Synthesis and properties of oligonucleotides containing 2'-deoxynebularine and 2'-deoxyxanthosine

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ABSTRACT

The preparation of synthetic oligonucleotides containing 2'-deoxynebularine (dN) and 2'-deoxyxanthosine (dX) is described. The thermal stabilities of duplexes containing dX, dN, and 2'-deoxyinosine (dI) base-paired with the four natural bases have been measured. Xanthine base pairs have stabilities at pH 5.5 that are similar to those of dI-containing duplexes at neutral pH. When xanthine is paired with adenine or cytosine an unusual stabilization of the duplex structure is observed at acid pH. Incorporation of base mispairs opposite template xanthine sites were measured using Drosophila DNA polymerase α. The relative nucleoside incorporation rates are in the order: T>C>>A>G. These rates do not correlate with relative thermodynamic stabilities of base mispairs with xanthine obtained from Tm measurements: T>G>A>C. We suggest that DNA polymerase misinsertion rates are greatest when the base mispair can be formed in accordance with Watson-Crick as opposed to other base pairing geometries even though other geometries, e.g. wobble, may result in a more stable final DNA product.

INTRODUCTION

Synthetic oligonucleotides probes have proven very useful in the detection of cloned DNA sequences. When a partial protein sequence is available, a mixture of oligonucleotides presenting all possible corresponding DNA sequences can be successfully used as a probe for screening cloned DNA (1). The mixed probe approach may have two principal drawbacks when the complexity of the mixture is very high. First, the oligonucleotide probes must, for reasons of practicality, be synthesized together on the same resin. Thus, the products of the synthesis can never be adequately characterized. Second, since the exact coding sequence is not known, it is difficult to set appropriate "stringent conditions" for the hybridization and subsequent washings.

A universal base—one that could base pair equally well with any of the four natural bases—could resolve these two difficulties. A number of compounds have been tested as possible universal bases, including 1,4-anhydro-2-deoxy-D-ribitol, 1-phenyl-β-1,4-anhydro-2-deoxy-D-ribitol (2), 2'-deoxyinosine (3,4), 3-β-D-2'-deoxyribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine (5) and 1-(2'-deoxy-β-D-ribofuranosyl)benzoimidazole (6). Recently, a probe containing deoxyinosine at the positions of ambiguity was used in the isolation of the cholecystokinin gene from a human genomic library (7).

In this paper we discuss the incorporation of two base analogues into synthetic oligonucleotides: 9-(β-D-2'-deoxyribofuranosyl)purine (2'-deoxynebularine, dN, 1) and 2'-deoxyxanthosine (dX, 2). We examine the thermal stabilities of duplexes containing these analogues base-paired with the four natural bases. We compare these stabilities with those of similar duplexes containing 2'-deoxyinosine (dI, 3) or 2'-deoxyguanosine.

The properties of xanthine-containing DNA duplexes are of added interest because xanthine may be present in DNA as the product of the spontaneous deamination of guanine. Thus far, the only information on xanthine base-pairing has been inferred from the behavior of polyxanthyllic acid in presence of other homopolymers (8-12). Our data show that the least stable xanthine base pair at pH 7.5 is xanthine-cytosine. Thus, the presence of xanthine in DNA could lead to transitions and transversions if DNA polymerases are capable of using xanthine as a template to incorporate deoxynucleoside triphosphates (dNTP) into copied DNA. In this paper, we present data that indicate that a eukaryotic polymerase incorporates dNTP substrates opposite a xanthine template site in vitro.
Table 1: Melting temperatures of purine-containing duplexes. The error in Tm is about ±0.8 °C.

<table>
<thead>
<tr>
<th>SEQUENCE</th>
<th>Tm (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGAGGC</td>
<td>35</td>
</tr>
<tr>
<td>GCCTCCG</td>
<td></td>
</tr>
<tr>
<td>GGCNCCG</td>
<td>29</td>
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<td>19</td>
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<td>GCCGCCG</td>
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<td>CGGNNGGC</td>
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<td>GCCACCG</td>
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<tr>
<td>CGGNNGGC</td>
<td>13</td>
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<tr>
<td>GCCCCCG</td>
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</tr>
<tr>
<td>CGGAGGC</td>
<td>12</td>
</tr>
<tr>
<td>GCCCCCG</td>
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</table>

* 0.15 M NaCl, 0.05 M sodium phosphate pH 7.5

RESULTS

To study the effect of the introduction of 2'-deoxynebularine on the stability of DNA duplexes, we prepared a series of heptanucleotides (I-VI).

I CGGNGGC     III GCCACCG     V GCCGCCG
II CGGAGGC     IV GCCCCCG     VI GCCTCCG

2'-Deoxynebularine (1) was obtained by reductive deamination of 3',5'-O-diacetyl-2'-deoxyadenosine, followed by deacetylation (13). The 5'-hydroxyl group was protected by treatment with 4,4'-dimethoxytrityl chloride in pyridine (14) and the resulting DMT-derivative phosphorylated with cyclohexylammonium 2-chlorophenyl 2-cyanoethyl phosphate (15). The presence of 2'-deoxynebularine in oligonucleotide I was confirmed by enzymatic degradation of an aliquot with snake venom phosphodiesterase and alkaline phosphatase and subsequent HPLC analysis of the digest. The melting temperatures of the purine-containing duplexes are shown in Table 1. Absorbance data were collected at 275 nm where the hyperchromicity associated with the melting process is maximal (16). With the exception of
the duplex containing a thymine-purine base pair, the melting temperatures of the purine-containing duplexes are quite low compared to that of the perfectly-matched duplex. The stability of the thymine-purine duplex could be explained by the formation of a hydrogen bond between the N-3 proton of thymine and the N-1 of the purine. In an analogous manner it might be expected that a hydrogen bond would form between N-1 of purine and the N-1 proton of guanine. The duplex containing a guanine-purine base pair has a lower melting temperature, however, possibly due to steric interference between the guanine 2-amino group and the purine or simply purine-purine interactions.

We examined the effects of xanthine on duplex stability using a series of nonadecanucleotides (A-G). Oligonucleotide B represents a natural sequence from the intron in the actin gene of *Saccharomyces cerevisiae*. Oligonucleotides A, C, D contain single base differences in position 9, and oligonucleotides E, F and G represent the complementary sequence with guanine, hypoxanthine, and xanthine opposite position 9 of the coding strand.

A: 5'GTCTCATGTAATAACATCG 3'  
B: 5'GTCTCATGTAATAACATCG 3'  
C: 5'GTCTCATGTAATAACATCG 3'  
D: 5'GTCTCATGTAATAACATCG 3'  
E: 3'CAGAGTACATGATTTGTAGC 5'  
F: 3'CAGAGTACATATTTGTAGC 5'  
G: 3'CAGAGTACATXATTGTAGC 5'  
H: 5'TGTAAAGCXGATTTGCTG 3'  
I: 3'CTAAACCAGCG 5'  
J: 3'CAGAGTACATGATTTGTAGC 5'

At first, we prepared 2'-deoxyxanthosine by the deamination of dG with nitrous acid (17) but we could not obtain 5'-O-DMT-dX. 2'-deoxyxanthosine is presumably ionized in pyridine (pK xanthosine 5.5, ref. 18-20), and we speculate that the resulting deoxyribosylxanthinate ion either reacted with DMT-Cl to give some unstable complexes or was simply too insoluble to react cleanly. For these reasons, we decided to protect the xanthine moiety with the p-nitrophenylethyl (NPE) protecting group, which was developed for the protection of O-6 of guanine(21). Using dG as our starting material we developed the synthetic scheme shown in Fig. 1, which gives NPE-protected dX in approximately 23% yield.
The key step in our synthetic scheme was the protection of the 2-amino group of the deoxyguanosine diacetate with the DMT group followed by the introduction of the NPE group at O-6 by the Mitsunobu reaction. The DMT group is easily removed in the next step to yield the free amino 6-O-protected dG. This product was deamminated with nitrous acid to produce diacetylated O-6 protected dX. (During the preparation of this manuscript a procedure has been published for the direct protection of the O-6 position of guanine without protection of the amino group (22)). The 5'-hydroxyl of the purified O-6-NPE-dX was protected by treatment with 4,4'-dimethoxytrityl chloride in pyridine. Reaction of the 5'-protected species with chloro-(N,N-diisopropylamino)methoxyphosphine gave the desired phosphoramidite derivative.
Table 2: Melting temperatures of duplexes containing xanthine, hypoxanthine, and guanine. The error in Tm is about ±0.3°C.

<table>
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<tr>
<th>Duplex</th>
<th>Base pair</th>
<th>Tm (°C)</th>
<th>ΔTm Tm (°C)</th>
<th>ΔTm Tm (°C)</th>
<th>pH7.5- pH5.5</th>
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<tr>
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<td>C-G</td>
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<td>0</td>
<td>59.7</td>
<td>0</td>
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<tr>
<td>A-G</td>
<td>A-X</td>
<td>51.9</td>
<td>8.8</td>
<td>55.5</td>
<td>4.2</td>
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<tr>
<td>B-G</td>
<td>C-X</td>
<td>51.8</td>
<td>8.9</td>
<td>52.8</td>
<td>6.9</td>
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<tr>
<td>C-G</td>
<td>G-X</td>
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<td>6.0</td>
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<tr>
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<td>T-X</td>
<td>55.5</td>
<td>5.2</td>
<td>54.9</td>
<td>4.8</td>
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<tr>
<td>A-F</td>
<td>A-I</td>
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<td>4.9</td>
<td>54.9</td>
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<tr>
<td>B-F</td>
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<td>3.0</td>
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<td>3.2</td>
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<tr>
<td>C-F</td>
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<td>53.7</td>
<td>7.0</td>
<td>52.6</td>
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<td>A-E</td>
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<td>C-E</td>
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<td>D-E</td>
<td>T-G</td>
<td>53.3</td>
<td>7.4</td>
<td>53.1</td>
<td>6.6</td>
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</table>

* 0.15 M NaCl, 0.05 M sodium phosphate pH 7.5
$ 0.15 M NaCl, 0.05 M sodium phosphate/citric acid pH 5.5
ΔTm = Tm(C-G) - Tm(base pair)

The NPE group used to protect 2'-deoxyxanthosine can be quantitatively removed by treatment with 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine within 60 min at room temperature (data not shown). During the DBU treatment, some of the linkages between oligonucleotides and the resin are cleaved (DMT-T-resin treated with 0.5 M DBU in pyridine for two hrs at room temperature releases 18% of its total bound DMT-T). Nevertheless, we prefer to deprotect the xanthine while the oligonucleotide is still bound to the resin, because it makes the removal of the DBU much easier. An aliquot of the purified xanthine-containing oligonucleotide was labelled using [Y-32P]ATP and T4 polynucleotide kinase and sequenced by the Maxam-Gilbert method (23). At the position corresponding to xanthine, we observed a very strong A reaction (which is consistent with the finding that dX depurinates spontaneously in unbuffered aqueous solution) and a relatively strong reaction with KMnO4 (24) (which is consistent with the structural similarity of xanthine to thymine) (data not shown). To verify the presence of fully-deprotected 2'-deoxyxanthosine in oligonucleotide G an aliquot was digested with snake venom phosphodiesterase and alkaline phosphatase, and the resulting
Fig. 2. Autoradiogram of a polyacrylamide gel showing base mispairs formed opposite template xanthine by Drosophila DNA Polymerase α. The gel autoradiogram is overexposed so that the faint bands are clearly observed. Individual lanes in the gel are labelled by letters identifying the nucleotide added by polymerase (see Experimental Section for reaction conditions): -, dNTP substrates absent from reaction mix; A, dATP; G, dGTP; C, dCTP; T, dTTP; U, dUTP; AP, dAPTP. Row labels are: P, unextended 32P-labelled single stranded primer molecules; X, primer molecules extended by one nucleotide incorporated opposite template base X; C, primer molecules extended by two nucleotides incorporated opposite template bases X and C. The template strand contains an additional seven nucleotides located 5' to C (see template sequence in text).

The mixture was analysed by HPLC. The chromatograph indicated the presence of dX, but not NPE-dX, in the digest (data not shown).

We have determined the melting temperatures (Tm) at two different pH's of twelve duplexes obtained by combination of oligonucleotides A-D with oligonucleotides E-G (see Table 2). At physiological pH, the most stable base pair in the xanthine series is X-T followed by X-G; X-A and X-C are considerably less stable. At pH 5.5, duplexes containing X-T and X-G mismatches melt about 1° lower than they do at pH 7.5, while those containing X-A and X-C mismatches melt 3.5° and 1° higher than they do at pH 7.5, respectively. Melting temperatures of duplexes containing hypoxanthine and guanine are consistent with previously published data (4,25,26).

Purified DNA Polymerase α from Drosophila melanogaster was used to incorporate dNTP substrates opposite xanthine located at a unique site on a synthetic DNA template strand (oligomer H). Each gel band along row X in Fig. 2 corresponds to the addition of a single nucleotide opposite the template xanthine site. The
relative band intensities in row X are a measure of the number of primer molecules extended by a single nucleotide, and are therefore proportional to the nucleotide insertion rates opposite the template xanthine site. The small differences in electrophoretic mobility for the extended oligonucleotide primers (dodecamers) are expected for short single stranded DNA oligomers differing in composition by a single nucleotide.

The relative incorporation rates of the four common dNTP substrates opposite X by Drosophila Polymerase α are T>C>>A>G (Fig. 2). The presence of a band opposite the template base C (insertion of dGTP, lane G) shows that DNA synthesis is not terminated following nucleotide incorporation at a template X site. The fraction of primer extensions beyond the X site during the 20 min incubation period is about 20%. Based on measurements of the relative band densities within the linear range of the X-ray film (data not shown), we find that compared to the incorporation of T opposite X (T·X base mispair), the rates of forming C·X, A·X, and G·X mispairs by the Drosophila Polymerase are approximately 60%, 17%, and 14% respectively.

We also measured the incorporation rates for the nucleotides dUTP and dAPTP (9-(β-D-2'-deoxyribofuranosyl)-2-aminopurine triphosphate). Since dUTP incorporation occurred no more readily than dTTP incorporation, and since dUTP was present as less than 0.5% of our dCTP preparation (HPLC, data not shown), we conclude that the measured dCTP incorporation is real and not an artifact of dUTP contamination of the substrate. 2-aminopurine, a base analogue of adenine, exhibits base-pairing ambiguities characteristic of both A (27, 28) and G (29). Mispairs formed by the insertion of AP opposite X occur at roughly one-half the rate of those involving A or G opposite X.

Drosophila Polymerase α has no detectible 3'-exonuclease proofreading activity (30). Once a nucleotide has been incorporated opposite X, it cannot be excised. Gel density patterns using Klenow fragment (which does have 3'-exonuclease activity) were similar to those in Fig. 2, except that removal of nucleotides opposite X and extensive degradation of the primer strand was observed following lengthy incubation periods (data not shown).
DISCUSSION

Several authors have suggested that a base analogue must fulfill two requirements to serve as a universal base: it must lack hydrogen-bonding groups and it must be able to stack well in the double helix (2,6). In water, base-stacking is believed to be the major source of DNA duplex stability (31). The formation of hydrogen bonds between bases during DNA naturation is counterbalanced by the loss of hydrogen bonds between the bases and the solvent. In this view, 2'-deoxynebularine would appear to be an example of a nucleoside that meets the two requirements for a universal base mentioned above. However, our data show that the presence of dN in a DNA duplex produces a marked decrease in stability except when paired with dT, in which case it is plausible that a hydrogen bond is formed. Our results are in accord with the idea that base-base hydrogen bonding may be needed to anchor the bases in a geometry that is optimal for base-stacking.

Fig. 3. Postulated base-pairing structures involving xanthine.
2'-Deoxyxanthosine was chosen as a candidate for universal base for two reasons. First, it is a purine and is thus large enough to interact with both purines and pyrimidines across the double helix. Pyrimidines, by contrast, are probably too small to base-pair with other pyrimidines. Second, deoxyxanthosine has the following configuration of hydrogen-bonding groups: acceptor-donor-acceptor (Fig. 3). It was expected that the 6-keto acceptor and the N-1 donor could form pairs of hydrogen bonds with adenine, cytosine, and thymine (in the same manner that has been proposed for hypoxanthine (4)), while the N-1 donor and the 2-keto acceptor could form a pair of hydrogen bonds with guanine. Our data show that xanthine base pairs have stabilities at pH 5.5 that are similar to those of hypoxanthine base pairs at pH 7.5. Duplexes containing X•A and X•C were more stable at low than at high pH, whereas the perfectly matched and the hypoxanthine-containing duplexes were all destabilized by acidification. These effects can be explained by recalling that the pK of deoxyxanthosine is about 5.5, by analogy with the riboside. K. Roy and T. Miles (20) have demonstrated that neutral xanthosine exists in the diketo tautomeric form and that ionization results from proton loss at N-3 and gives a 6-keto-2-enolate anion. If the same pattern is assumed to hold for deoxyxanthosine present in a DNA duplex, then our experimental results can be explained in the following way. The low melting temperatures of the duplexes containing X•A and X•C mismatches may be due to the destabilizing effect of an unsolvated negative charge on the xanthine. Decreasing the pH tends to alleviate this problem, and the melting temperatures of these duplexes rise. It is possible that the destabilization of a negative charge on xanthine is partially compensated when dX is paired with dT or dG because of hydrogen bond formation between the enolate oxygen of dX and H-1 of dG or H-3 of dT (Fig. 3).

Xanthine base pairs are less stable than hypoxanthine base pairs at neutral pH. This makes dI more satisfactory as a universal base. Deoxyguanosine has been utilized opposite dC/dT ambiguities (32). As can be deduced from Table 2, dG is as effective a universal base as dI.
Xanthine can arise in DNA by the spontaneous deamination of guanine. Thus, the base-pairing properties of template X with incoming dNTP substrates during replication of DNA may be biologically important. The occurrence of X•A and X•G mispairs may lead to G•C→T•A and G•C→C•G transversion mutations and the occurrence of X•T mispairs may lead to G•C→A•T transitions. In Fig. 2, we see that the rates of formation for various base mispairs with X by DNA Polymerase α do not correlate directly with the thermal stabilities of oligonucleotide duplexes containing the mispairs (Table 2). The most stable mispair X•T (Tm 55.5 °C) is also the one synthesized most rapidly by the polymerase. However, the least stable mispair X•C is synthesized at a rate of 60% of that for X•T. The rate of X•C formation is 3-4 times greater than the rates of synthesizing X•G and X•A mispairs and 8 times greater than the rate for forming an X•AP mispair. Evidently, DNA polymerase imposes constraints on substrate-template interactions that can result in the more rapid formation of base mispairs having lower stability in the final product DNA. It is important to note that nucleotide misincorporation opposite X is not caused by a mechanism involving looping-out i.e. displacement of X. If displacement of X were occurring, then insertion of dGTP opposite the template C site (see fig. 2) should occur preferentially. In fact, the data show that dGTP is inserted with the lowest efficiency among the four common dNTP substrates.

Transition mismatches involving incorporation of a pyrimidine opposite X are favored over transversion mismatches which require that polymerase insert the larger purine nucleotide opposite X. The favored X•T and X•C mispairs can occur in Watson-Crick geometry (Fig. 3). DNA polymerase exerts significant discrimination against the insertion of either G or A opposite X (Fig. 2) even though X•G and X•A mispairs can be coupled by two hydrogen bonds and are observed to form a more stable duplex DNA structure (Table 2). We have recently found a similar result for base mispairs involving the mutagen 2-aminopurine. Here, the thermodynamically more stable AP•A wobble base pair (16,33) is incorporated into DNA at a 50-100-fold
lower rate than the less stable AP•C mispair (29) which we have recently shown to have a protonated Watson-Crick configuration (34).

EXPERIMENTAL SECTION
Abbreviations not previously defined are as follows: Bz: benzoyl, DCM: dichloromethane, DMSO: dimethylsulphoxide, DMT: dimethoxytrityl, EtOH: ethanol, ibu: isobutyryl, and MeOH: methanol.

5'-O-DMT-N-protected(A\textsubscript{Bz}, C\textsubscript{Bz}, G\textsubscript{ibu}, T) deoxyribonucleoside-3'\textendashO-2-chlorophenylphosphates and 3'-O-(N,N-diisopropylamino)methoxy phosphoramidites were purchased from Bachem and American Bionetics respectively. 2'-deoxynebularine was prepared following the procedure described by Nair and Chamberlain (13). Oligonucleotide syntheses were done in a Bachem manual synthesizer and in an automatic Microsyn 1460 synthesizer (Systec). Deoxyribonucleoside triphosphates were purchased from P-L Biochemicals. dAPTP was synthesized as described previously (35). Melting temperatures were measured in a Perkin-Elmer Lambda 3 B spectrophotometer equipped with a temperature controller and a Perkin-Elmer 3600 data station. The concentration of each strand was 0.40 ± 0.02 O.D. units at 260 nm (approximately 16 μg/mL). Melting temperatures were estimated by taking the second derivative of the melting curve and locating the point of inflection where the second derivative changes sign (16).

5'-O-(4,4'-dimethoxytrityl)-2'-deoxynebularine.

2'-deoxynebularine (1.9 g, 8.3 mmol) and 4,4'-dimethoxytrityl chloride (2.8 g, 8.3 mmol) were added to a 50 mL round-bottom flask containing a magnetic stirring bar. Pyridine (15 mL) was added, with stirring. A solution formed, from which a precipitate soon appeared. After 1 hr, a small sample of the reaction mixture was removed and partitioned between dichloromethane (0.5 mL) and water (0.5 mL). TLC of the organic layer (90:10 DCM/EtOH) showed one major spot (Rf 0.4). The reaction was concentrated in vacuo to an oil, which was then coevaporated twice with toluene. The residue was taken up in dichloromethane and the resulting suspension was washed with...
water, saturated sodium bicarbonate (2x) and water again. The organic solution was treated with anhydrous sodium sulphate and evaporated to dryness in vacuo, and the oily product was loaded onto a silica gel column (1.5x30 cm). The column was washed through with DCM (250 mL), and the product was then eluted with 94:6 DCM/MeOH. Column fractions were analyzed by TLC, and those containing the product were combined and evaporated to a foam. Yield 3.1 g (5.7 mmol, 70%).

^1^H-NMR (DMSO-d_6, 90 MHz) δ (ppm): 9.0 (1H, s) H-6; 8.7 (1H, s) H-2; 8.6 (1H, s) H-8; 6.3-7.3 (13H, m) aromatic; 6.4 (1H, t) H-1'; 4.4 (1H, m) H-3'; 3.9 (1H, m) H-4'; 3.7 (6H, s) OCH; 3.6 (2H, m) H-5'; 2.4-3.0 (2H, m) H-2'.

5'-O-(4,4'-dimethoxytrityl)-2'-deoxynebularine 2-chlorophenyl cyanoethyl phosphate.

5'-O-(4,4'-dimethoxytrityl)-2'-deoxynebularine (0.32 g, 0.6 mmol) was phosphorylated with cyclohexylammonium 2-chlorophenyl 2-cyanoethyl phosphate (0.44 g, 1.2 mmol), 2,4,6-trisopropylbenzenesulfonyl chloride (0.73 g, 2.4 mmol) and 1-methylimidazole (0.39 g, 0.48 mmol) following the procedure described by K. Yamada and R. Dohmori (15). Yield 0.44 g (0.56 mmol, 94%).

6-O-[2-(4-nitrophenyl)ethyl]-3',5'-di-O-acetyl-2'-deoxyguanosine.

N-(4,4'-dimethoxytrityl)-3',5'-di-O-acetyl-2'-deoxyguanosine (obtained from dG as described in refs. 14,36) (3.5 g, 5.35 mmol), coevaporated twice with 1,4-dioxane, was reacted in 27 mL of that solvent with triphenylphosphine (4.91 g, 18.7 mmol), diethylazodicarboxylate (3.26 mL, 18.7 mmol) and 2-(4-nitrophenyl)ethanol (3.13 g, 18.7 mmol) under a dry nitrogen atmosphere. There was strong evolution of heat. After 1 hr., TLC (98.5:1.5 DCM/MeOH) showed only one spot that was positive for DMT (Rf=0.38), which was taken to be the product. The reaction was stopped with water, and the mixture was evaporated to dryness. The mass spectrum indicated the presence of the product (M+1=803). The crude was loaded onto a silica gel column, which was eluted with DCM, but the product could not be recovered completely free of impurities. For this reason, further purification was deferred until after the detritylation. All column fractions containing the desired product were pooled and evaporated to dryness. The residue

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was dissolved in 9:1 DCM/MeOH (50 mL) and kept on ice. A cold solution of benzenesulfonylic acid (2 g) in DCM/MeOH 9:1 (50 mL) was added. After 10 min, the flask was removed to a dry ice bath. TLC (98.5:1.5 DCM/MeOH) then confirmed that detritylation was complete. The reaction mixture was neutralized with saturated sodium bicarbonate (100 mL). The aqueous layer was washed with DCM (75 mL), and the organic layers were combined and washed with saturated sodium bicarbonate (2 x 75 mL) and then water (2 x 75 mL). The organic solution was dried over magnesium sulphate and concentrated to an oil in vacuo. The desired product was recovered by silica gel chromatography, employing a one-step gradient: 1) 99.5:0.5 DCM/pyridine; 2) 97.5:2.0:0.5 DCM/MeOH/pyridine. The product was a yellow powder, 1.8 g (67%). TLC (95:5 DCM/MeOH): Rf = 0.59. UV (MeOH): max. 251, 279 nm. \(^1\)H-NMR (270 MHz, CDC\(_3\), 60 °C): \(\delta\) (ppm) 8.14 (d, 2H) aromatic, 7.73 (s, 1H) H-8, 7.46 (d, 2H) aromatic, 6.25 (t, 1H) H-1', 5.44 (m, 1H) H-3', 4.85 (s, 2H) 2-amino, 4.75 (t, 2H) 1-methylene, 4.45 (m, 1H) H-4', 4.30 (m, 2H) H-5', 3.26 (t, 2H) 2-methylene, 2.98 (m, 1H) H-2', 2.51 (m, 1H) H-2', 2.11 (s, 3H) 3'-acetyl, 2.06 (s, 3H) 5'-acetyl. \(^1\)C-NMR (CDC\(_3\)) \(\delta\) (ppm): 170.6 3'-acetyl carbonyl, 170.3 5'-acetyl carbonyl, 161.0 C-6, 159.2 C-2, 153.6 C-4, 146.9 aromatic, 146.0 aromatic, 137.7 C-8, 129.9 aromatic, 123.7 aromatic, 116.1 C-5, 84.5 C-4', 82.3 C-1', 74.6 C-3', 66.2 1-methylene, 63.8 C-5', 36.7 2-methylene, 35.2 C-2', 21.0 3'-acetyl methyl, 20.8 5'-acetyl methyl.

6-O-[2-(4-nitrophenyl)ethyl]-3',5'-di-O-acetyl-2'-deoxyxanthosine.

6-O-[2-(4-nitrophenyl)ethyl]-3',5'-di-O-acetyl-2'-deoxyguanosine (1.7 g, 3.4 mmol) was suspended in hot acetone (7 mL) and poured into a solution of sodium nitrite (5 g) in water (15 mL). Glacial acetic acid (7.5 mL) was added, and the mixture was agitated vigorously for 2 hrs, until the formation of nitrogen had ceased. TLC (95:5 DCM/MeOH) showed the desired product (Rf 0.36) along with three impurities (Rf's 0.29, 0.53, 0.72). The reaction mixture was neutralized with sodium bicarbonate and extracted with ethyl acetate (3 x 50 mL). The ethyl acetate solution was washed with saturated sodium bicarbonate (2 x 50 mL) and water (50 mL), dried over magnesium sulphate and evaporated to dryness. The residue was loaded onto silica gel, and the pure product was
obtained by using a one-step gradient: 1) 98.5:1.0:0.5 DCM/MeOH/pyridine; 2) 97.5:2.0:0.5 DCM/MeOH/pyridine. The product, a yellow solid, weighed 807 mg (47%). UV (MeOH): max. 246, 270 nm.

$^1$H-NMR (270 MHz, CDCl$_3$) δ (ppm): 8.17(d,2H) aromatic, 7.84(s,1H) H-8, 7.50(d,2H) aromatic, 6.35(t,1H) H-1', 5.36(m,1H) H-3', 4.89(t,2H) 1-methylene, 4.41(m,1H) H-4', 4.36(m,2H) H-5', 3.30(t,2H) 2-methylene, 2.68(m,2H) H-2', 2.14(s,3H) 3'-acetyl, 2.12(s,3H) 5'-acetyl. $^{13}$C-NMR (CDCl$_3$) δ (ppm): 170.6 3'-acetyl carbonyl, 170.2 5'-acetyl carbonyl, 162.0 C-6, 159.0 C-6, 150.7 C-4, 147.0 aromatic, 145.4 aromatic, 137.8 C-8, 130.0 aromatic, 123.8 aromatic, 116.3 C-5, 85.1 C-4', 82.6 C-1', 74.1 C-3', 67.6 1-methylene, 63.6 C-5', 37.9 2-methylene, 35.1 C-2', 20.9 3'-acetyl methyl, 20.8 5'-acetyl methyl.

6-O-[2-(4-nitrophenyl)ethyl]-2'-deoxyxanthosine.

6-O-[2-(4-nitrophenyl)ethyl]-3',5'-di-O-acetyl-2'-deoxyxanthosine (807 mg, 1.61 mmol) was dissolved in methanol (25 mL). 25% aqueous ammonia (25 mL) was added, and the turbid solution that formed was stirred for 6 hrs. TLC (95:5 DCM/MeOH) showed no starting material, and the reaction mixture was dried down in vacuo and then coevaporated several times with xylene to remove the acetamide. The residue was kept overnight in a desiccator under high vacuum. Yielded 660 mg (98%), free of acetyl protons in the $^1$H-NMR spectrum. Homogenous by TLC (90:10 DCM/MeOH), Rf=0.35. UV (MeOH): max 243, 273 nm. $^1$H-NMR (270 MHz, d$_6$-DMSO, 60 °C) δ (ppm): 8.16(s,1H) H-8, 8.14(d,2H) aromatic, 7.62(d,2H) aromatic, 6.25(t,1H) H-1', 4.76(t,2H) 1-methylene, 4.38(m,1H) H-3', 3.86(m,1H) H-4', 3.57(m,2H) H-5', 3.27(t,2H) 2-methylene, 3.17 (broad) hydroxyls, 2.58(m,1H) H-2', 2.26(m,1H) H-2'.

5'-O-(4,4'-dimethoxytrityl)-6-O-[2-(4-nitrophenyl)ethyl]-2'-deoxyxanthosine.

6-O-[2-(4-nitrophenyl)ethyl]-2'-deoxyxanthosine (652 mg, 1.56 mmol) was treated with 4,4'-dimethoxytrityl chloride (582 mg, 1.72 mmol) in anhydrous pyridine (25 mL), under a dry nitrogen atmosphere. After standard work up (see above), the oil was purified on a silica gel column eluted with: 1) 99.5:0.5 DCM/pyridine; 2) 97.5:2.0:0.5 DCM/MeOH/pyridine; 3) 95.5:4.0:0.5
DCM/MeOH/pyridine. The desired product was obtained as a yellow powder, 643 mg (57%). Homogeneous by TLC (90:10 DCM/MeOH) Rf=0.56. UV (MeOH): max. 235, 269 nm.

5'-O-(4,4'-dimethoxytrityl)-6-O-[2-(4-nitrophenyl)ethyl]-2'-deoxyxanthosine-3'-O-(N,N-diisopropylamino)-methoxyphosphate.

5'-O-(4,4'-dimethoxytrityl)-6-O-[2-(4-nitrophenyl)ethyl]-2'-deoxyxanthosine (326 mg, 0.45 mmol) was treated with 0.4 mL of diisopropylethylamine (1.8 mmol) and 0.13 mL of chloro-(N,N-diisopropylamino)methoxyphosphine (0.67 mmol) under a dry nitrogen atmosphere as described in ref. 37. The resulting product was desiccated and stored under nitrogen at -20 °C. It weighed 410 mg and was used without further purification. Homogenous by TLC (45:45:15 DCM/EtOAc/triethylamine, Rf 0.8).

Oligonucleotide synthesis.

All the oligonucleotides were synthesized using standard solid-phase procedures on a 1-2 μmol scale. The synthesis of the oligonucleotide containing purine (I) was carried out in a manual synthesizer using phosphotriester methodology (37) and poly(styrene-co-1% divinylbenzene) as a solid support. The rest of the oligonucleotides were synthesized on controlled-pore glass by successive additions of the appropriate N-diisopropyl 2-cyanoethoxy nucleoside phosphoramidites in an automatic synthesizer. The DMT-oligonucleotidyl-resins were deprotected (38,39) and the resulting DMT-oligonucleotides purified by reversed-phase chromatography, treated with 80% acetic acid and rechromatographed (38). The purity of the products was confirmed by gel electrophoresis (20% acrylamide, 7M urea).

The xanthine-containing oligonucleotides (G,H) were deprotected as follows: the resin was treated first with triethylammonium thiophenolate to cleave the phosphate methyl esters (2 hr, r.t.), second with 0.5 M DBU in pyridine (2 hr, r.t.) to remove the NPE group, and third with concentrated ammonia (16 hr, 65 °C) to cleave the oligonucleotide from the resin and remove the rest of the protecting groups. The resulting DMT-oligonucleotides were purified by reversed-phase chromatography as described above.

Enzymatic digestions of oligonucleotides.

The polymers (0.5 O.D. units at 260 nm) were incubated in
50 mM tris-HCl pH 8.0 and 10 mM magnesium chloride with snake venom phosphodiesterase (0.4 μg) and bacterial alkaline phosphatase (0.4 μg) in a total volume of 20 μL at 37 °C for 5 hr. The resulting mixture was diluted and analysed by HPLC. Column: Radial-pak C-18. Flow rate 2 mL/min. Solvent A: 0.01 M ethylenediammonium diacetate pH 7.5. Solvent B: solvent A/acetonitrile (1:1). A 20 min linear gradient from 0% B to 50% B and 5 min isocratic at 50% B. The retention times observed under these conditions were: \( \text{dX} \) 5.5 min, \( \text{dC} \) 7.0 min, \( \text{dG} \) 8.8 min, \( \text{dT} \) 9.3 min, \( \text{dN} \) 10.0 min, \( \text{dA} \) 10.8 min, O-NPE-dX 22.2 min.

**dNTP incorporation rates for the xanthine-containing template.**

Single-stranded primer oligonucleotide (polymer I) was labelled at its 5'-terminus using 5000 Ci/mmol\([\text{V}^{32}\text{P}]\)-ATP (obtained from ICN Radioisotopes Division) and T4 polynucleotide kinase (New England Biolabs). Highly purified Drosophila DNA Polymerase \( \alpha \) was kindly given to us by Dr. I. R. Lehman, Stanford University.

DNA synthesis reactions were run in parallel in the presence of equimolar concentrations (550 μM) of either dTTP, dATP, dCTP, dUTP, or dAPTP. Reaction mixtures (10 μL) containing 50 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, 10 mM MgCl₂, 10 μg bovine serum albumin, 40 mM KCl, 550 μM dNTP, 12 ng annealed DNA primer-template (polymer H + labelled polymer I), and 1 unit of Drosophila DNA Polymerase \( \alpha \), were incubated at 37 °C for 20 min. The reactions were terminated by adding 20 μL of a solution consisting of 20 mM EDTA in formamide followed by heating at 100 °C for 5 min to denature the primer-template DNA. The extended \( \text{32P} \) labelled primer strands were analysed by electrophoresis on a 16% polyacrylamide gel, 41 cm long. The gels were dried in vacuo on Whatman 3MM filter paper. Autoradiograms were made with Kodak GPB X-ray film and band intensities were measured using a Hoefer GS300 densitometer. The relative \( G \cdot X \) rate was computed by summing the band intensities opposite X and G.

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