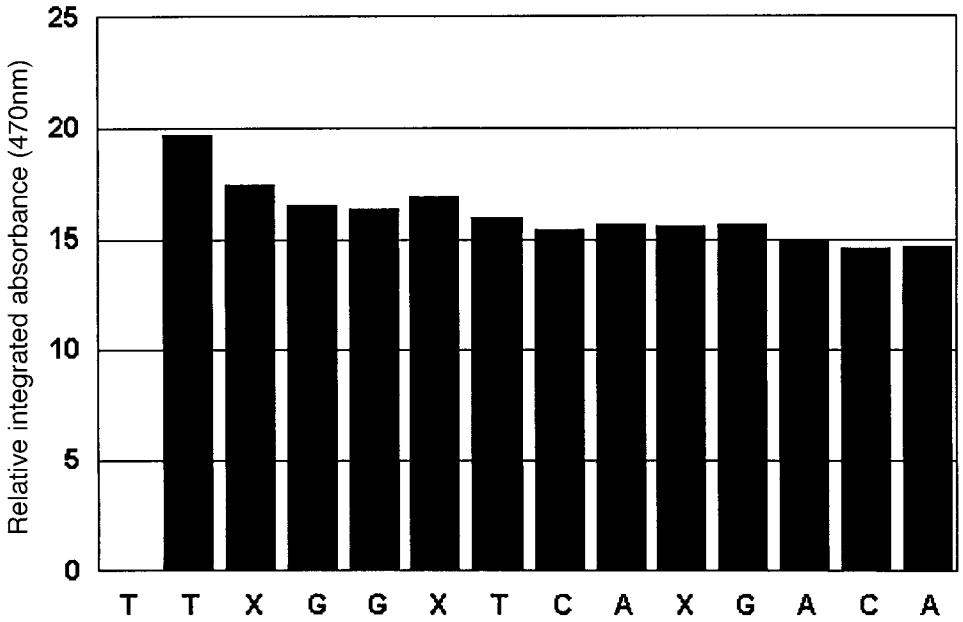


Figure. *Integrated absorbance* (470 nm) *monitoring trityl-group release for each coupling step in the synthesis of oligonucleotide 5′-d(ACAGXACTXGGXTT)-3′* (**13**)

species displayed the characteristic UV absorbance spectrum of 2′-deoxyisoguanosine ($isoG_d$) and, when quantified as $isoG_d$, accounted for the final third of the expected X_d positions. The peaks in the HPLC analysis of the enzymatic-digestion products corresponding to X_d and the unexpected nucleoside were collected and further analyzed by mass spectrometry. The peaks were confirmed as arising from X_d and $isoG_d$, respectively, indicating that a displacement of O^6-[2-(4-nitrophenyl)ethyl] on 1/3 of the protected X_d positions by ammonia had taken place during deprotection. The corresponding product of an analogous displacement at position O^2, *i.e.* G_d, was not observed.

Deprotected product oligonucleotides were purified in two steps by anion-exchange HPLC followed by reversed-phase HPLC. The purity of oligonucleotides treated in this manner was confirmed by anion-exchange HPLC in 20 mM NaOH and eluting with a NaCl solution gradient. These conditions give a different separation profile than at neutral pH because of the ionization of G_d and T_d at high pH. Product oligonucleotides were also subjected to enzymatic digestion with snake venom phosphodiesterase and alkaline phosphatase; the peaks observed during reversed-phase HPLC of the resulting nucleoside mixture were checked for expected UV spectra with a photodiode-array detector and were integrated to verify the correct relative nucleoside composition. Finally, the oligonucleotides were analyzed by MALDI-TOF mass spectrometry.

The four oligodeoxynucleotide duplexes **14**·**15**, **14**·**16**, **14**·**17**, and **14**·**18** made by *Eritja et al.* pairing X_d opposite each of the four standard bases [1] were synthesized from phosphoramidite **8** and characterized as described above. The melting temperatures (T_ms) of the four duplexes were determined under the conditions described in

Table. *Duplex Melting Temperatures* (T_ms) *for Oligodeoxynucleotides Containing* X_d

	pH 7.5		pH 5.5		ΔT_{m1}	ΔT_{m2}
	T_{m1} [°][a])	T_{m2} [°][b])	T_{m1} [°][a])	T_{m2} [°][b])		
14·15	51.9	52.8 ± 0.7	55.5	55.0 ± 0.7	3.6	2.3
14·16	51.8	49.7 ± 0.4	52.8	51.9 ± 0.4	1	2.2
14·17	54.7	54.6 ± 0.4	53.9	53.5 ± 0.5	− 0.8	− 1.1
14·18	55.5	55.4 ± 0.6	54.9	54.5 ± 0.5	− 0.6	− 0.9

[a]) From [1]; error of ± 0.3°. [b]) Indicated errors are for 95% confidence.

the previous determinations at pH 7.5 and pH 5.5 (see *Table*). The T_ms determined here agree fairly well with the previous values, having the same ranking from highest to lowest. The agreement is especially good for those duplexes whose melting temperatures are pH-independent, *i.e.* for **14·17** and **14·18**). However, the values for the other two duplexes *i.e.* **14·15** and **14·16**, disagree somewhat.

5'-d(ACAGXACTXGGXTT)-3' (**13**)

3'-d(CAGAGTACATXATTGTAGC)-5' (**14**)

5'-d(GTCTCATGTAATAACATCG)-3' (**15**)

5'-d(GTCTCATGTACTAACATCG)-3' (**16**)

5'-d(GTCTCATGTAGTAACATCG)-3' (**17**)

5'-d(GTCTCATGTATTAACATCG)-3' (**18**)

Experimental Part

General. All chemicals were purchased from *Aldrich Chemical* or *Fisher Scientific* and were used without further purification. Pyridine, Et_3N, and MeCN were dried over 4 Å molecular sieves. Silica gel was purchased from *Acros Organics* (0.035 – 0.07 mm, pore diameter *ca.* 6 nm). 'Evaporation' refers to removal of volatile solvents with a rotary evaporator and membrane pump. NMR Spectra: *Varian-XL300* spectrometer at 300 MHz referenced to $SiMe_4$ (^{1}H), at 75.4 MHz referenced to $CDCl_3$ (^{13}C), and at 121.4 MHz referenced to H_3PO_4 (^{31}P). The University of Florida Department of Chemistry Mass Spectrometry Laboratory performed high-resolution MS analyses with a *Finnigan-MAT-95Q* apparatus and ESI analyses with a *Finnigan-LCQ* ion trap. The University of Florida ICBR Protein Chemistry Core performed MALDI-TOF-MS analyses on a *PerSeptive Biosystems Voyager*.

3′,5′-Di-O-*acetyl-2′-deoxy*-O^2,O^6-*bis-[2-(4-nitrophenyl)ethyl]xanthosine* (**10**). To a soln. of **9** (9.34 mmol, 4.68 g) in anh. benzene (160 ml), Ag_2CO_3 (1.5 equiv., 14.0 mmol, 3.87 g) was added. The mixture was refluxed (1 h) and then cooled (50°), and 2-(4-nitrophenyl)ethyl iodide (2 equiv., 18.68 mmol, 5.17 g) was added. The mixture was stirred under Ar (15 h, 50°). A precipitate formed and was removed by filtration. The solvent was evaporated and the residue purified by CC (silica gel, $CHCl_3$/acetone 8:2): **10** (5.67 g, 93%). ^{1}H-NMR ($CDCl_3$): 2.07 (*s*, Ac); 2.15 (*s*, Ac); 2.61 (*m*, 1 H−C(2′)); 3.00 (*m*, 1 H−C(2′)); 3.28 (*m*, 2 PhCH_2CH$_2$O); 4.35 (*m*, 1 H−C(4′), 2 H−C(5′)); 4.62 (*m*, PhCH$_2$CH_2O); 4.80 (*m*, PhCH$_2$CH_2O); 5.52 (*m*, H−C(3′)); 6.40 (*dd*, H−C(1′)); 7.19 (*d*, 4 H, Ph); 7.49 (*m*, 4 H, Ph); 7.98 (*s*, H−C(8)). ^{13}C-NMR ($CDCl_3$): 20.4; 20.6; 34.7; 34.8; 36.8; 63.5; 66.5; 67.2; 73.9; 82.0; 84.2; 117.5; 123.5; 129.6; 129.7; 139.2; 145.5; 145.9; 146.4; 146.5; 152.7; 160.5; 161.1; 170.0; 170.1.

2′-Deoxy-O^2,O^6-*bis-[2-(4-nitrophenyl)ethyl]xanthosine* (**11**). Compound **10** (8.72 mmol, 5.67 g) was dissolved in warm MeOH (480 ml), and the soln. was cooled to r.t. Aq. NH_3 soln. (25%, 480 ml) was added at a rate such that the mixture remained clear. The mixture was stirred at r.t. (24 h) and a solid began to crystallize. The solvent was evaporated and the residue co-evaporated with xylene (5 × 40 ml) to remove acetamide. The residue was co-evaporated with MeCN (3 × 40 ml) to remove traces of H_2O and dried under vacuum overnight.

The light yellow solid obtained can be used in the next step without further purification. If desired, however, the solid can be further purified by CC (silica gel, $CHCl_3$/acetone 4:1, then $CHCl_3$/MeOH 82.5:17.5). ^{1}H-NMR ((D_6)DMSO): 2.30 (*m*, 1 H–C(2′)); 2.71 (*m*, 1 H–C(2′)); 3.26 (*m*, 2 PhC*H*$_2$CH$_2$O); 3.57 (*m*, 2 H–C(5′)); 3.88 (*m*, H–C(4′)); 4.43 (br. *s*, OH); 4.61 (*m*, 2 PhCH$_2$C*H*$_2$O); 4.77 (*m*, 2 PhCH$_2$C*H*$_2$O); 4.97 (br. *s*, OH); 5.36 (*m*, H–C(3′)); 6.31 (*dd*, H–C(1′)); 7.62 (*m*, 4 H, Ph); 8.18 (*m*, 4 H, Ph); 8.37 (*s*, H–C(8)). ^{13}C-NMR ((D_6)DMSO): 34.2; 34.3; 2 signals superimposed by DMSO, 61.6; 66.4; 67.0; 70.7; 83.4; 87.8; 116.8; 123.34; 130.2; 140.7; 146.2; 146.5; 146.8; 153.1; 160.1; 160.6.

*2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-*O^2,O^6*-bis-[2-(4-nitrophenyl)ethyl]xanthosine* (**12**). To a soln. of **11** in anh. pyridine (150 ml), $(MeO)_2$TrCl (1.2 equiv., 10.5 mmol, 3.54 g) was added. The mixture was stirred (22 h), then H_2O (3 ml) added, and the soln. evaporated. Aq. $NaHCO_3$ soln. (70 ml) and AcOEt (70 ml) were added. The org. layer was dried (Na_2SO_4) and evaporated and the residue purified by CC (silica gel, $CHCl_3$/acetone 9:1, then 9:2) to give **12** (5.26 g, 70% over 2 steps). Yellow foam. ^{1}H-NMR ($CDCl_3$): 2.57 (*m*, 1 H–C(2′)); 2.77 (*m*, 1 H–C(2′)); 3.10–3.44 (*m*, 2 PhC*H*$_2$CH$_2$O, 2 H–C(5′)); 3.72 (*s*, 2 MeO); 4.18 (*m*, H–C(4′)); 4.50–4.84 (*m*, 2 PhCH$_2$C*H*$_2$O, H–C(3′)); 6.48 (*t*, H–C(1′)); 6.75 (*d*, 4 H, $(MeO)_2$*Tr*); 7.10–7.50 (*m*, 13 H, Ph, $(MeO)_2$*Tr*); 8.01 (*s*, H–C(8)); 8.07 (*m*, 4 H, Ph). ^{13}C-NMR ($CDCl_3$): 34.5; 39.9; 54.6; 63.6; 66.3; 66.8; 71.3; 83.6; 85.9; 86.1; 112.6; 117.0; 123.1; 123.6; 126.4; 127.3; 127.6; 128.7; 129.4; 129.5; 135.1; 135.2; 136.1; 139.1; 144.2; 145.4; 145.7; 146.1; 146.2; 148.6; 152.6; 158.0; 160.3; 160.7.

*2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-*O^2,O^6*-bis-[2-(4-nitrophenyl)ethyl]xanthosine 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**8**). A soln. of **12** (6.06 mmol, 5.26 g) in anh. MeCN (140 ml) was placed in an ice bath, and ^{i}Pr$_2$NEt (4 equiv., 24.2 mmol, 3.13 g, 4.23 ml) was added, followed by dropwise addition of 2-cyanoethyl diisopropylphosphoramidochloridite (9.09 mmol, 2.15 g, 2.03 ml, 1.5 equiv.). The reaction was monitored by TLC (silica, AcOEt/$CHCl_3$/Et$_3$N 45:45:10; R_f 0.70) and was complete within 5 min. The mixture was further stirred (10 min). Then aq. $NaHCO_3$ soln. (100 ml) was added, the mixture extracted (CH_2Cl_2), the org. layer dried (Na_2SO_4) and evaporated, and the residue purified by CC (silica gel, AcOEt/CH_2Cl_2/Et$_3$N 70:30:1). The resulting yellow foam was dissolved in CH_2Cl_2 (10 ml; freshly distilled from K_2CO_3) and gradually added dropwise to a vigorously stirred pentane soln. (75 ml). A precipitate was isolated by pouring off the solvent and placed under vacuum to give the two diastereoisomers of **8** (5.46 g, 85%). Pale yellow foam. ^{1}H-NMR ($CDCl_3$): 1.08–1.20 (*m*, 2 *Me*$_2$CH); 2.45, 2.60 (2*t*, CH$_2$C*H*$_2$CN); 2.64–2.84 (*m*, 2 H–C(2′)); 3.18 (*t*, C*H*$_2$CH$_2$CN), 3.24–3.38 (*m*, 2 H–C(5′), PhC*H*$_2$CH$_2$O); 3.73 (*s*, 2 MeO); 3.51–3.84 (*m*, 2 Me$_2$C*H*, PhC*H*$_2$CH$_2$O); 4.24 (*m*, H–C(4′)); 4.53 (*m*, PhCH$_2$CH$_2$O); 4.79 (*m*, H–C(3′), PhCH$_2$CH$_2$O); 6.41 (*t*, H–C(1′)); 6.78 (*m*, 4 H, (Me_2O)*Tr*); 7.13–7.50 (*m*, 13 H, Ph, $(MeO)_2$*Tr*); 7.96, 7.98 (2*s*, H–C(8)); 8.07–8.16 (*m*, 4 H, Ph). ^{13}C-NMR ($CDCl_3$): 20.3; 24.5; 35.1; 39.8; 43.1; 43.3; 55.2; 58.0; 58.1; 58.2; 58.4; 63.3; 63.5; 66.7; 67.3; 73.5; 73.7; 74.0; 74.1; 74.2; 83.9; 85.4; 85.8; 86.4; 113.1; 117.3; 117.6; 123.7; 126.9; 127.8; 128.0; 129.8; 129.9; 135.5; 139.3; 144.3; 145.7; 146.0; 146.7; 153.1; 158.5; 160.6; 161.2. ^{31}P-NMR ($CDCl_3$): 150.8, 150.9. HR-MS (FAB$^+$): 1069.4281 ($C_{56}H_{62}O_{12}N_8P^+$, *MH*$^+$; calc. 1069.4225).

Oligonucleotide Synthesis. Oligonucleotides were prepared on an *Expedite-8900* synthesizer with reagents from *Glen Research* and 1-μmol columns (*Perseptive Biosystems*). Standard conditions were used for the synthesis, except for an extended coupling time for **8** (3 min), an activator soln. of 1*H*-imidazole-4,5-dicarbonitrile in MeCN (0.25M), and a deblocking soln. of $CHCl_2COOH$ in CH_2Cl_2 (2.5%). Trityl-group release was monitored at 470 nm.

Oligonucleotide Deprotection. Solid support (1-μmol scale) bearing synthetic 'trityl off' oligonucleotide was incubated in conc. aq. NH_4OH soln. for 24–40 h at r.t. The soln. was removed, and H_2O (200 μL) was added to the residue. The resulting suspension was filtered through a 0.2-μm filter. An aliquot (20 μl) was removed to a 1.5-ml microfuge tube and lyophilized to dryness. The residue was dissolved in *N*,*N*,*N*′,*N*′-tetramethylguanidinium 2-nitrobenzaldoximate soln. (50 μl, 0.3M in H_2O/dioxane 1:1) and placed in a heating block (70°, 18 h). The progress was monitored during the deprotection by the high-pH anion-exchange technique detailed above. The reaction mixture was quenched with 0.6M sodium citrate, pH 5.2 (50 μl, made from 0.6M solns. of sodium citrate/citric acid 4:1). AcOEt (100 μl) was added and the org. layer removed and discarded. A small amount of 0.6M sodium citrate, pH 5.2 (2.5 μl) was added, and the soln. was again extracted with AcOEt (100 μl). After placing the soln. under a stream of air for a short time to remove any residual AcOEt, the oligonucleotide soln. was purified and desalted by HPLC.

HPLC Purification and Analysis of Oligonucleotides. Oligonucleotides were purified by anion-exchange HPLC on a *Waters* system consisting of a *600S* controller, a *616* pump, and a *486* tunable absorbance detector with a *Dionex* column (*DNA-Pac PA-100*, 4 × 250 mm). Typical linear gradient: flow rate 0.5 ml/min; solvent *A*, 20 mM $K_xH_yPO_4$ (pH 7.0); solvent *B*, 20 mM $K_xH_yPO_4$, 1M NaCl (pH 7.0); solvent *C*, MeCN; *C* constant at 20%, 80% *A* to 55% *A* (5 min), 55% *A* to 48% *A* (25 min). The oligonucleotide was collected for reversed-phase

purification and desalting on a *Waters-PrepLC* system with a *486* tunable absorbance detector and a *PrepLC* 25-mm module containing a single *PrepPak* cartridge (*Prep NovaPak HR* C_{18}, 6 μm, 60 Å, 25 × 100 mm). A linear binary gradient was used: flow rate 5.1 ml/min; solvent *A*, 25 mM $(Et_3NH)OAc$ (pH 7.0); solvent *B*, MeCN; 100% *A* (3 min), 100% *A* to 70% *A* (27 min).

Characterization of Oligonucleotides. HPLC Analysis of purified oligonucleotides was done with the *Dionex* anion exchange column on the *Waters-616* system described above: flow rate 0.5 ml/min; solvent *A*, 20 mM NaOH; solvent *B*, 20 mM NaOH, 1M NaCl; 90% *A* to 70% (2 min), 70% *A* to 63% *A* (30 min). This peak corresponding to the desired oligonucleotide accounted for > 95% of the integrated A_{260} in each chromatogram. Enzymatic digestion to component nucleosides of a small amount of oligonucleotide (0.1 OD_{260} units) was performed as previously described to verify composition [15]. An *Absorbosphere* C_{18} reversed-phase column (*Alltech Associates*) was used to separate nucleosides with the following linear binary gradient: flow rate 0.5 ml/min; solvent *A*, 25 mM $(Et_3NH)OAc$, pH 7; solvent *B*, MeCN; 100% *A* (3 min), 100% *A* to 87% *A* (25 min), 87% *A* to 20% *A* (20 min). Order of eluting nucleosides: C_d, X_d, $isoG_d$, G_d, T_d, A_d. The relative nucleoside composition was calculated on the basis of the following extinction coefficients ($M^{-1} \cdot cm^{-1}$) at 260 nm: 7300 (C_d), 8400 (X_d), 4600 ($isoG_d$), 11700 (G_d), 8800 (T_d), 15400 (A_d). Oligonucleotides containing X_d were then analyzed by MALDI-TOF-MS. A typical result was seen for oligonucleotide **14**: MALD-TOF-MS: 5855 ($[M+H]^+$; cal., 5854). This technique, however, could not reliably resolve oligonucleotides differing by a single mass unit, as would be present in oligonucleotides in which a variable position contained either X_d or $isoG_d$. Thus, after oligonucleotide **13** was subjected to NH_4OH at high temp. and extended incubation as described above, the HPLC peaks corresponding to the component nucleosides were collected. Electrospray-ionization MS (ESI)-MS was then used to confirm the identities of the components assigned as $isoG_d$ (ESI-MS (pos.): 268 ($[M+H]^+$) and X_d (ESI-MS (neg.): 267 ($[M-H]^-$); ESI-MS (pos.): 269 ($[M+H]^+$)).

Duplex Melting. Duplex melting was done with a *Varian-Cary-1-Bio* spectrophotometer equipped with a 6 × 6 cell changer and a *Varian-Cary* temperature controller. These duplex melting experiments mimic those of *Eritja et al.* [1]. An extinction coefficient (ε) at 260 nm was determined for each oligonucleotide as $\varepsilon_{oligo} = [\Sigma \varepsilon_i \cdot n_i(B_i)]/H_{oligo}$, where $\varepsilon_i = \varepsilon$ of nucleoside *i* listed above, $n_i(B_i)$ = number *n* of nucleotides of base *i*. A hyperchromicity factor was determined for each oligonucleotide by the equation $H_{oligo} = A_{260}(85°)/A_{260}(20°)$. A melting-buffer soln. at pH 7.5 was made from 0.05M sodium phosphate/0.15M NaCl. A pH 5.5 melting buffer soln. was made by adjusting the pH 7.5 soln. to pH 5.5 with a soln. of 0.05M citric acid/0.15M NaCl. Each strand was introduced in melting-buffer soln. at 1.6 μM concentration in 1-cm cuvettes, and the absorbance of the sample was monitored at 260 nm. The duplex was heated and cooled for 5 cycles at $1° \cdot min^{-1}$, regulating the temp. from a cuvette with an internal probe. The first cycle was discarded, and the T_m was calculated as the maximum of dA_{260}/dT *vs. T* for the remaining heating and cooling cycles. Results: see *Table*.

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