4642–4648 Nucleic Acids Research, 1995, Vol. 23, No. 22

The effects of the ring fragmentation product of thymidine C5-hydrate on phosphodiesterases and klenow (exo⁻) fragment

Tracy J. Matray, Karin J. Haxton and Marc M. Greenberg*

Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA

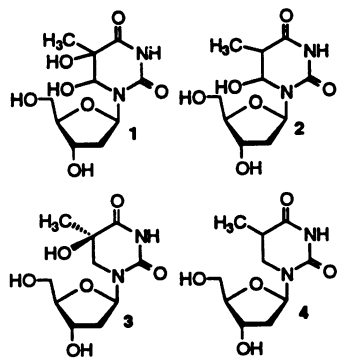
Received August 9 1995; Revised and Accepted October 12, 1995

ABSTRACT

***N*-(2-Deoxy-β-D-erythro-pentofuranosyl)-*N*-3-(*2R*-hydroxyisobutyric acid)urea (α-*R*-hydroxy-β-ureidoisobutyric acid, 8) was site specifically incorporated into a series of oligonucleotides via the ammonolysis of biopolymers containing *5R*-thymidine C5-hydrate (3). α-*R*-hydroxy-β-ureidoisobutyric acid (8) inhibits snake venom phosphodiesterase, λ exonuclease and Klenow (exo⁻) fragment. Kinetic measurements for insertion of nucleotides opposite 8 by Klenow (exo⁻) fragment indicate that this lesion is instructive.**

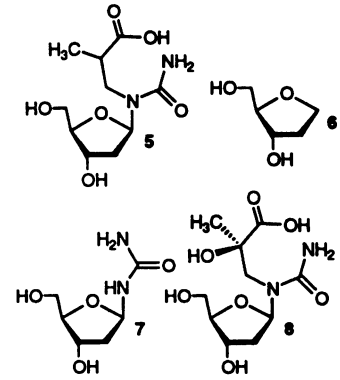
INTRODUCTION

Exposure of pyrimidine nucleosides to γ-radiolysis, as well as radiomimetic agents (e.g. Fe•EDTA and Cu•phenanthroline) and alkylating agents results in the formation of a variety of lesions (1–3). Substantial alterations are imparted upon the pyrimidine moiety by the reactive species (e.g. hydrogen atom, hydroxyl radical) produced by these agents, via their reaction with the double bond. The spectrum of modified nucleosides formed is similar regardless of whether the pyrimidine nucleoside is present as a monomer or in a biopolymer. Although many lesions are repairable, these products have finite lifetimes within cells. Consequently, a variety of lesions can be present during the transfer of genetic information. The presence of modified nucleosides within DNA raises the issue as to how such structural modifications affect the function of enzymes (e.g. polymerases, phosphodiesterases) which routinely act upon nucleic acids.



Scheme 1.

Thymidine glycol (**1**), the regioisomeric hydrates (**2** and **3**) and dihydrothymidine (**4**) are formed under anaerobic conditions, which mimic the hypoxic nature of cancer cells. Thymidine C6-hydrate (**2**) and thymidine C5-hydrate (**3**) are also formed via UV irradiation (4,5). Thymidine glycol exerts a significant block to extension by polymerases, whereas dihydrothymidine does not affect polymerase activity (6–8). Both the glycol and dihydrothymidine inhibit a variety of diesterases, but dihydrothymidine does not strongly inhibit snake venom phosphodiesterase (9). Recently presented evidence demonstrates that thymidine C6-hydrate (**2**) also blocks replication of DNA templates containing this lesion (5). To our knowledge there is no report on the effect of thymidine C5-hydrate (**3**) on polymerase enzymes. However, we recently incorporated **3** site specifically into chemically synthesized oligonucleotides and intend to report shortly on its effects on polymerase enzymes (10). The dihydrothymidine derivatives (**1–4**), having lost their aromatic character are more susceptible to fragmentation via hydrolysis. *N*-(2-Deoxy-β-D-erythro-pentofuranosyl)-*N*-3-(isobutyric acid)urea (β-ureidoisobutyric acid, **5**) is the hydrolysis product of 5,6-dihydrothymidine (**4**). Unlike the cyclic nucleoside (**4**) from which it is derived, **5** strongly inhibits translesional synthesis by polymerase enzymes containing little or no 3′→5′ exonuclease activity *in vitro*. The ureido derivative (**5**) has also been shown to be a lethal lesion *in vivo* (7,8).



Scheme 2.

Fragmentation products such as **5** have been categorized as 'noninstructive lesions'. Other noninstructive lesions include the abasic (**6**) and urea (**7**) damage products (11–13). Extensive kinetic analysis of translesional synthesis across from **6** was carried out

* To whom correspondence should be addressed

using synthetic templates and it was shown that polymerase enzymes prefer to incorporate deoxyadenosine across from **6**. This preference is often referred to as the 'A rule' (12,14). Furthermore, kinetic analysis revealed that selective incorporation of deoxyadenosine across from **6** is largely attributable to a more favorable V_{max} than K_m. To our knowledge, a similar analysis has not been carried out for either **5**, **7** or **8**. *N*-(2-Deoxy-β-D-erythro-pentofuranosyl)-*N*-3-(2*R*-hydroxyisobutyric acid)urea (α-*R*-hydroxy-β-ureidoisobutyric acid, **8**) is the respective fragmentation product formed from 5*R*-thymidine C5-hydrate (**3a**) upon hydrolysis. Herein, we report on the site specific incorporation of **8** into chemically synthesized oligonucleotides and its effect on diesterases and the Klenow (exo$^-$) fragment *in vitro*.

MATERIALS AND METHODS

T4 polynucleotide kinase was from New England Biolabs. *Escherichia coli* DNA polymerase Klenow (exo$^-$) fragment was from United States Biochemical and terminal deoxynucleotidyl transferase was from Gibco BRL. Snake venom phosphodiesterase and λ exonuclease were from Boehringer Mannheim. [γ-^{32}P]ATP (110 TBq/mmol) and [α-^{32}P]ddATP (110 TBq/mmol) were obtained from Amersham. Commercially available DNA synthesis reagents were obtained from Glen Research (Sterling, VA).

Oligonucleotides with or without modification were synthesized on an ABI 380B DNA synthesizer. Oligonucleotides containing only naturally occurring nucleotides were synthesized using standard protocols and commercially available reagents (e.g. β-cyanoethylphosphoramidites). Deprotection was carried out in concentrated NH_4OH at 55°C for 12 h. Oligonucleotides containing α-*R*-hydroxy-β-ureidoisobutyric acid (**8**) were synthesized using allyloxy phosphoramidites (10,15). Synthesis cycles and deprotection procedures employed were as previously described. Crude, deprotected oligonucleotides containing **8** were treated with NH_4OH at 55°C for 3 h. All oligonucleotides were purified on 20% polyacrylamide denaturing gels.

Electrospray mass spectrometry was carried out on **14** using a Fisons VG-Quattro. Samples were prepared for analysis by precipitation from NH_4OAc (1.5 M, pH 5.5) twice. NMR spectra were collected on either a Bruker 270 MHz or Bruker 300 MHz spectrometer. UV-melting temperature measurements were performed on a Beckman Quartz spectrometer equipped with a Gilford 2527 thermo programmer. Measurements were recorded at a heating rate of 0.5 or 1.0°C/min. Oligonucleotides (~0.8 OD) were hybridized by heating to 95°C for 10 min and cooling to room temperature slowly in Tris buffer (10 mM, pH 7.0) and NaCl (100 mM). Liquid scintillation counting was carried out using a Tri-Carb 1500 Liquid Scintillation Counter. Liquid scintillation cocktail used was Ultima Gold XR® by Packard.

N-(2-Deoxy-β-D-erythro-pentofuranosyl)-*N*-3-(2*R*-hydroxyisobutyric acid)urea (α-*R*-hydroxy-β-ureidoisobutyric acid; 8)

Concentrated NH_4OH (50 ml) was added to **3** (42 mg, 0.16 mmol) in a 100 ml reaction tube. The tube was sealed and the mixture was stirred at 55°C for 4 h. The NH_4OH was removed under vacuum and the resulting foam was purified via flash chromatography (MeOH:EtOAc; 3:7), yielding 40 mg (89%) of **8**. ^{1}H NMR (d_6-DMSO) δ6.54 (bd s, 2H), 5.88 (m, 1H), 4.98 (bd s, 1H), 4.0 (m, 1H), 3.59–3.45 (m, 3H), 3.43–3.32 (m, 3H), 3.11 (bd s, 1H), 2.27–2.20 (m, 1H), 1.71–1.65 (m, 1H), 1.09 (s, 3H). The carboxylic acid proton was too broad to be detected. MS (FAB): 279.1 (M + H), 301.1 (M + Na).

Snake venom phosphodiesterase (SVPD) digestion

The substrate (**11a,b**) was labeled at the 5′-end using [γ-^{32}P]ATP and T4 polynucleotide kinase via a standard protocol (16). Snake venom phosphodiesterase digestions as a function of time were carried out at room temperature in 70 µl of solution containing 5′-^{32}P labeled **11**, 0.5 mU of SVPD and reaction buffer (15 µM $MgCl_2$, 100 mM Tris-acetate, pH 8.8). The reaction was initiated by addition of enzyme and after various time intervals (1, 5, 10, 20, 30, 60 min) 10 µl of the reaction mixture was removed and quenched by addition to 20 µl of EDTA (20 mM) in formamide loading buffer. The mixture was immediately vortexed and heated at 95°C for 5 min. SVPD digestions of **11a** were carried out using varying amounts of enzyme (0.03, 0.3, 3, 15 and 30 mU) using the same buffer for 45 min

λ Exonuclease digestion

Oligonucleotide templates (the strand containing **8** or thymidine respectively) used to form duplexes **12a,b** (100 pmol) were phosphorylated using nonradioactive dATP and T4 polynucleotide kinase via a standard protocol (16). Following precipitation, the phosphorylated oligonucleotide was labeled at its 3′-terminus by treating with terminal deoxynucleotidyl transferase (60 U) and [α-^{32}P]ddATP (40 µCi) in a buffered solution containing potassium cacodylate (0.1 M), $CoCl_2$ (2 mM) and DTT (0.1 mM). Oligonucleotides were precipitated and hybridized via standard protocols (16).

The digests were done at room temperature in 70 µl of a solution containing control duplex (**12b**) or the modified duplex (**12b**), 14 U of λ exonuclease and reaction buffer (5 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5). Aliquots (10 µl) of the reaction mixture were removed at various times and quenched as described above.

Kinetics of insertion opposite α-*R*-hydroxy-β-ureidoisobutyric acid (8)

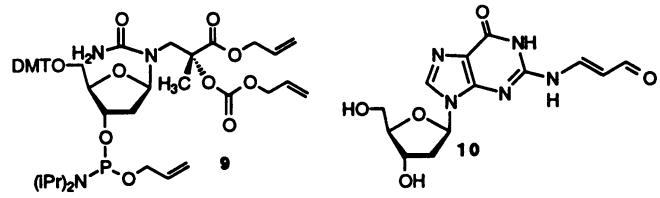
The primer was labeled at the 5′-end using [γ-^{32}P]ATP and T4 polynucleotide kinase via a standard protocol (16). Following precipitation, the labeled oligonucleotides were separated from excess [γ-^{32}P]ATP by elution through a Sephadex G-25 spin column. The requisite duplex (**13a,b**) was formed via hybridizing labeled primer to the respective template (2 equivalents) 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. Enzymatic extension experiments were carried out at 25°C by addition of 5 µl of a cocktail buffer solution containing enzyme•DNA complex, to 5 µl of a solution containing various concentrations of a single dNTP. The enzyme•DNA cocktail typically contained template-primer (5–100 nM) and Klenow (exo$^-$) fragment (0.005–0.5 U) in a buffer containing HEPES (0.4 M, pH 6.6), Tris–HCl (0.4 mM, pH 7.0), EDTA (0.02 mM) and BSA (0.8 mg/ml). dNTP solutions contained Tris (100 mM, pH 8.0), $MgCl_2$ (10 mM) and mercaptoethanol (3 mM). dATP solutions varied in concentration from 0.04 to 2 µM. dCTP, dGTP and TTP cocktails ranged from 0.1 to 4.0 mM. Reaction times (90 s–2.5 h) were adjusted to achieve ~20% primer extension at the highest concentration of dNTP. Reactions were quenched by

addition of 20 µl of formamide loading buffer containing 20 mM EDTA. The quenched reactions (30 µl) were counted via Cerenkov radiation and equal amounts of radiation were loaded into the wells of 0.4 mm thick denaturing gels (20% acrylamide, 19:1 monomer/bis ratio, 45% urea). Gels were run for 5 h at 2000 V and exposed to X-ray film. The autoradiogram obtained was used to locate bands, which were cut out, eluted in scintillation cocktail (5 ml) for 12 h and counted via liquid scintillation counting.

RESULTS AND DISCUSSION

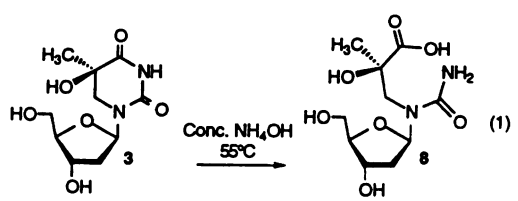
Modified oligonucleotide synthesis and characterization

The structural effects of modified nucleosides on nucleic acids and their effects on the enzymes that act on biopolymers containing these lesions is an important problem. Consequently, several methods have been developed for the site specific incorporation of non-native nucleosides into oligonucleotides. These methods include: post synthetic modification of oligonucleotides (6), enzymatic incorporation of nucleotide triphosphates (17) and *de novo* chemical synthesis (10), wherein the modified nucleoside is incorporated as its respective phosphoramidite derivative. Each of these strategies has its own advantages and disadvantages. We utilize chemical synthesis to site specifically incorporate modified nucleosides into oligonucleotides, by combining the palladium [Pd(0)] labile phosphoramidites developed by Noyori with our own photolabile oligonucleotide synthesis supports (10,15,18).



Scheme 3.

In order to synthesize oligonucleotides containing **8**, we had the option of specifically synthesizing the respective phosphoramidite (**9**) or modifying an oligonucleotide containing **3** post-synthetically. The latter approach was recently reported for the incorporation of malondialdehyde adduct **10** into an oligonucleotide and has also been utilized for the preparation of biopolymers containing **5** (7,19). Based upon our prior success at chemically synthesizing oligonucleotides containing **3** (10), we elected to pursue the latter strategy for the site specific incorporation of **8** into oligonucleotides. The successful implementation of this strategy requires that **3** be cleanly converted to **8**. This was demonstrated first at the monomeric level and subsequently within the biopolymer.



Equation 1.

α-*R*-Hydroxy-β-ureidoisobutyric acid (**8**) was isolated via column chromatography from *5R*-5,6-dihydro-5-hydroxythymidine (**3**), following treatment with concentrated NH_4OH for 4 h at 55°C. Fragmentation product **8** did not cyclize to **3** upon standing in H_2O at room temperature for 48 h. While oligomerization of neat **8** was observed upon standing, it is important to note that oligomerization involves the primary hydroxyl group and is therefore limited to the monomeric species.

5'-CAT AXG CAT GTA GCT GAT
11a X=8
11b X=T

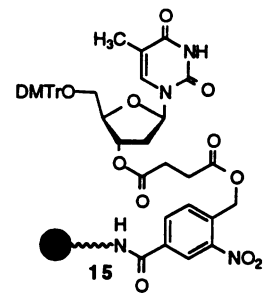
5'-TGA CTG CAT AXG CAT GTA GAC GAT GTG CAT
3'-ACT GAC GTA TAC GTA CAT CTG CTA CAC GTA
12a X=8
12b X=T

5'-CAT AXG CAT GTA GAC GAT GTG CAT
3'- C GTA CAT CTG CTA CAC GTA
13a X=8
13b X=T

5'-CAT A8A CAT GTA GAC GAT GTG CAT
14

Scheme 4.

Facile hydrolysis of **3** was taken advantage of during the synthesis of oligonucleotides containing **8**. *5R*-5,6-Dihydro-5-hydroxythymidine (**3**) was site specifically incorporated into oligonucleotides as described previously (10,18). Oligonucleotides were synthesized on photolabile, solid phase synthesis support **15** (18). Alkaline treatment of the biopolymer (NH_4OH, 55°C, 3 h) prior to gel electrophoresis purification yielded oligonucleotides containing **8**. In order to demonstrate the homogeneity of the biopolymer produced, **14** was characterized by electrospray mass spectrometry. Deconvolution of the variously charged species revealed that the major product consisted of the *m/z* = 7412 expected for **14**. Minor products included oligonucleotides containing a single allyloxy or allyloxy carbonyl group, as well as a small amount of oligonucleotide containing **3**.



Scheme 5.

An oligonucleotide containing **8** was characterized further via the determination of the T_m of a duplex formed by hybridization to the complementary strand which contained a deoxyadenosine across from **8** (**12a**). Duplex **12a** melted at 65°C; whereas the respective duplex containing thymidine in place of **8** (**12b**) melted at 69°C. This is a relatively small depression compared to shorter duplexes that contain noninstructive lesions, such as **6**, **7** and propanediol which induce considerable (12–20°C) depressions of T_m (13,20).

Enzymatic digestion using phosphodiesterases

Snake venom phosphodiesterase digests 3'-hydroxy containing oligonucleotides in the 3'→5' direction, releasing 5'-phosphorylated nucleotides. Subjection of **11b** to 0.5 mU of SVPD for 1 min

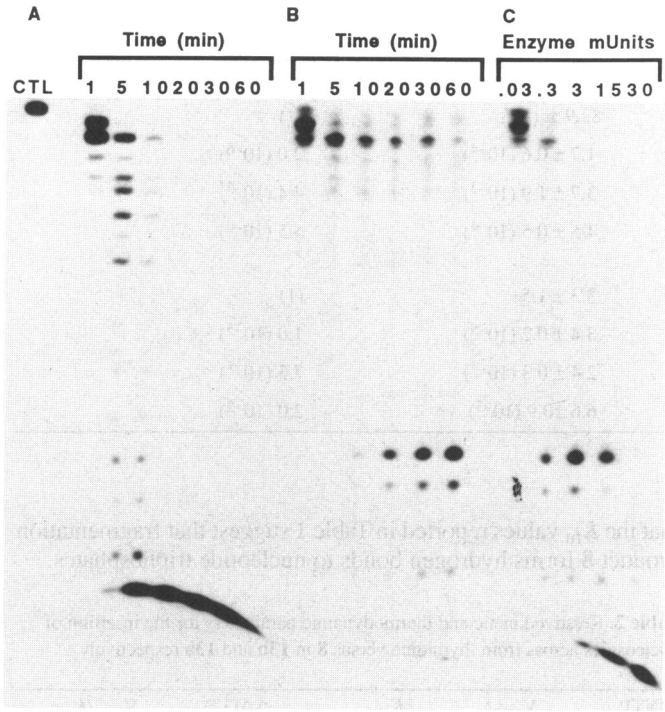


Figure 1. Snake venom phosphodiesterase digestion of **11**. (**A**) **11b**; 0.5 mU SVPD (**B**) **11a**; 0.5 mU SVPD (**C**) **11a**; reaction time, 45 min.

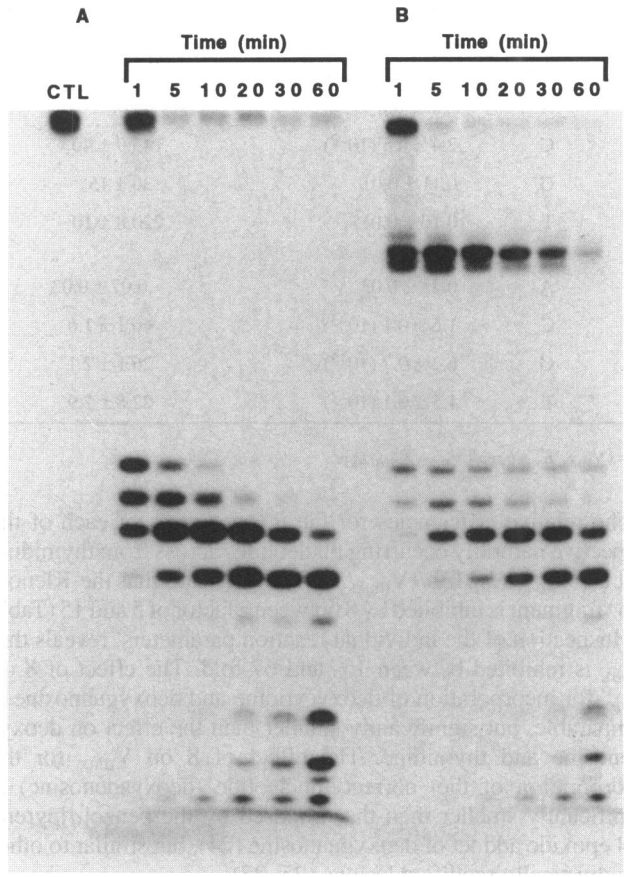


Figure 2. λ Exonuclease digestion of **12**. All reactions contain 14 U enzyme. (**A**) **12b** (**B**) **12a**.

produces a ladder of fragments ranging from undigested **11b** (top) to mononucleotide. Within 5 min, **11b** is fully digested to mononucleotide. Digestion of the radiolabeled oligonucleotide containing **8** (**11a**) by SVPD produced a ladder which was inhibited at two sites (Fig. 1). The position of the bands corresponding to the sites of SVPD inhibition were determined by counting down the ladder from the intact control. This indicates that SVPD was inhibited from hydrolyzing the phosphodiesters bound to both the 3′-hydroxyl and the 5′-hydroxyl of **8** in **11a**. Increasing the amount of enzyme revealed that SVPD could read through the lesion (**8**, Fig. 1c).

λ Exonuclease is functionally distinct from SVPD in that it preferentially hydrolyzes duplex DNA containing 5′-phosphate ends in the 5′→3′ direction (21). However, the two enzymes are affected similarly by the presence of **8** within a biopolymer substrate (Fig. 2). Neither oligonucleotide duplex is digested completely by λ exonuclease. One possible explanation for this is that as the duplex becomes shorter, some of it may dehybridize, preventing further digestion. The presence of **8** in duplex **12a** results in inhibition of hydrolysis of both the 3′ and 5′ phosphate groups of **8**. However, **8** does not terminate the activity of λ exonuclease, because (as was observed for SVPD) increasing the amount of enzyme (up to 20 U) enables λ exonuclease to read through **8** (data not shown).

Inhibition of both exonucleases on either side of lesion **8** is distinct from the effects of benzo[*a*]pyrene diol epoxide modified oligonucleotides, which retard hydrolysis at only one phosphate. The phosphate at which hydrolysis is hindered is dependent upon the stereochemistry of the alkylating agent (22). To our knowledge, similar experiments to those reported herein involving **8** have not been conducted for similar lesions (e.g. **1** and **4**). The effects of **1** and **4** on SVPD were obtained using dinucleotides, for which only a 5′-phosphate was present (9). Therefore, it is not possible to conclude that **8** behaves differently from other lesions that involve dearomatization of the thymine moiety.

Translesional synthesis using klenow (exo⁻) fragment

The kinetics of insertion for individual nucleotides across from **8** (**13a**) were examined using *E.coli* DNA polymerase I [Klenow (exo⁻) fragment]. For comparison, identical experiments were carried out with the exception that thymidine replaced **8** (**13b**). All experiments were carried out using a 'standing start' procedure by hybridizing a 24 nucleotide long template to a 19 nucleotide long primer which was labeled at its 5′-terminus (14). Reaction velocities were determined via measurement of the band intensities by liquid scintillation counting for unextended and extended oligonucleotides (14,23). Experimental conditions [e.g. reaction time, (dNTP), units of enzyme] were adjusted in order to limit extension of the primer to <20%. Qualitatively, inhibition of translesional synthesis by **8** is reflected in the larger quantities of each of the three reaction variables mentioned above, compared to the conditions employed when thymidine was present in the template opposite the insertion site. V_{max} and K_m values were obtained from Hanes–Woolf plots of these data. Sample plots are shown in Figure 3 and values reported in Table 1 are the result of at least three individual kinetic runs.

Table 1. Kinetic parameters of nucleoside insertion reactions catalyzed by Klenow (exo⁻) fragment using **13**

X	dNTP	V_{max} (% min^{-1})	K_m (μM)	V_{max}/K_m	Misinsertion frequency (F)[a]
T	A	11.6 ± 0.8	0.14 ± 0.03	82.9 ± 18.7	(1)
T	C	2.4 ± 0.5 (10^{-2})	143.9 ± 40	1.7 ± 0.6 (10^{-4})	2.0 (10^{-6})
T	G	0.11 ± 0.01	30 ± 15	3.7 ± 1.9 (10^{-3})	4.4 (10^{-5})
T	T	0.10 ± 0.01	220.8 ± 10	4.5 ± 0.5 (10^{-4})	5.5 (10^{-6})
8	A	0.23 ± 0.03	0.07 ± 0.03	3.3 ± 1.5	(1)
8	C	1.6 ± 0.1 (10^{-3})	46.1 ± 1.6	3.4 ± 0.2 (10^{-5})	1.0 (10^{-5})
8	G	6.3 ± 0.7 (10^{-3})	26.1 ± 2.1	2.4 ± 0.3 (10^{-4})	7.3 (10^{-5})
8	T	1.5 ± 0.1 (10^{-3})	22.8 ± 2.9	6.6 ±0.9 (10^{-5})	2.0 (10^{-5})

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

The relative efficiencies for the incorporation of each of the respective naturally occurring nucleotides across from thymidine and **8** [(V_{max}/K_m)**13b**/(V_{max}/K_m)**13a**] indicate that the Klenow exo⁻ fragment is inhibited by **8** between a factor of 5 and 15 (Table 2). Inspection of the individual reaction parameters, reveals that V_{max} is inhibited between 15- and 67-fold. The effect of **8** on V_{max} for incorporation of deoxycytidine and deoxyguanosine is comparable, but significantly smaller than the effect on deoxyadenosine and thymidine. The effect of **8** on V_{max} for the incorporation of the 'correct' nucleotide (deoxyadenosine) is significantly smaller than that imposed by the benzo[*a*]pyrene diol epoxide adduct of deoxyguanosine (24), but similar to other less drastically modified lesions (25–27).

In contrast, (by neglecting differences in k_2) the relative effect of **8** on K_m is <0.7 kcal/mol for the incorporation of deoxyadenosine, deoxycytidine and deoxyguanosine. Simply put, the Klenow (exo⁻)•DNA complex binds the individual dNTPs (excluding TTP) with essentially the same relative affinity, regardless of whether the template contains the native nucleoside thymidine or **8** at the insertion point. Interestingly, thymidine triphosphate is bound more strongly (~1.3 ± 0.1 kcal/mol) by the Klenow (exo⁻)•DNA complex containing **8** (**13a**) than it is by **13b** (Table 2).

Despite the inordinately strong binding of thymidine triphosphate when **8** is present in the Klenow (exo⁻)•DNA complex, the efficiency (V_{max}/K_m) with which the enzyme inserts thymidine opposite **8** compared to incorporation of deoxyadenosine (misinsertion frequency, Table 1) is essentially identical to that measured when thymidine is present in the template. In fact, the misinsertion frequency of Klenow (exo⁻) fragment is essentially identical for each of the respective three nucleotide triphosphates regardless of whether the template contains thymidine or **8**. It is tempting to speculate on the structural reason for the fidelity exhibited by the Klenow (exo⁻) fragment during translesional synthesis across from **8**. Consideration only of the fidelity of the Klenow fragment would lead one to conclude that the observed inhibition is a consequence of the 'A rule' and that **8** represents an example of a noninstructive lesion (12,14). However, such a conclusion is inconsistent with the variation of K_m as a function of triphosphate substrate. Furthermore, even though dATP is preferentially inserted opposite an abasic lesion (6), the enzyme•DNA complex binds this substrate significantly more weakly than does the comparable complex containing thymidine (12). This is not the case for templates containing **8**. We believe that the K_m values reported in Table 1 suggest that fragmentation product **8** forms hydrogen bonds to nucleotide triphosphates.

Table 2. Relative kinetic and thermodynamic parameters for the insertion of nucleosides across from thymidine versus **8** in **13b** and **13a** respectively

dNTP	V_{max}	K_m	$\Delta\Delta G°$[a]	V_{max}/K_m
A	50 ± 7.4	2.0 ± 1.0	0.4 ± 0.3	25.1 ± 13.1
C	15 ± 3.2	3.1 ± 0.9	0.7 ± 0.2	4.9 ± 1.8
G	17 ± 2.4	1.2 ± 0.6	0.1 ± 0.3	15 ± 7.8
T	67 ± 8.1	9.7 ± 1.3	1.3 ± 0.1	6.9 ± 1.2

[a](kcal/mol).

The relatively strong binding of thymidine by **8** is consistent with two dimensional NMR experiments which suggest that the structurally similar urea fragment (**7**) forms hydrogen bonds to thymidine within a DNA duplex (13). Presumably, the substituted urea of **8** can participate in a hydrogen bonding pattern (16) analogous to that suggested for the thymine•**7** pairing. Selectivity in the binding of deoxyadenosine over deoxyguanosine and deoxycytidine by nucleoside fragmentation products is not as well precedented in the literature. Examination of molecular models allows us to speculate as to the structural reason for the maintenance of fidelity for the translesional synthesis across from **8** by Klenow (exo⁻) fragment. The ureido compound can adopt a conformation in which the hydrogen bond donor and acceptors are nearly superimposable in the xy plane to the analogous hydrogen bonding groups in thymidine (17). While the carboxylic acid carbonyl which is analogous to O4 in thymidine is in a different position along the z axis, a slight rotation enables **8** to bind to adenine. Alternatively, a hydrogen bonding pattern analogous to that present in a dA•T base pair can be achieved, but at greater expense to the alignment of the phosphate backbone (18).

CONCLUSIONS

α-*R*-hydroxy-β-ureidoisobutyric acid (**8**) inhibits snake venom and λ phosphodiesterases at both 3′ and 5′ phosphates which serve as a link between **8** and the remainder of the biopolymer. This inhibition pattern is thus far unique to **8**. However, this point should not be overemphasized, because comparable experiments

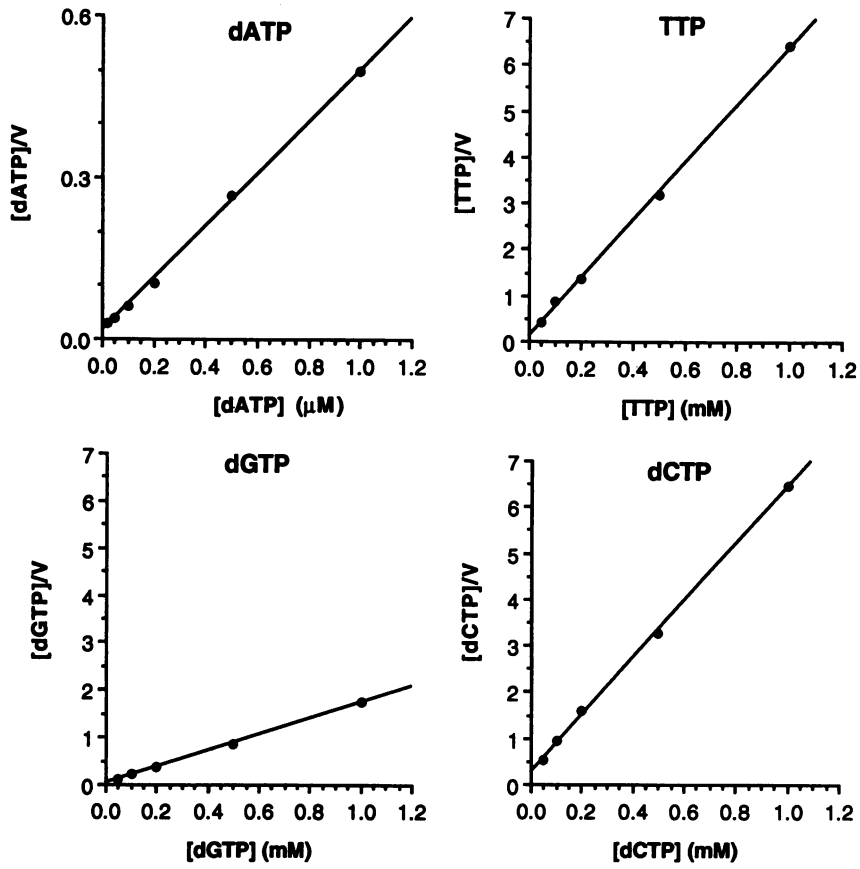
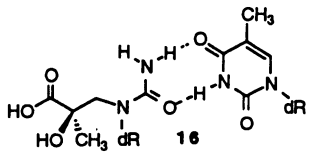
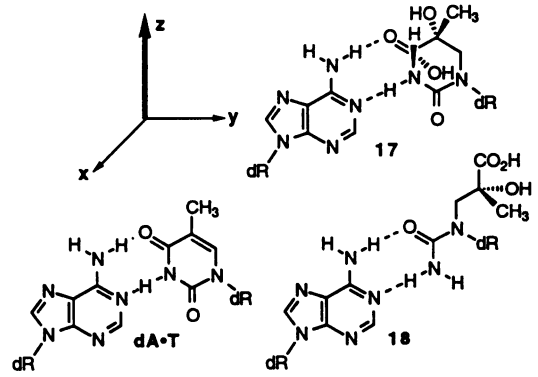


Figure 3. Hanes–Woolf plots of nucleotide triphosphate insertion opposite **8** using Klenow (exo$^-$) fragment.



Scheme 6



Scheme 7

involving other structurally similar lesions have not been undertaken.

Fragmentation product **8** is similar in structure to other lesions which have been categorized as noninstructive lesions (e.g. **5**–**7**). Noninstructive lesions generally inhibit polymerase enzymes; yet, of these other noninstructive lesions, the effects on polymerase enzyme kinetics have only been reported for **6**. α-*R*-hydroxy-β-ureidoisobutyric acid (**8**) inhibits translesional synthesis by Klenow (exo$^-$) fragment, but does not exhibit kinetic parameters that are indicative of a noninstructive lesion. With the exception of the binding of thymidine triphosphate by the Klenow (exo$^-$)•DNA complex, K_m follows a trend that is very similar to that of thymidine. These results, in conjunction with a T_m of a duplex containing **8** suggest that this fragmentation product forms hydrogen bonds. This structural feature, as well as the effect of **8** on base stacking await spectroscopic verification. The structural basis for the ability of templates containing **8** to discriminate between dNTPs is unclear. Nonetheless, our results suggest that other DNA damage products (e.g. **5** and **7**) not previously thought to do so, may function as instructive lesions. These latter issues warrant further investigation.

ACKNOWLEDGEMENTS

We are grateful for partial support of this research by the National Institutes of Health (GM-46534). Mass spectra were obtained on

instruments supported by the National Institutes of Health shared instrumentation grant GM-49631. K.J.H. is an undergraduate research participant.

REFERENCES

1 von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology.* Taylor and Francis, New York.
2 Dizdaroglu, M., Aruoma, O. I. and Halliwell, B. (1990) *Biochemistry*, **29**, 8447–8451.
3 Aruoma, O. I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1989) *J. Biol. Chem.*, **264**, 20 509–20 512.
4 Doetsch, P. W., Zastawny, T. H., Martin, A. M. and Dizdaroglu, M. (1995) *Biochemistry*, **34**, 737–742.
5 Ganguly, T. and Duker, N. J. (1992) *Mutat. Res.*, **293**, 71–77.
6 Clark, J. M. and Beardsley, G. P. (1987) *Biochemistry*, **26**, 5398–5403.
7 Ide, H., Petrullo, L. A., Hatahet, Z. and Wallace, S. S. (1991) *J. Biol. Chem.*, **266**, 1469–1477.
8 Evans, J., Maccabee, M., Hatahet, Z., Courcelle, J., Bockrath, R., Ide, H. and Wallace, S. (1993) *Mutat. Res.*, **299**, 147–156.
9 Weinfeld, M., Soderlind, K.-J. M. and Buchko, G. W. (1993) *Nucleic Acids Res.*, **21**, 621–626.
10 Matray, T. J. and Greenberg, M. M. (1994) *J. Am. Chem. Soc.*, **116**, 6931–6932.
11 Takeshita, M., Chang, C.-N., Johnson, F., Will, S. and Grollman, A. P. (1987) *J. Biol. Chem.*, **262**, 10 171–10 179.
12 Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J. and Goodman, M. F. (1987) *J. Biol. Chem.*, **262**, 6864–6870.
13 Gervais, V., Guy, A., Téoule, R. and Fazakerley, G. V. (1992) *Nucleic Acids Res.*, **20**, 6455–6460.
14 Goodman, M. F., Creighton, S., Bloom, L. B. and Petruska, J. (1993) *Crit. Rev. Biochem. Mol. Biol.*, **28**, 83–126.
15 Hayakawa, Y., Wakabayashi, S., Kato, H. and Noyori, R. (1990) *J. Am. Chem. Soc.*, **112**, 1691–1696.
16 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17 Hatahet, Z., Durmal, A. A. and Wallace, S. S. (1993) *Nucleic Acids Res.*, **21**, 1563.
18 Greenberg, M. M. and Gilmore, J. L. (1994) *J. Org. Chem.*, **59**, 746–753.
19 Reddy, G. R. and Marnett, L. J. (1995) *J. Am. Chem. Soc.*, **117**, 5007–5008.
20 Vesnaver, G., Chang, C.-N., Eisenberg, M., Grollman, A. P. and Breslauer, K. J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3614–3618.
21 Little, J. W. (1967) *J. Biol. Chem.*, **242**, 679–686.Little, J. W. (1967) *J. Biol. Chem.*, **242**, 679–686.
22 Mao, B., Li, B., Amin, S., Cosman, M. and Geacintov, N. E. (1993) *Biochemistry*, **32**, 11 785–11 793.Mao, B., Li, B., Amin, S., Cosman, M. and Geacintov, N. E. (1993) *Biochemistry*, **32**, 11 785–11 793.
23 Boosalis, M. S., Petruska, J. and Goodman, M. F. (1987) *J. Biol. Chem.*, **262**, 14 689–14 696.
24 Shibutani, S., Margulis, L. A., Geacintov, N. E. and Grollman, A. P. (1993) *Biochemistry*, **32**, 7531–7541.
25 Shibutani, S., Takeshita, M. and Grollman, A. P. (1991) *Nature*, **349**, 431–434.
26 Shibutani, S., Bodepudi, V., Johnson, F. and Grollman, A. P. (1993) *Biochemistry*, **32**, 4615–4621.
27 Dosanjh, M. K., Galeros, G., Goodman, M. F. and Singer, B. (1991) *Biochemistry*, **30**, 11 595–11 599.