Inhibition of Klenow Fragment (exo$^-$) Catalyzed DNA Polymerization by (5*R*)-5,6-Dihydro-5-hydroxythymidine and Structural Analogue 5,6-Dihydro-5-methylthymidine†

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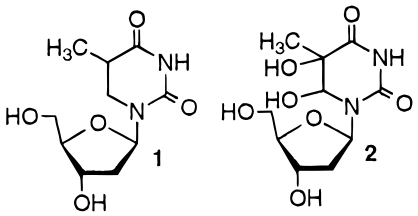
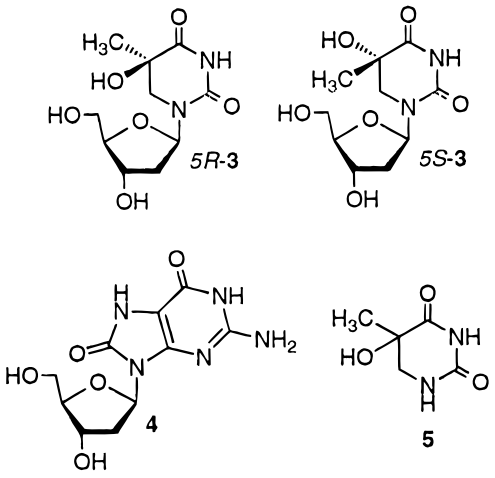
ABSTRACT: Oligonucleotides containing *5R*-5,6-dihydro-5-hydroxythymidine (*5R*-**3**) and structural analogue 5,6-dihydro-5-methylthymidine (**9**) at defined sites were chemically synthesized via a method that obviates the use of NH_4OH. Oligonucleotides prepared by this method were used to examine the effects of *5R*-**3** and **9** on the fidelity of Klenow (exo$^-$) *in vitro*. The presence of lesions *5R*-**3** and **9** in DNA templates was shown to inhibit polymerization of primers hybridized to these templates. Inhibition was observed for both translesional synthesis and extension one nucleotide past the lesion, with the latter being more pronounced. The fidelity of Klenow (exo$^-$) was reduced only slightly when utilizing substrates containing either dihyropyrimidine nucleotide. These results provide the first experimental verification of computational studies carried out on the effects of **3** on DNA templates, and are consistent with a structural model in which the C5-methyl group of *5R*-**3** adopts a pseudoaxial orientation resulting in a disruption in base stacking.

Dihydropyrimidines are formed in nucleic acids as a result of the action of a variety of agents including γ-radiolysis, redox active metal ions, and UVA and UVB photolysis (*1*−*6*). In general, the dihydropyrimidine lesions are substrates for endonuclease repair enzymes, which implies that, if they are left intact, these lesions would be deleterious to cells (*1*, *7*). Despite their excision by repair mechanisms, the finite lifetime of these lesions in genomic DNA requires that their influence on the accuracy of polymerases be addressed. When present during periods of polymerase activity, these nucleotide lesions can give rise to mutations and can be lethal to cells (*8*). Elucidation of the effects of modified nucleotides at the molecular level is facilitated by the preparation of biopolymers containing these lesions. Using this approach, dihydrothymidine (**1**) and thymidine glycol (**2**) have been shown to be weak and strong inhibitors of *in vitro* nucleic acid synthesis, respectively (*9*−*11*). The results of *in vitro* experiments were affirmed by *in vivo* studies in which it was found that dihydrothymidine lesions (**1**) are nonlethal, whereas ≈1 molecule of **2** in single-stranded transfecting DNA is sufficient to inactivate cells (*9*).



Until recently, 5,6-dihydro-5-hydroxythymidine (**3**) had not been chemically synthesized or site specifically incorporated into oligonucleotides (*12*−*14*). Consequently, computational studies have served as the sole source of data concerning the effects of **3** on DNA structure and polymerase enzyme activity (*15*, *16*). These computational experiments suggest novel structural explanations for the origin of polymerase inhibition by **3** and other dihydropyrimidines which have been experimentally tested for the first time in the current work.



In contrast to lesions such as 8-oxodeoxyguanosine (**4**), which is believed to inhibit polymerases during translesional synthesis by presenting an altered hydrogen bonding pattern to the enzyme, the source of enzyme inhibition caused by dihydropyrimidines is believed to be the nonplanarity of the nucleobases (*15*−*18*). *Ab initio* calculations on 5-hydroxy-5,6-dihydrothymine (**5**) indicate that this molecule prefers to adopt a half-chair conformation in which the larger of the two substituents at C5 (the methyl group) is pseudoaxial and the hydrogen bonding components maintain a nearly coplanar arrangement. The X-ray crystal structure of *5S*-**3** confirms the coplanarity of the cyclic hydrogen bonding groups in the dihydropyrimidine ring, but indicates that in

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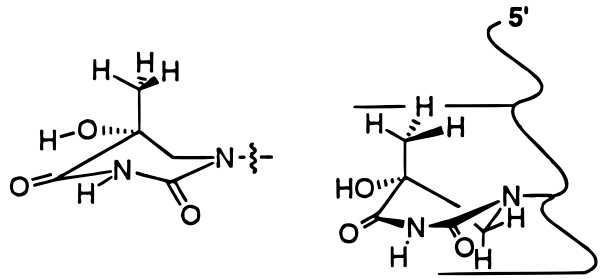
Scheme 1



the solid state the methyl group at C5 is pseudoequatorial in this molecule (*19*). Calculations also suggest that, when placed in duplex DNA, the pseudoaxial methyl group in the *5R* diastereomer of 5,6-dihydro-5-hydroxythymidine (*5R*-**3**) significantly disrupts base stacking via steric interactions with the nucleobase of the nucleotide that is bonded to the 5′-phosphate of *5R*-**3** (Scheme 1) (*15*). Due to the helical twist of duplex DNA, the structural perturbation induced by the respective pseudoaxial methyl group in *5S*-**3** is expected to be significantly smaller than that produced by the epimeric thymidine C5-hydrate. As a consequence of these structural deviations, the presence of *5R*-**3** in DNA templates is predicted to significantly inhibit the extension of primers by polymerases past base pairs containing this lesion. Similar calculations on dodecamers containing **1** and **2** also suggest that a pseudoaxial substituent at C5 is a controlling factor in polymerase inhibition. The lack of polymerase inhibition and cell death induced by **1** is attributed to the adoption of a pseudoequatorial orientation by the methyl group at C5, resulting in minimal perturbation of duplex DNA. In contrast, the methyl group at C5 of either *cis* diastereomer of **2** occupies a pseudoaxial position, which is proposed to be the determining structural factor in the inhibitory effects and cytotoxicity attributed to this lesion. However, while calculations predict that the hydroxyl group at C6 in **2** does not significantly contribute to the effect of this lesion on polymerase activity, the experimental data do not allow one to exclude involvement of this substituent. In addition to being biologically relevant in its own right, 5,6-dihydro-5-hydroxythymidine (**3**) is a suitable candidate to experimentally test the proposed structure-based effects of dihydropyrimidine lesions because it lacks the substituent at C6 in thymidine glycol (**2**).

MATERIALS AND METHODS

General Methods. ^{1}H NMR spectra were recorded at 300 or 270 MHz. IR spectra were obtained using a Perkin-Elmer Series 1600 FT-IR spectrophotometer. Electrospray mass spectra were obtained using a Fisons VG Quattro instrument. Oligonucleotide synthesis was carried out on an ABI 380B oligonucleotide synthesizer. Synthesis and isolation of oligonucleotides containing *5R*-**3** and **9** were carried out as previously described (*13*, *14*, *20*). Photolabile solid phase oligonucleotide synthesis supports and allyloxy protected phosphoramidites were prepared as described previously (*20*, *21*). Long-chain alkyl amine controlled pore glass support (CPG)[1] was purchased from Sigma. All other DNA synthesis reagents were obtained from Glen Research. Oligonucleotides were purified and radioactively labeled using standard procedures (*22*). Enzyme kinetic studies were carried out as previously described (*23*−*25*). Band intensities were quantitated using a Molecular Dynamics Phosphorimager equipped with ImageQuant Software (Version 3.3). T4 polynucleotide kinase was from New England Biolabs. Klenow (exo$^-$) was from USB.

3′,5′-O-Bis(tert-butyldimethylsilyloxy)-5,6-dihydro-5-methylthymidine. (16). To a solution of **15** (300 mg, 0.63 mmol) in THF (3 mL) at −78 °C was added s-BuLi (0.73 M cyclohexane solution, 2.19 mL, 1.6 mmol) over 5 min (*26*). After 1 h, DMPU (240 mg, 1.9 mmol) was added. The solution was warmed to −30 °C and quenched with iodomethane (90 mg, 0.63 mmol). The reaction was warmed to room temperature and stirred for 2 h, at which time it was quenched with saturated NH_4Cl (1 mL). The mixture was poured into ether (50 mL), washed with brine (50 mL), and dried over Na_2SO_4. Column chromatography (EtOAc:CH_2Cl_2; 1:19) gave **16** as a white foam (0.25 g, 81%). ^{1}H NMR ($CDCl_3$) δ 7.48 (bd s, 1H), 6.30 (t, 1H, J = 7 Hz), 4.34−4.30 (m, 1H), 3.79−3.76 (m, 1H), 3.73−3.64 (m, 2H), 3.31 (d, 1H, J = 13 Hz), 3.04 (d, 1H, J = 13 Hz), 1.94−1.90 (m, 2H), 1.21 (s, 3H), 1.18 (s, 3H), 0.88 (s, 9H), 0.86 (s, 9H), 0.05−0.04 (m, 12H). IR (film) 3206, 3085, 2929, 2857, 1704, 1481, 1253, 1222, 1121, 1095, 813, 777 cm^{-1}. Anal. Calcd. for $C_{23}H_{46}N_2O_5Si_2$: C, 56.75; H, 9.52; N, 5.75. Found: C, 56.88; H, 9.47; N, 5.84.

5′-O-Dimethoxytrityl-5,6-dihydro-5-methylthymidine (17). An equimolar mixture (0.5 M) of glacial acetic acid and TBAF (1.2 mL, 0.61 mmol) was added via syringe to **16** (0.05 g, 0.10 mmol) dissolved in THF (0.5 mL). After 36 h, THF (5 mL) and H_2O (1 mL) were added, and the resulting solution was concentrated *in vacuo*. The residue was dissolved in H_2O (50 mL) and extracted with a 1:1 (v:v) mixture of CH_2Cl_2:pyridine (3 × 25 mL). The organic layers were combined and dried over $MgSO_4$. The crude residue was dried from pyridine (2 × 5 mL). 4,4′-Dimethoxytrityl chloride (0.038 g, 0.11 mmol), was added to crude nucleoside in pyridine (1 mL) at 4 °C. After 12 h, additional 4,4′-dimethoxytrityl chloride was added (0.03 g, 0.09 mmol) and the solution was allowed to stir for an additional 24 h. The reaction was quenched by addition of MeOH (1 mL), then poured into $NaHCO_3$ (50 mL), and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and purified by column chromatography (hexanes:EtOAc; 3:2) to yield **17** (0.036 g, 63%) as a white foam. ^{1}H NMR ($CDCl_3$) δ 7.59 (bd s, 1H), 7.35−7.14 (m, 9H), 6.71 (d, 4H, J = 9 Hz), 6.31 (t, 1H, J = 6 Hz), 4.41−4.36 (m, 1H), 3.86−3.81 (m, 1H), 3.72 (s, 6H), 3.29−3.17 (m, 3H), 3.01 (d, 1H, J = 13 Hz), 2.17−1.99 (m, 2H), 1.03 (s, 3H), 1.01 (s, 3H). ^{13}C ($CDCl_3$) δ 175.1, 158.6, 152.1, 144.6, 135.6, 130.0, 128.1, 127.9, 127.0, 113.2, 86.6, 84.3, 83.3, 72.6, 63.6, 55.2, 47.8, 37.6, 37.3, 22.2, 21.9. IR (film) 3217, 3068, 2930, 1694, 1607, 1508, 1485, 1393, 1250, 1177, 1034 cm^{-1}. Exact mass (FAB) calcd. for $C_{32}H_{36}N_2O_7$ (M^+) 560.2523, found 560.2522.

gem-Dimethyl Phosphoramidite (14). 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (33 mg, 0.14 mmol) was added to **17** (0.040 g, 0.072 mmol) and diisopropylethylamine (41 mg, 0.32 mmol) dissolved in CH_2Cl_2 (1 mL) at 0 °C. The reaction was warmed to room temperature, and stirred for 30 min, at which time EtOAc (30 mL) was added. The solution was washed with $NaHCO_3$ (50 mL) brine (50

[1] Abbreviations: CPG, controlled pore glass; Ddtc, sodium *N*,*N*-diethyldithiocarbamate; DMPU, *N*,*N*-dimethylpropylurea; ESMS, electrospray mass spectrometry; GC/MS, gas chromatography/mass spectrometry; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran.

mL) and dried over Na_2SO_4. Column chromatography (EtOAc: CH_2Cl_2; 1:4) gave **14** (0.03 g, 60%) as a white foam. ^{1}H NMR ($CDCl_3$) δ 7.41−7.37 (m, 2H), 7.31−7.20 (m, 8H), 6.82−6.78 (m,4H), 6.37−6.31 (m, 1H), 4.62-4.51 (m, 1H), 4.02−3.96 (m, 1H), 3.78 (s, 6H), 3.67−3.45 (m, 4H), 3.39−3.01 (m, 4H), 2.59 (t, 1H, J = 6 Hz), 2.39 (t, 1H, J = 6 Hz), 2.23−2.15 (m, 2H), 1.16−1.01 (m, 18H). ^{31}P NMR ($CDCl_3$) δ 148.3, 148.1. IR (film) 3211, 3075, 2966, 2932, 1703, 1607, 1509, 1462, 1250, 1179, 1034, 828, 728 cm^{-1}.

Deprotection of Oligonucleotides. In a typical procedure, the CPG support containing the allyloxy protected oligonucleotides (≈3 mg, ≈67 nmol) was added to a solution of $Pd_2(dba)_3$•$CHCl_3$ (8 mg, 7.5 μmol), $P(C_6H_5)_3$ (20 mg, 75 μmol), and 1.2 M n-$C_4H_9NH_2$-HCOOH (1:1) in THF (sparged with N_2 for 30 min) (*21*). After vortexing well, the mixture was heated at 55 °C for 5 h with vortexing approximately every 15−30 min. The CPG support was filtered and washed well with THF. After air drying, the CPG supports were subjected to a second treatment analogous to that described above. After filtering, the CPG supports were washed successively with THF, acetone, 0.1 M Ddtc (2 × 2 mL for 15 min), acetone, and water. The cleaned CPG supports were transferred to a Pyrex tube containing 3 mL of a 9:1 mixture (by volume) of CH_3CN:H_2O. The mixture was sparged with N_2 for ≈20 min and then photolyzed [band pass photolysis (λ_{max} = 400 nm)] for 2 h (with stirring) to cleave the oligonucleotide from the CPG supports (*20*). Following the prescribed workup, the oligonucleotide was purified by denaturing polyacrylamide gel electrophoresis (20% polyacrylamide) and desalted using a reverse phase purification cartridge.

Klenow Fragment (exo$^-$) Kinetic Experiments. Primers labeled at the 5'-end using [γ -^{32}P] dATP and T4 polynucleotide kinase were purified by elution through a Sephadex G-25 spin column. Labeled primers were hybridized to the appropriate template (2 equiv) in a buffer consisting of NaCl (100 mM) and Tris (10 mM, pH 7.0) by heating to 95 °C for 5 min and cooling to room temperature over 1 h. Kinetic experiments were initiated by the addition of 5 μL of a DNA/enzyme solution to 5 μL of a nucleotide triphosphate solution. The DNA/enzyme cocktail typically contained template-primer (50−100 nM), HEPES (0.4 M, pH 6.6), Tris-HCl (0.4 mM, pH 7.0), EDTA (0.02 mM), BSA (0.8 mg/mL), and Klenow fragment (exo$^-$) (0.005−0.5 unit). dNTP solutions included Tris (100 mM, pH 8.0), $MgCl_2$ (10 mM), and β-mercaptoethanol (3 mM). Reaction conditions (time, [dNTP], number of units of enzyme) are listed individually below. The reactions were quenched by the addition of 20 μL of formamide loading buffer containing 20 mM EDTA. Equal amounts (9 μL) of the quenched reactions were loaded into the wells of denaturing gels (20% acrylamide, 19:1 monomer/bis ratio, 45% urea) and run for 5 h at 2000 V using a BRL Model S2 gel electrophoresis apparatus (40 cm long). The gel was then exposed to a phosphorimaging screen, and band intensities were quantified using a phosphorimager. Kinetic parameters were calculated as described in detail elsewhere (*24*, *25*).

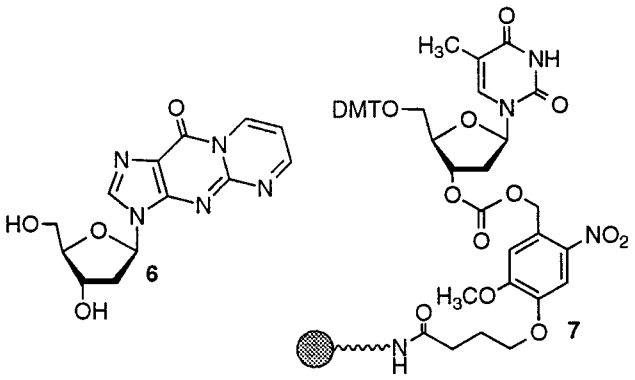
*Reaction Conditions for Kinetic Analysis of Nucleotide Insertion Opposite 5R-***3** *and* **9**. Kinetics of insertion were measured in reactions incubated for control (X = T) [dNTP/time (min)]: A/1.5, C/30, G/9, T/5; translesional (X = *5R*-**3** and **9**): A/2.5, C/90, G/45, T/30. Each set of triphosphate reactions used differing amounts of Klenow fragment (exo$^-$) to achieve approximately 25% maximum extension: (X = T) [dNTP/enzyme (units)] A/0.005, C/0.1, G/0.05, T/0.1; (X = 5*R*-**1**) A/0.005, C/0.5, G/0.25, T/0.5. Triphosphate solutions were 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM for dCTP, dGTP, and TTP insertion reactions (X = T, *5R*-**3**, and **9**). For correct base pair formation (dATP): (X = T) 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 μM; (X = *5R*-**3** and **9**) 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0 μM solutions were used.

*Reaction Conditions for Kinetic Analysis of Extension of a dA•5R-***3**(**9**) *Base Pair.* Kinetics of extension were measured in reactions incubated for: control (X = T) [dNTP/time (min)] A/10, C/5, G/45, T/4; (X = *5R*-**3** and **9**) A/100, C/90, T/20. Each set of triphosphate reactions used differing amounts of Klenow fragment (exo$^-$) to achieve approximately 25% maximum extension: (X = T) [dNTP/enzyme (units)] A/0.1, C/0.05, G/0.25, T/0.005; (X = *5R*-**3** and **9**) A/0.3, C/0.5, T/0.1. Triphosphate solutions were 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM for dCTP, dGTP, and TTP insertion reactions (X = T, *5R*-**1**, and **9**) except for dCTP (X = T), which used 0.1, 0.2, 0.5, 0.75, 1.0, and 2.0 mM. For correct base pair formation (TTP): (X = T, *5R*-**1**, and **9**) 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0 μM solutions were used.

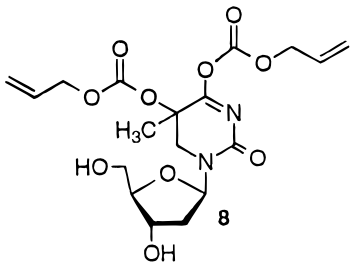
RESULTS

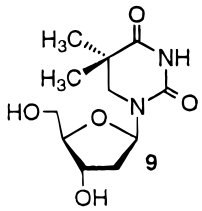
*Synthesis of Oligonucleotides Containing 5R-***3** *and* **9**. Investigation of the effect of alkaline labile nucleosides such as **3** on polymerase enzyme activity required the development of a synthetic method for their site-specific incorporation into oligonucleotides. Oligonucleotides containing nucleoside lesions incorporated at defined sites have been prepared via enzymatic incorporation of the respective nucleotide triphosphate of the lesion (*10*, *27*), postsynthetic modification of chemically synthesized oligonucleotides (*11*, *28*), and *de novo* chemical synthesis of oligonucleotides in which the modified nucleosides are introduced in the form of the appropriately activated monomer (*13*, *14*, *17*, *18*, *23*).

Enzymatic incorporation of nucleotides is limited to those modified nucleotides that are accepted as substrates by polymerase enzymes. Similarly, postsynthetic chemical modification of oligonucleotides is limited to those lesions that can be selectively formed (e.g., **2**) within a biopolymer using reagents that will not react at other sites within the biopolymer (*11*, *28*). This approach is also limited with respect to the sequence of the biopolymer that can be prepared, since only a single modifiable nucleotide can be present in the oligonucleotide during the modification reaction. Although a strategy that allows one to prepare biopolymers of any sequence by ligating oligonucleotides was recently reported, it could be difficult to prepare large amounts of material by this method (*27*).



De novo chemical synthesis of oligonucleotides is the most versatile method for the site-specific incorporation of modified nucleosides into biopolymers. Examples of modified nucleosides that have been incorporated during automated oligonucleotide synthesis include the 8-oxopurine nucleosides (e.g., **4**) and the malondialdehyde adduct of deoxyguanosine (**6**) (*17*, *29*). Incorporation of modified nucleosides into biopolymers by conventional synthesis is often limited to those lesions that are compatible with the strongly alkaline deprotection conditions (e.g., concentrated aqueous ammonia) that are typically employed during this process. Despite a report to the contrary, we found that dihydropyrimidines (e.g., **1**−**3**) are unstable to aminolysis, resulting in complete fragmentation of the dihydropyrimidine ring within 30 min at 55 °C (*13*, *14*, *23*, *30*, *31*).





We recently reported a general strategy for incorporating alkaline-sensitive nucleotides into biopolymers that is compatible with automated chemical oligonucleotide synthesis (*13*, *14*, *20*, *23*, *26*). Deprotection with concentrated aqueous ammonia is obviated by utilizing photolabile solid phase synthesis supports (e.g., **7**) in conjunction with palladium labile phosphate and amino protecting groups (*21*). The oligonucleotides obtained from these solid phase synthesis supports following purification by polyacrylamide gel electrophoresis contain 3′-hydroxyl termini, which enables them to be employed as substrates in enzymatic reactions that require this functionality.

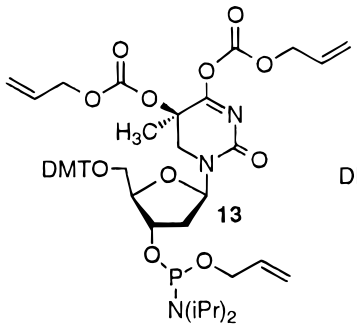
5'- CAT ATG CAT GTA GAC GAT GTG CAT
10

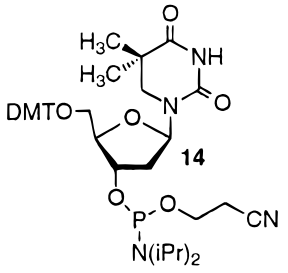
5'-CAT A(*5R*-**3**)G CTT GTT GTC GAT GTG CTT
11

5'-CAT A**9**G CTT GTT GTC GAT GTG CTT
12

Successful incorporation of *5R*-**3** into chemically synthesized oligonucleotides was originally determined via gel electrophoresis and GC/MS (following formic acid digestion and persilylation) (*13*). Furthermore, HPLC analysis revealed that **3** was the sole product formed upon subjection of **8** to the Pd(0) deprotection conditions. However, ESMS analysis revealed that the Pd(0) deprotection did not proceed to completion. In light of these results, the Pd(0) deprotection conditions were optimized on **10** which was prepared using allyloxy-protected phosphoramidites, before carrying out kinetic studies with oligonucleotides containing *5R*-**3** and **9** (*21*). The effectiveness of the Pd(0) deprotection was analyzed using ESMS. Oligonucleotide **10** was subjected to two Pd(0) treatments (4 h, 55 °C), followed by photolytic cleavage as described previously (*20*). ESMS analysis revealed a mixture of molecules consisting of the fully deprotected product [**10**, *m/z* = 7390.0 (observed); *m/z* = 7391.9 (calculated)] and higher molecular weight products (Figure 1). The major impurity corresponds to an oligonucleotide containing a single extraneous allyl group [*m/z* = 7431.0 (observed); *m/z* = 7431.9 (calculated)]. The other impurities present contain between two (*m/z* = 7472.0 (calculated)] and four allyl groups [*m/z* = 7552.1 (calculated)]. Treatment of this mixture of oligonucleotides with concentrated aqueous ammonia at 55 °C for 12 h yields fully deprotected **10** and a small amount of oligonucleotide containing a single allyl group (data not shown). Three cycles of the Pd(0) deprotection process did not result in any further improvement in the homogeneity of **10**. The overall yield of the Pd(0) deprotection was approximated based upon the relative intensities of each molecular ion present. For these purposes, the isolation of oligonucleotides containing one or more allylamine groups (as suggested above) are treated in the same manner as if the respective protecting groups were unreactive. Based upon the presence of 4 palladium labile protecting groups in **10**, we estimate the overall yield of the deprotection reaction to be approximately 97%.

During the synthesis of **11**, *5R*-thymidine C5-hydrate (*5R*-**3**) was incorporated into oligonucleotides as the (bis)-allyloxycarbonyl-protected phosphoramidite (**13**) (*13*, *14*). The *gem*-dimethyl analogue of *5R*-**3** (**9**) was introduced into **12** as the β-cyanoethyl phosphoramidite (**14**).





Phosphoramidite **14** was obtained in a straightforward manner from the (bis)silyl ether of dihydrothymidine (**15**, Scheme 2) (*26*, *32*). Thymidine was incorporated using the commercially available β-cyanoethyl-protected phosphoramidite, whereas the other three naturally occurring nucleosides were introduced as the respective allyloxy phosphoramidites (*21*). The β-cyanoethyl groups were cleaved after Pd(0) treatment using neat diisopropylamine for 48 h at room temperature. Analysis of the ESMS spectra obtained for **11** and **12** indicated approximately the same extent of overall deprotection (≈97%) as determined for **10** (Figure 2).

*Inhibition of Klenow Fragment (*exo$^-$*) by 5R-***3** *and* **9**. Klenow fragment from *Escherichia coli* DNA polymerase I catalyzes the polymerization of DNA in the 5′ to 3′ direction, but lacks the 5′ to 3′ exonuclease activity of the holoenzyme. Klenow fragment (exo$^-$) was specifically chosen as a model DNA polymerase enzyme for these studies because it also lacks the proofreading ability (3′ to 5′ exonuclease activity) of the parent enzyme. Use of Klenow fragment (exo$^-$) enables us to examine the polymerase activity of the enzyme without complications due to additional enzyme activities.

Prior to examining the effects of modified nucleotides on the activity of Klenow fragment (exo$^-$), we needed to determine whether the residual protecting groups present on

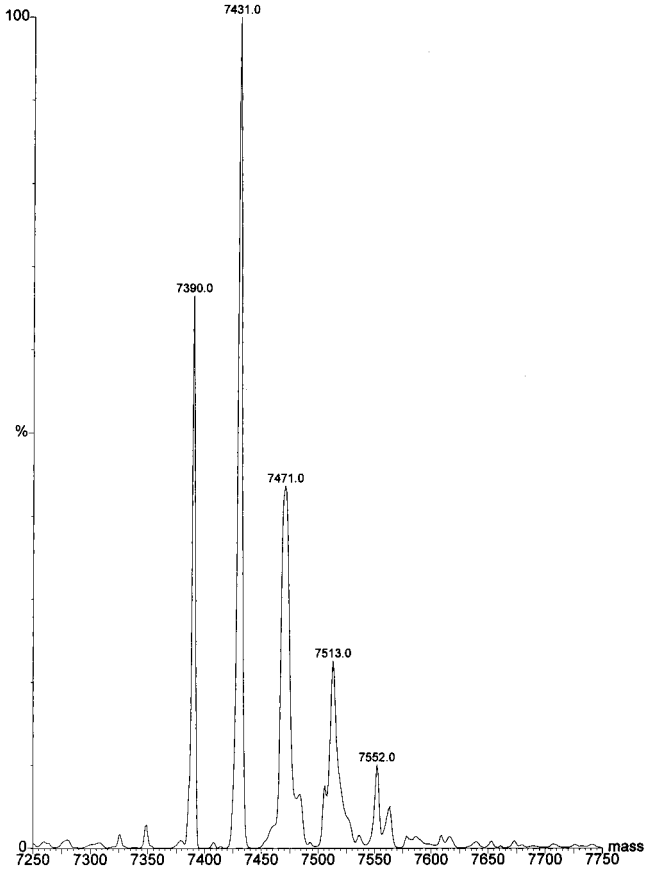


FIGURE 1: ESMS spectrum of **10**. Calculated *m/z* of fully deprotected oligonucleotide: 7391.8.

the Pd(0) deprotected oligonucleotides influenced the enzymatic extension of primers hybridized to these templates. Consequently, we compared the extension kinetics of duplexes using a template (**10**) that was deprotected with Pd(0) versus one of identical sequence that was synthesized using commercially available β-cyanoethyl phosphoramidites and deprotected using standard NH_4OH protocols. In each case, single nucleotide extension of a 19 nucleotide primer that was hybridized to **10** was examined. Incorporation of dATP and misinsertion of dGTP across from the thymidine at position five of **10** were examined (Table 1). The kinetic parameters (V_{max} and K_m) were determined from a series of relative velocity measurements carried out at varying dNTP (substrate) concentrations. The individual velocity measurements were extracted from the absolute intensities of the unextended and singly extended radiolabeled primer (*23*−*25*). The kinetic parameters for individual nucleotide incorporation (dATP and dGTP) were independent (within experimental error) of the method by which the template oligonucleotide was prepared (Table 1).

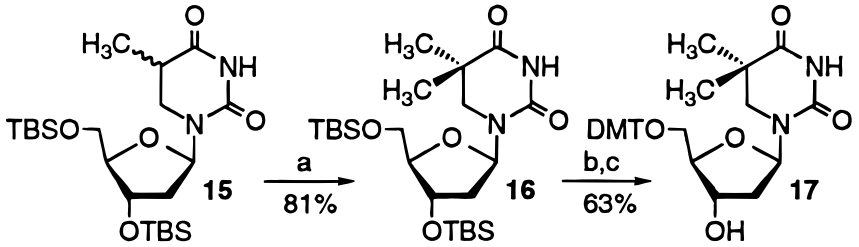
Having verified that the residual protecting groups did not unduly influence the activity of polymerase·DNA complexes, the kinetics of translesional synthesis across from *5R*-**3** and **9** were examined under Michaelis−Menten conditions (Table 2). Both modified nucleotides inhibited the enzyme, with the reduction in V_{max}/K_m ranging between 13 and 49 when compared to the efficiency with which the respective nucleotide triphosphate is incorporated opposite thymidine. The extent of inhibition for incorporation of individual nucleotides opposite *5R*-**3** and **9** was very similar, never differing by more than a factor of 2.5. Furthermore, oligonucleotide·enzyme complexes containing *5R*-**3** and **9** bound the four commonly occurring dNTPs (K_m) with approximately the same affinity as the respective complex containing thymidine. Finally, while *5R*-**3** and **9** inhibit translesional synthesis by Klenow fragment (exo$^-$), the frequency with which the enzyme inserts nucleotides other than deoxyadenosine (misinsertion frequency) across from these lesions is essentially identical to the error rate when thymidine is present in the template.

Klenow (exo$^-$) was inhibited to a significantly greater extent when single nucleotide extension past a *5R*-**3**·dA or **9**·dA base pair was examined (Table 3). Deoxyguanosine incorporation across from the purine nucleotide in the template was too slow to measure in these experiments. However, incorporation of thymidine or deoxycytidine across from deoxyadenosine is inhibited due to the presence of *5R*-**3** in the DNA template (V_{max}/K_m), 80- and 280-fold, respectively. The source of the inhibition is the velocity of the extension reaction, which is inhibited by a factor of 130 and 530 for thymidine and deoxycytidine, respectively. In fact, the K_m of the three nucleotide triphosphates is equal to or stronger when *5R*-**3** replaces thymidine in the template. Despite the significant inhibition of Klenow fragment (exo$^-$) catalyzed extension past *5R*-**3**·dA and **9**·dA base pairs, dATP is the only nucleotide for which the misinsertion frequency is moderately increased relative to the template containing thymidine. Klenow fragment (exo$^-$) is 36 times more likely to insert deoxyadenosine across from deoxyadenosine when the preceding base pair in the template is a *5R*-**3**·dA base pair than when a native T·dA base pair is present. However, it is only about 10 fold more likely to do so when a **9**·dA base pair is present.

DISCUSSION

Oligonucleotides containing alkaline labile nucleotides were synthesized via a general method that avoids the use

Scheme 2



[a]Key: a) sec-BuLi (2.5 eq.), DMPU (3 eq.), THF, -78°C then CH_3I (1.0 eq.)
b) TBAF (0.5 M), AcOH (0.5 M), THF, 25°C c) DMTCl, pyridine, 0°C

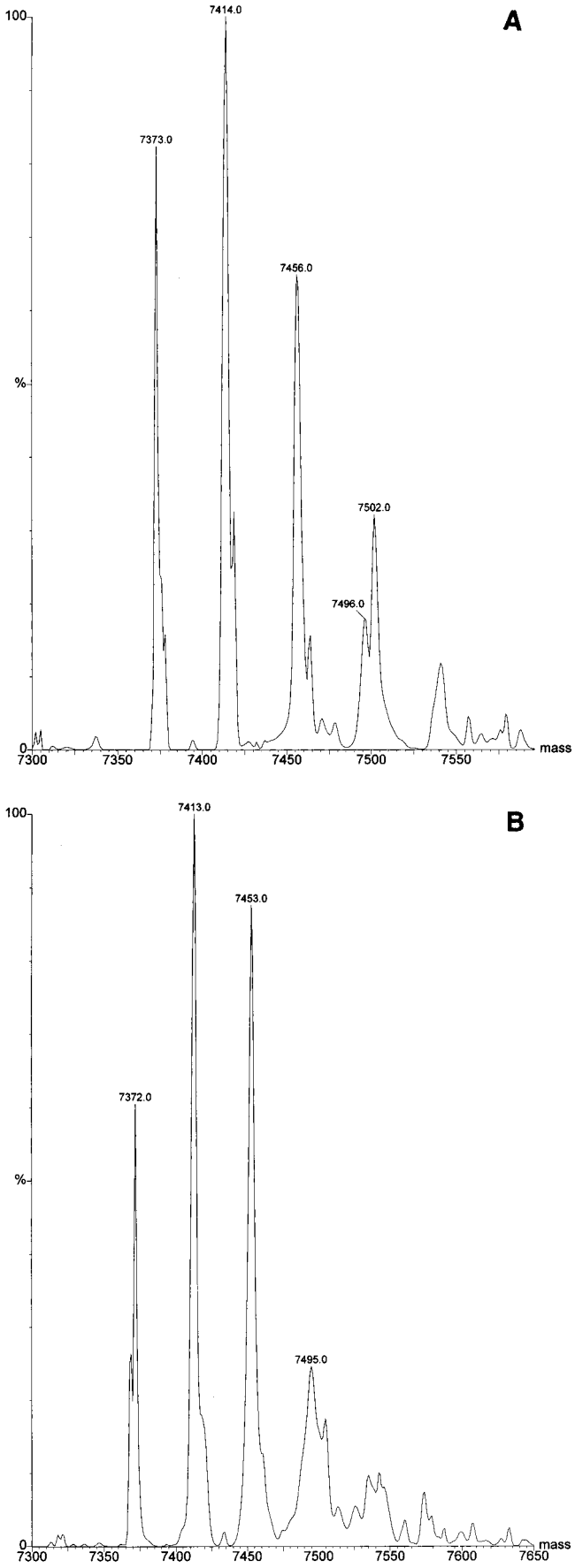


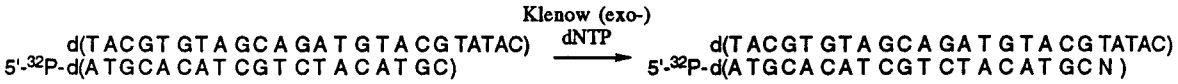
FIGURE 2: ESMS spectra of the following: (a) **11**. Calculated *m/z* of fully deprotected oligonucleotide: 7373.8 (b) **12**. Calculated *m/z* of fully deprotected oligonucleotide: 7371.8.

of concentrated aqueous ammonia. The deprotection reaction proceeds in approximately 97% yield. Due to the large number of allyloxy protecting groups in an oligonucleotide (~40 in a typical 24mer), a significant fraction of oligonucleotide molecules contain remnants of between one and three protecting groups. These side products probably escaped detection previously due to the methods employed for their characterization, and the fact that they are cleaved upon subsequent aminolysis (data not shown) (*13*, *14*, *21*). The allyl groups are believed to be bonded to the exocyclic amines of the nucleobases and not to the phosphate diesters. This proposal is based upon the fact that incomplete phosphate deprotection would affect the migratory aptitude of oligonucleotides to a much greater extent than an allyl group on an amine, as the latter is uncharged under the electrophoresis conditions regardless of whether the exocyclic amines are protected (*33*−*35*). The oligonucleotides synthesized via the method described above give rise to single tight bands upon gel electrophoresis, consistent with the above proposal. We suggest that the allylamine side products are formed via nucleophilic attack of the released nucleobase amines on the π-allyl complex of palladium that is believed to be formed during reactions of this type. This hypothesis is consistent with synthetic methods where Pd(0) is used to form allylamines (*36*, *37*).

The presence of a small percentage (≈3−4%) of protecting groups (or remnants thereof) on each exocyclic amine was expected to destabilize duplexes of oligonucleotides containing these groups. However, such biopolymers are capable of forming Watson−Crick-type base pairs with the appropriate complementary oligonucleotides (*35*). Of more relevance for the experiments carried out herein is the fact that, although a fraction of the oligonucleotide molecules contain a single randomly distributed allylamine substituted nucleotide, control experiments (Table 1) reveal that these chemical impurities do not affect Klenow (exo$^-$) activity. These experiments indicate that any effects on polymerase activity observed using DNA templates containing *5R*-**3** (**11**) and **9** (**12**) can be ascribed to the modified nucleotide.

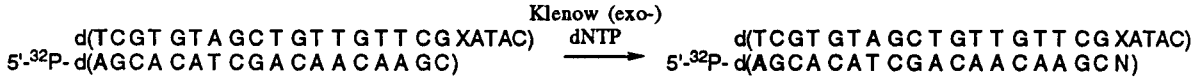
The modest inhibition imparted by *5R*-**3** and **9** during translesional synthesis is consistent with computer calculations and the X-ray structure of the *5S*-**3**, which would lead one to predict that the dihydropyrimidine moieties of the nucleosides adopt conformations in which the functional groups capable of forming hydrogen bonds are positioned in a very similar manner to the respective groups found in thymidine. The measured K_m values during translesional synthesis of DNA templates containing *5R*-**3** and **9** (Table 2) are also consistent with this structural picture of modified nucleosides. The overall small effect of *5R*-**3** and **9** on translesional synthesis by Klenow (exo$^-$) is most evident by the negligible increase (<2 for any given dNTP) of the misinsertion frequency (Table 2). Furthermore, the similarity of inhibition by *5R*-**3** and **9** is consistent with the predictions of computer calculations that the inhibition induced by *5R*-**3** is due to a steric effect on the structure of the DNA duplex and not an alteration of normal hydrogen bonding patterns due to the hydroxyl group at C5 (*15*, *16*). This behavior is in direct contrast to that observed for an enzyme·DNA complex containing the fragmentation product of *5R*-**3** (**18**), which experienced a more favorable K_m for TTP than did the respective complex containing thymidine in the template (23 versus 221 μM) (*23*).

Table 1: Comparison of Kinetic Parameters of Nucleotide Insertion Reactions between Templates Deprotected with NH_4OH and Pd(0)



deprotection method	dNTP	V_{max} (% min^{-1})	K_m (μM)
NH_4OH	A	35.5 ± 3.5	0.58 ± 0.17
NH_4OH	G	0.16 ± 0.004	35.3 ± 8.1
Pd(0)	A	31.5 ± 4.2	0.83 ± 0.3
Pd(0)	G	0.18 ± 0.008	25.2 ± 7.7

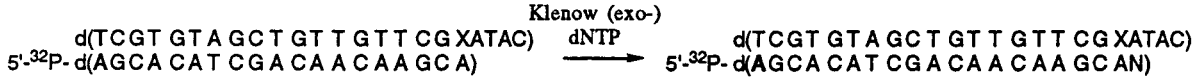
Table 2: Kinetic Parameters of Translesional Nucleotide Insertion Reactions Catalyzed by Klenow Fragment (exo$^-$)



X	dNTP	V_{max} (% min^{-1})	K_m (μM)	V_{max}/K_m	misinsertion frequency (F)[a]
T	A	42.4 ± 2.1	0.28 ± 0.03	151.4 ± 19.8	(1)
T	C	3.6 ± 0.1 (10^{-2})	284.8 ± 42	1.3 ± 0.2 (10^{-4})	8.6 (10^{-7})
T	G	0.28 ± 0.06	37.7 ± 7.1	7.4 ± 2.1 (10^{-3})	4.9 (10^{-5})
T	T	0.31 ± 0.01	211.3 ± 20	1.5 ± 0.2 (10^{-3})	9.9 (10^{-6})
5R-**3**	A	23.4 ± 3.4	3.6 ± 0.7	6.5 ± 1.6	(1)
5R-**3**	C	2.3 ± 0.4 (10^{-3})	225.6 ± 39	1.0 ± 0.2 (10^{-5})	1.5 (10^{-6})
5R-**3**	G	6.3 ± 0.1 (10^{-3})	33.0 ± 7.7	1.9 ± 0.4 (10^{-4})	2.9 (10^{-5})
5R-**3**	T	1.0 ± 0.1 (10^{-2})	231.8 ± 54	4.3 ± 1.0 (10^{-5})	6.6 (10^{-6})
9	A	23.3 ± 4.1	3.4 ± 0.8	6.8 ± 1.9	(1)
9	C	1.3 ± 0.1 (10^{-3})	326.8 ± 42	4.0 ± 0.6 (10^{-6})	5.9 (10^{-7})
9	G	9.4 ± 0.3 (10^{-3})	61.0 ± 3.5	1.5 ± 0.1 (10^{-4})	2.2 (10^{-5})
9	T	9.1 ± 0.2 (10^{-3})	156.0 ± 17	5.8 ± 0.6 (10^{-5})	8.5 (10^{-6})

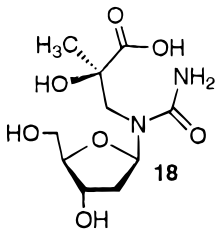
[a] $F = (V_{max}/K_m)dNTP/(V_{max}/K_m)_{dATP}$.

Table 3: Kinetic Parameters of Nucleotide Extension Reactions Catalyzed by Klenow Fragment (exo$^-$)



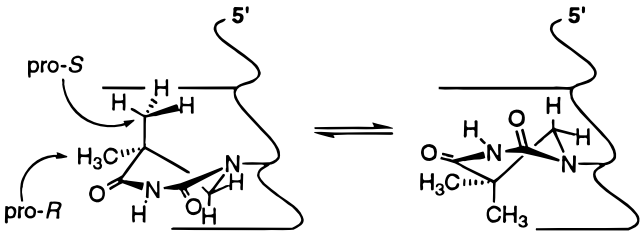
X	dNTP	V_{max} (% min^{-1})	K_m (μM)	V_{max}/K_m	misinsertion frequency (F)[a]
T	T	9.2 ± 2.3	0.8 ± 0.2	11.5 ± 4.1	(1)
T	C	0.35 ± 0.01	143.9 ± 7.9	2.4 ± 0.1 (10^{-3})	2.1 (10^{-4})
T	G	7.6 ± 0.4 (10^{-3})	83.8 ± 17.1	9.1 ± 1.9 (10^{-5})	7.9 (10^{-6})
T	A	0.12 ± 0.01	403.5 ± 35.6	3.0 ± 0.4 (10^{-4})	2.6 (10^{-5})
5R-**3**	T	0.07 ± 0.01	1.7 ± 0.9	4.1 ± 2.2 (10^{-2})	(1)
5R-**3**	C	6.6 ± 0.5 (10^{-4})	22.1 ± 5.6	3.0 ± 0.8 (10^{-5})	7.3 (10^{-4})
5R-**3**	G	nd[b]	nd	nd	nd
5R-**3**	A	1.6 ± 0.1 (10^{-3})	41.9 ± 5.3	3.8 ± 0.5 (10^{-5})	9.3 (10^{-4})
9	T	0.05 ± 0.01	1.9 ± 0.9	2.8 ± 1.5 (10^{-2})	(1)
9	C	2.2 ± 0.1 (10^{-3})	146.7 ± 22.4	1.5 ± 0.2 (10^{-5})	5.4 (10^{-4})
9	G	nd	nd	nd	nd
9	A	1.9 ± 0.2 (10^{-3})	179.4 ± 18.5	1.1 ± 0.3 (10^{-5})	3.9 (10^{-4})

[a] $F = (V_{max}/K_m)_{dNTP}/(V_{max}/K_m)_{TTP}$. [b] nd = not determined.



The observed inhibition of extension past *5R*-**3** and **9** by Klenow (exo$^-$) is also consistent with calculations that predict significantly greater inhibition for this process than for translesional synthesis. Similar predictions were made and qualitatively verified for the effect of thymidine glycol (**2**) on DNA polymerase activity (*11*). The reduction in V_{max} for correct nucleotide insertion was far more significant for extension of templates containing *5R*-**3** and **9** (less than 1% of V_{max} for templates containing thymidine) than for translesional synthesis (~55%). The reduction in V_{max} for incorrect nucleotide insertion in templates containing *5R*-**3** and **9** is also greater for extension than for translesional synthesis, albeit smaller relative to the reduction observed for correct nucleotide insertion. These results are fully consistent with the expected disruption in base stacking due to the axial substituent at C5 in both *5R*-**3** and **9**. For two of the three nucleotides examined (Table 3), the effect on

Scheme 3



V_{max} by *5R*-**3** is more significant than that of **9**. This is consistent with an expected larger difference in energy between the two conformational isomers of *5R*-**3** than between those of **9**. The conformational isomers of **9** are expected to be close in energy, in which a methyl group is pseudoequatorial in each half chair conformation (Scheme 3). In addition, electronic overlap is not an issue due to the absence of the C5-hydroxyl group. Hence, **9** exists more often than *5R*-**3** in a conformation in which a methyl group does disrupt base stacking with the deoxyadenosine bound to the lesion's 5′-phosphate. When the pro-*R* methyl group in **9** is pseudoaxial, one would expect a smaller degree of inhibition for the same reasons that *5S*-**3** is predicted to be a weaker inhibitor than *5R*-**3** (*15*, *16*). The effects of *5R*-**3** and **9** on K_m during extension of templates containing these modified nucleotides by Klenow (exo$^-$) are more difficult to interpret. However, the overall trend is consistent with a smaller perturbation introduced into DNA templates by **9** than by *5R*-**3**. This is reflected in K_m values for dCTP and dATP incorporation that more closely parallel those observed for templates containing thymidine.

Finally, while the combined effects on V_{max} and K_m fortuitously result in a slightly larger overall inhibition (V_{max}/K_m) on Klenow (exo$^-$) by **9** than *5R*-**3**, we believe that the greater structural perturbation introduced by *5R*-**3** as compared to **9** is reflected in the overall misinsertion frequency for extension past the appropriate *5R*-**3**·dA base pair. Both modifications exhibit the largest elevation of misinsertion relative to templates containing thymidine when dATP is substrate. However, *5R*-**3** is more than twice as likely to induce Klenow (exo$^-$) to misinsert dATP than **9** is. A similar trend is observed for dCTP misinsertion, where Klenow (exo$^-$) is 3.4 times more likely to incorporate this deoxynucleotide across from deoxyadenosine when *5R*-**3** is present in the template than when thymidine is present and 2.5 times more likely to incorporate dC when **9** is present.

Conclusions. Using a combination of chemical synthesis and enzyme kinetics, we have provided the first experimental data on the effect of *5R*-5,6-dihydro-5-hydroxythymidine (*5R*-**3**) on polymerase enzyme activity. Experiments on *5R*-**3** and structural analogue **9** support computational studies which predict that lesions such as thymidine C5-hydrate inhibit polymerase enzymes by disrupting base stacking within DNA. These *in vitro* studies reveal that *5R*-**3** moderately inhibits Klenow (exo$^-$) during translesional synthesis but serves as a significant block for extension of primers by the polymerase. However, in contrast to other nucleotide lesions (e.g., **4**), thymidine C5-hydrate does significantly reduce the fidelity of Klenow (exo$^-$) *in vitro*. The kinetic results obtained involving *5R*-**3** and **9** indicate that these molecules:

(1) Present an array of hydrogen bonding groups that are very similar to those present in thymidine.

(2) Inhibit DNA polymerase activity via a disruption in base stacking.

In addition, the synthetic strategy described provides a general method for the synthesis of oligonucleotides containing alkaline labile nucleotides. The oligonucleotides obtained are of sufficient purity to employ in kinetic studies. However, we anticipate that other experiments (i.e., NMR) will require more homogeneous oligonucleotides. Efforts are underway to achieve the small incremental increase in yields of oligonucleotide deprotection (97−>99%) that are needed to make these experiments possible.

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