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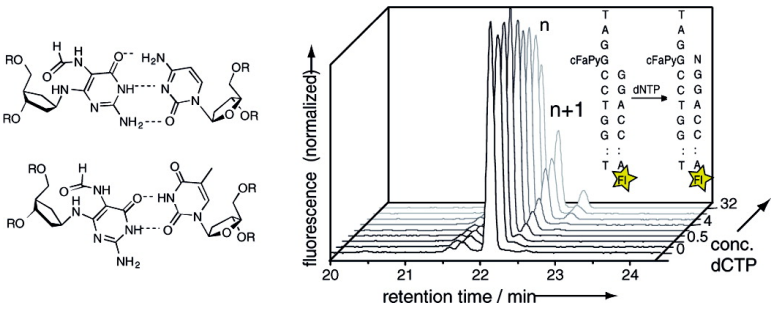

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Base Pairing and Replicative Processing of the Formamidopyrimidine-dG DNA Lesion

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Abstract: The 2,6-diamino-4-hydroxy-5-formamidopyrimidine of 2′-deoxyguanosine (FaPydG) is one of the major DNA lesions found after oxidative stress in cells. To clarify the base pairing and coding potential of this major DNA lesion with the aim to estimate its mutagenic effect, we prepared oligonucleotides containing a cyclopentane based analogue of the DNA lesion (*c*FaPydG). In addition, oligonucleotides containing the cyclopentane analogue of 2′-deoxyguanosine (*c*dG), and oligonucleotides containing 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were synthesized. The thermodynamic stability of duplexes containing these building blocks and all canonical counterbases were determined by concentration dependent melting-point measurements (van't Hoff plots). The data reveal that *c*FaPydG greatly destabilizes a DNA duplex ($\Delta\Delta G^{\circ}_{298K} \approx 2-4$ kcal mol^{-1}). The optimal base pairing partner for the *c*FaPydG lesion is dC. Investigation of duplexes containing dG and *c*dG shows that the effect of substituting the deoxyribose by a cyclopentane moiety is marginal. The data also provide strong evidence that the FaPydG lesion is unable to form a base pair with dA. Our computational studies indicate that the *syn*-conformation required for base pairing with dA is energetically unfavorable. This is in contrast to 8-oxodG for which the *syn*-conformation represents the energetic minimum. Kinetic primer extension studies using *S. cerevisiae* Pol η reveal that *c*FaPydG is replicated in an error-free fashion. dC is inserted 2−3 orders of magnitude more efficiently than dT or dA, showing that FaPydG is a lesion which retains the coding potential of dG. This is also in contrast to 8-oxodG, for which base pairing with dC and dA was established.

Introduction

DNA is constantly damaged by reactive oxygen species (ROS), which are either formed directly in the cell during aerobic respiration or generated after the exposure of cells to certain chemical agents. The resulting base lesions interfere with the replication fidelity and therefore endanger the integrity of the genetic information. Oxidatively generated lesions are one main reason for mutagenesis and are in addition believed to be involved in the aging process.[1−3]

One of the most prevalent DNA lesions formed after the attack of DNA by ROS is the 2′-deoxyguanosine-based ring-opened 2,6-diamino-4-hydroxy-5-formamidopyrimidine DNA lesion (FaPydG).[4] This hydrolysis product of the nucleobase dG is generated in addition to the well studied 8-oxo-7,8-dihydro-2′-deoxyguanosine lesion (8-oxodG) from a common radical precursor[5,6] (Scheme 1a). Both lesions were isolated from DNA of cells exposed to oxidative stress or γ-irradiation, which underpins their biological relevance.[7,8]

For both guanine derived lesions, it is known that the Watson−Crick hydrogen bonding mode with cytosine is largely retained. Both lesions, however, possess in principle the ability to participate in alternative base pairing modes. For 8-oxodG, it is well documented that this lesion forms a stable base pair with dC in the *anti*-conformation. 8-oxodG, however, can rotate around the glycosidic bond to adopt a *syn*-conformation.[9] This *syn*-conformation allows the lesion to form a Hoogsteen base pair with dA as depicted in Scheme 1b. A similar dual base pairing behavior was recently postulated for the FaPydG lesion. Investigations by our group with a FaPydG analogue (*c*FaPydG, see below) and by Ide et al.[10] with a methylated derivative of the FaPydG lesion, however, question the ability of the FaPydG lesion to base pair with dA and claim instead a base pairing potential with dT.

The problem of any study with the FaPydG lesion is the β-FaPydG lesion readily anomerizes to give the α-FaPydG lesion under conditions generally required for DNA synthesis. Whether this anomerization is of biological relevance is unclear. Due to rapid anomerization of the lesion under these chemical synthesis conditions, it has only been possible to incorporate

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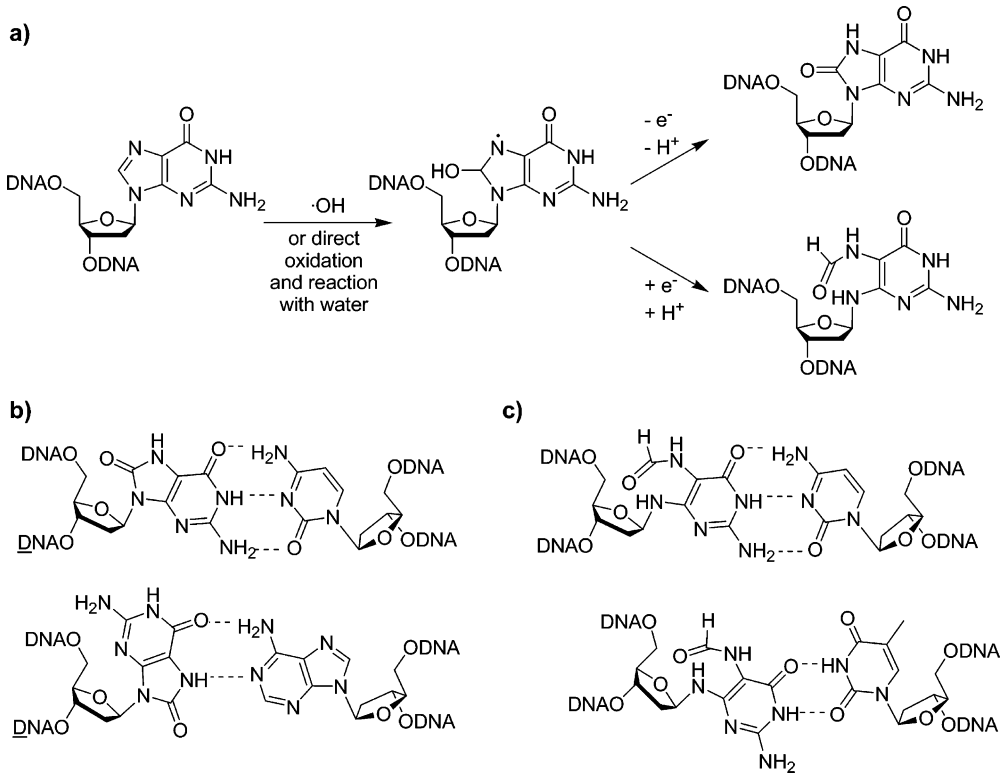
Scheme 1. (a) Formation of the Lesions 8-oxodG and FaPydG, (b) Pairing of *anti*-8-oxodG with dC (Watson−Crick) and *syn*-8-oxodG with dA (Hoogsteen), (c) Proposed Pairing of FaPydG with dC (Watson−Crick) and dT ("Wobble" Base Pair)



an α/β-mixture of the FaPydG lesion into DNA.[11,12] The exact α to β ratio in these synthetic DNA strands is unknown, although a recent detailed enzymatic investigation provided evidence that the β-form may be the prevalent anomer in duplex DNA after annealing.[13] Hydrolysis of the glycosidic bond at elevated temperatures is another problem associated with the FaPydG lesion.

Both other research groups[14] und ourselves[15] have recently reported about the development of nonhydrolyzable and epimerizable β-analogues of the FaPydG lesion. We reported synthesis of a bioisosteric cyclopentane analogue of the FaPydG lesion. This analogue (*c*FaPydG) features a cyclopentane unit instead of the naturally occurring 2-deoxyribose moiety (O to CH_2 modification). This analogue is kinetically stable, which allowed us to obtain cocrystal structures of DNA containing this lesion in complex with the formamidopyrimidine DNA glycosylase repair enzyme[16] (Fpg) and with a high fidelity DNA polymerase.[17]

In this study, we present a detailed analysis of the base pairing and coding potential of β-FaPydG employing the cyclopentane *c*FaPydG analogue using melting point and primer extension experiments. A comparative study of dG and its cyclopentane analogue *c*dG was undertaken in order to estimate the effect of the O to CH_2 "chemical mutation". The conclusions of this study were complemented by computational investigations.

Results and Discussion

Synthesis: For the synthesis of the FaPydG analogue *c*FaPydG (Scheme 2), we started with the enantiomerically pure cyclopentylamine **1**, which was prepared in six steps from (1*R*,4*S*)-2-azabicyclo[2.2.1]hept-5-ene-3-one using the synthetic route recently communicated by Cullis and co-workers.[18] The pyrimidinone **2** was prepared from 4,6-dichloro-2-pyrimidinylamine as published.[19,20] The critical coupling step between these two compounds was accomplished using dimethylformamide as solvent at 70 °C under strictly anhydrous conditions in order to avoid hydrolysis of the base labile acetyl protecting group. The configuration of compound **3** was proven by X-ray crystallography. Crystallization was achieved by vapor diffusion of water into a saturated solution of **3** in water/DMF 1:1 over several weeks (Figure 1).

Protection of the hydroxyl groups with TBDMS−Cl yielded compound **4**. Reduction of the nitro group furnished the highly air-sensitive amine **5**, which was subsequently handled under inert gas. Formylation was possible with EDC and formic acid with fair yields. The subsequent deprotection of the TBDMS groups proved to be difficult due to parallel cleavage of the acetyl protecting group under basic fluoride deprotection conditions such as TBAF in THF. The formamide group on the other hand was labile under most acidic deprotection conditions. The cleavage reaction was finally possible with pyridine buffered pyridine−HF complex in ethyl acetate.

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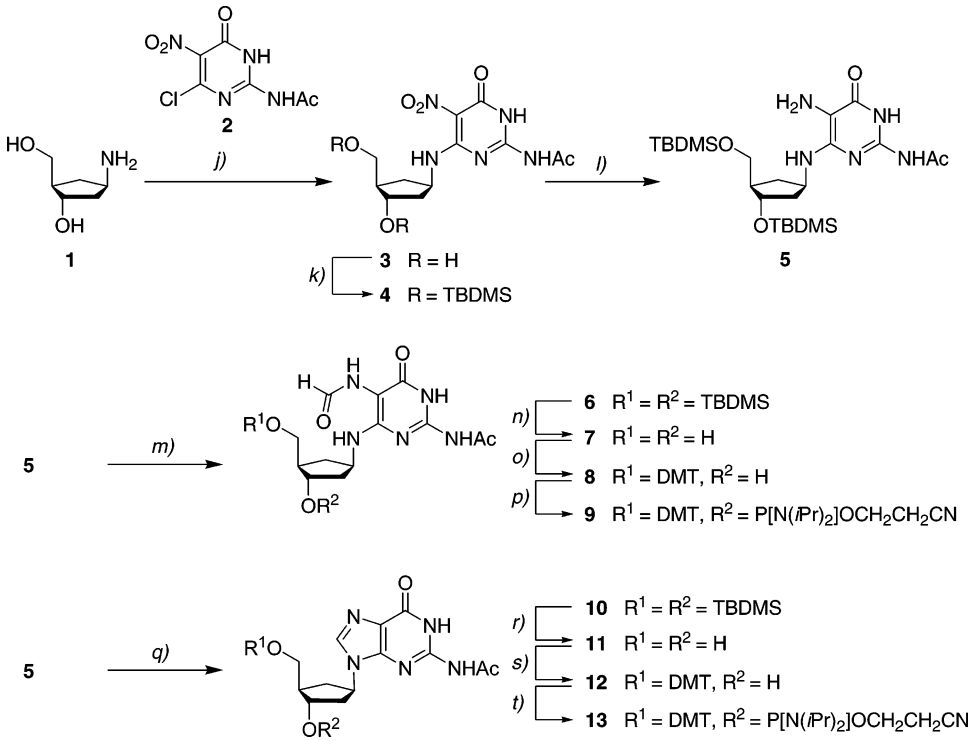
Scheme 2. Synthesis of the Carbocyclic Analogues of β-FaPydG (β-*c*FaPydG) and 2′-deoxyguanosine (*c*dG)[a]



[a] (j) DIPEA, DMF, 70 °C, 90 min, 86%, (k) TBDMSCl, imidazole, DMF, 25 °C, 3 h, 79%, (l) Pd/C, H_2, EtOH, 25 °C, 3 h (m) HCOOH, DIPEA, EDC, DMF, 25 °C, 48 h, 60% for two steps, (n) HF·pyridine, pyridine, EtOAc, 25 °C, 20 h, 94%, (o) DMTCl, pyridine, DMAP, 0−20 °C, 3 h, 56%, (p) P[N(^{i}Pr)$_2$]$_2$OCH$_2$CH$_2$CN, diisopropylaminotetrazolide, CH_2Cl_2, 25 °C, 20 h, 53%, (q) diethoxymethyl acetate, 25 °C, 20 h, then 100 °C, 75 min, 37%, (r) HF·pyridine, EtOAc, 25 °C, 20 h, 85%, (s) DMTCl, pyridine, 20 °C, 2.5 h, 85%, (t) ClPN(^{i}Pr)$_2$OCH$_2$CH$_2$CN, DIPEA, THF, 25 °C, 2 h, 52%.

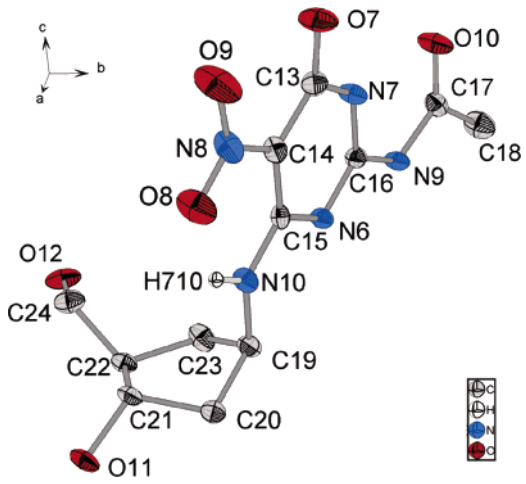


Figure 1. X-ray crystal structure of compound **3**.

The DMT protecting group was introduced using standard conditions. The phosphoramidite was finally prepared with 3-(bis(diisopropylamino)phosphanooxy)propanenitrile and diisopropylaminotetrazolide.[21]

To determine whether the replacement of the deoxyribose by a cyclopentane ring system significantly affects the biophysical characteristics of nucleobases in DNA, we prepared the carbocyclic 2′-deoxyguanosine (*c*dG) derivative **13** to compare with dG.

Compound **13** was synthesized from the air-sensitive diamino precursor **5**. Purine formation proceeded in moderate yields using diethoxymethyl acetate.[22] The silyl protecting groups were removed with pyridine−HF in ethyl acetate. Conversion of **11** into the phosphoramidite **13** was possible using standard procedures. Full experimental procedures for the synthesis of **9** and **13** are given in the Supporting Information. Incorporation of the *c*FaPydG building block into oligonucleotides was possible using standard conditions for coupling and deprotection. For capping, however, we had to replace phenoxyacetyl anhydride with the sterically more demanding pivaloylic anhydride in order to avoid transamidation of the formamidopyrimidine.[12] Cleavage of the fully assembled DNA from the solid support and of all base labile protecting groups was performed using a saturated solution of ammonia in water/ethanol 1:3 at 15 °C. Oligonucleotides containing 8-oxodG were synthesized with a commercially available 8-oxodG building block and deprotected using a saturated solution of ammonia in water/ethanol 1:3 and mercaptoethanol at 60 °C, as suggested by the manufacturer. All oligonucleotides were purified by reversed-phase HPLC. DMT-on oligonucleotides were deprotected after HPLC purification on a Sep-Pak cartridge (Waters) as described in the Supporting Information. All oligonucleotides prepared for this study are listed in Table 1.

To prove the correct incorporation of the *c*FaPydG into DNA, an HPLC−MS/MS experiment of the total enzymatic digest of oligonucleotide **d1** was performed. The HPL chromatogram showed five peaks, of which four could be assigned to the canonical nucleotides dA, dC, dG, and dT by their mass and fragmentation pattern. The fifth peak had the same retention time, mass, and fragmentation pattern as the fully deprotected

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Table 1. Synthesized Modified Oligonucleotides

name	sequence	$\epsilon_{260,\ calcd}$ (L μmol^{-1} cm^{-1})	M_{calcd} (amu)	M_{found}[a] (amu)
d1	5′GCGAT***c*FaPydG**TAGCG	0.1152	3413.0	3413.4
d2	5′TGCAGT***c*FaPydG**ACAGC	0.1255	3686.2	3686.3
d3	5′TAG***c*FaPydG**CCTGGTCATT	0.1355	4285.6	4285.5
d4	5′GCGAT**8-oxodG**TAGCG	0.1152	3413.1	3413.6
d5	5′TGCAGT**8-oxodG**ACAGC	0.1255	3686.3	3687.0
d6	5′TAG**8-oxodG**CCTGGTCATT	0.1355	4285.7	4285.1
d7	5′GCGAT***cd*G**TAGCG	0.1214	3395.2	3395.8
d8	5′TGCAGT***cd*G**ACAGC	0.1316	3668.3	3669.2
d9	5′TAG***cd*G**CCTGGTCATT	0.1416	4267.7	4268.2

[a] MALDI-TOF, hydroxypicolinic acid matrix, negative mode.

*c*FaPydG proving that *c*FaPydG was not modified during DNA synthesis and purification (See Supporting Information for details).

Thermodynamic Measurements: The thermodynamic parameters $\Delta H°$, $\Delta S°$, and $\Delta G°_{298K}$ of the dissociation process of oligonucleotide duplexes containing *c*FaPydG and 8-oxodG were derived from concentration dependent melting-point investigations using van't Hoff plots.[23] In total we determined van't Hoff plots for two different DNA double strands with dG, *c*dG, 8-oxodG, or *c*FaPydG at a defined position. Each strand was hybridized with four different counterstrands to form all possible base pairs. UV melting points of the duplexes were determined for concentrations ranging from 0.3 to 27 μM (Table 2).

We first observed the effect of replacing the 2-deoxyribose moiety with cyclopentane on the obtained $\Delta G°_{298K}$, $\Delta H°$, and $\Delta S°$ values. To this end oligonucleotides containing carbocyclic 2′-deoxyguanosine *c*dG were studied in comparison to strands containing dG.

As expected, the absolute value of $\Delta G°_{298K}$ at 25 °C for the melting process of duplexes containing dG at the site of interest paired with dC was highest. Duplexes containing a mismatch exhibited a strongly reduced $\Delta G°_{298K}$ ($\Delta\Delta G°_{298K} \approx 4$ kcal/mol). The carbocyclic *c*dG nucleotide exhibits a very similar behavior. The observed melting point differences between duplexes containing dG and *c*dG are very small (between 0.2 and 2.0 °C). The cyclopentane replacement generates a slightly more stable duplex due to a small increase of the absolute value of the dissociation enthalpy $\Delta H°$, counteracted by a small increase of $\Delta S°$. However, these effects are small leading to the conclusion that $\Delta G°_{298K}$ for duplexes containing dG and *c*dG are the same within the error margin of the experiment (see Table 2).

We next investigated the base pairing preferences of 8-oxodG and determined in accord with the literature that the 8-oxodG lesion features the highest absolute value of $\Delta G°_{298K}$ when base paired with dC and dA. The data therefore reflect the well-known phenomenon that 8-oxodG pairs with dC in the *anti*- and with dA in the *syn*-conformation as depicted in Scheme 1c; 8-oxodG:dG or 8-oxodG:dT-pairs behaved like dG:dG or dG:dT mismatches, respectively.

We finally studied the base pairing characteristics of the FaPydG lesion using the *c*FaPydG analogue. The presence of the *c*FaPydG lesion in DNA conferred rather large duplex destabilizations. The highest melting point, and hence the best interaction, is observed with dC in a *c*FaPydG:dC base pair. The absolute value of $\Delta G°_{298K}$ for the melting process of duplexes containing a *c*FaPydG:dC is, however, not much higher than for a dG:dT mismatch. The second best pairing interaction of *c*FaPydG is observed with dT as the counterbase. This base pair is only 1.9 (**d1**) to 2.6 (**d2**) kcal/mol less stable than the *c*FaPydG:dC base pair. *c*FaPydG opposite any purine base gives extremely strong destabilized duplexes (about 3.0−3.5 kcal/mol weaker paired than *c*FaPydG:dC). This destabilization is even higher than that measured for a normal mismatch. If we correct the data for the effect of the O → CH_2 chemical alteration, we obtain even stronger destabilizing effects. From these data it is evident, that *c*FaPydG induces a large destabilization of any DNA duplex regardless of the counterbase. The best interaction is obtained with dC. Some constructive interactions may also be possible with dT (Scheme 1c). The measured destabilization of β-*c*FaPydG facing dA is, in our experiments, so high that the formation of a *c*FaPydG:dA base pair has to be completely ruled out.

This conclusion contrasts results reported by Haraguchi and Greenberg[12] who observed that FaPydG is able to base pair with dA. The reason for the discrepancy may be that their seminal results were obtained with the already mentioned lesion α/β-mixture. Synthesis of a carbocyclic α-FaPydG derivative is underway to investigate this issue. The result of our study, however, is in agreement with earlier data obtained using a OHC−NCH_3-methylated analogue of FaPydG.[14]

Density Functional Calculations: The melting point data are at a first glance difficult to explain in light of the rather similar structures of the FaPydG and 8-oxodG lesions. Why does 8-oxodG base pair with dA and the structurally similar FaPydG not? To investigate this problem one has to ask whether FaPydG is able to rotate around the glycosidic bond into a stable *syn*-like conformation in order to form the required Hoogsteen base pair with dA. To analyze this question we performed theoretical studies. We calculated first the torsion potential of FaPydG and *c*FaPydG around the C1′−N bonds in order to clarify how much the data from the analogue deviate from the natural lesion. The results of the calculations are presented in Figure 2a. Each data point represents a full geometry optimization using B3LYP/6-31G* density functional calculation with a constraint on the investigated torsion angle.[24]

The potential functions of *c*FaPydG and FaPydG are as expected very similar, which supports again the validity of our model compound. Both compounds exhibit two local minima, which correspond to the *syn*- and the *anti*-conformation.[25] The *anti*-conformation, which allows Watson−Crick base pairing with dC, however, is in both cases about 6 kcal/mol more stable than the *syn*-conformation needed to establish the Hoogsteen binding mode with dA. Because of the calculated low barrier

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Table 2. Thermodynamic Melting Data of Duplexes Containing dG, *c*dG, 8-oxodG, and *c*FaPydG

	5′ GCGAT**X**TAGCG (**d1**) 3′ CGCTA**Y**ATCGC			5′ TGCAGT**X**ACAGC (**d2**) 3′ ACGTCA**Y**TGTCG		
base pair[a] X, Y	$\Delta G°_{298}$ (°C)	$\Delta H°$ [b] (kcal mol^{-1})	$\Delta S°$ [b] (cal mol^{-1} K^{-1})	$\Delta G°_{298}$ (°C)	$\Delta H°$ [b] (kcal mol^{-1})	$\Delta S°$ [b] (cal mol^{-1} K^{-1})
dG, dA	−9.0	−46 ± 1.1	−122 ± 3.8	−11.5	−68 ± 1.3	−189 ± 4.1
dG, dC	−14.2	−74 ± 1.5	−199 ± 4.7	−16.0	−85 ± 2.3	−231 ± 6.9
dG, dG	−9.9	−51 ± 0.7	−137 ± 2.2	−11.5	−68 ± 1.5	−190 ± 4.9
dG, dT	−10.3	−54 ± 1.8	−146 ± 5.7	−12.4	−73 ± 0.9	−204 ± 2.7
*c*dG, dA	−9.8	−49 ± 2.2	−130 ± 7.0	−11.5	−70 ± 1.3	−196 ± 12.6
*c*dG, dC	−13.5	−70 ± 2.6	−191 ± 8.1	−15.6	−87 ± 1.7	−238 ± 11.5
*c*dG, dG	−11.7	−62 ± 2.5	−170 ± 8.7	−13.0	−86 ± 1.7	−245 ± 10.8
*c*dG, dT	−10.5	−57 ± 2.6	−157 ± 9.6	−12.9	−82 ± 2.4	−232 ± 14.6
8-oxodG, dA	−12.6	−66 ± 1.4	−178 ± 4.3	−13.3	−74 ± 1.6	−204 ± 4.9
8-oxodG, dC	−13.1	−70 ± 1.1	−190 ± 3.5	−15.3	−85 ± 1.4	−234 ± 4.2
8-oxodG, dG	−9.3	−49 ± 0.5	−133 ± 1.5	−11.9	−75 ± 1.3	−211 ± 4.0
8-oxodG, dT	−9.9	−56 ± 1.4	−154 ± 4.6	−12.2	−78 ± 1.4	−221 ± 4.4
*c*FaPydG, dA	−7.0	−42 ± 2.0	−118 ± 7.0	−10.4	−64 ± 1.6	−181 ± 5.0
*c*FaPydG, dC	−10.2	−51 ± 1.0	−137 ± 3.2	−13.4	−80 ± 1.8	−222 ± 5.7
*c*FaPydG, dG	−6.9	−43 ± 2.0	−121 ± 6.8	−9.9	−61 ± 1.1	−173 ± 3.7
*c*FaPydG, dT	−8.3	−47 ± 1.0	−129 ± 3.4	−10.8	−68 ± 1.1	−193 ± 3.7

[a] Conditions: 150 mm NaCl, 10 mm Tris/HCl, pH 7.4, c_{oligo} = 0.3−27 μm. [b] The error is calculated by propagation of the standard error of the linear regression.

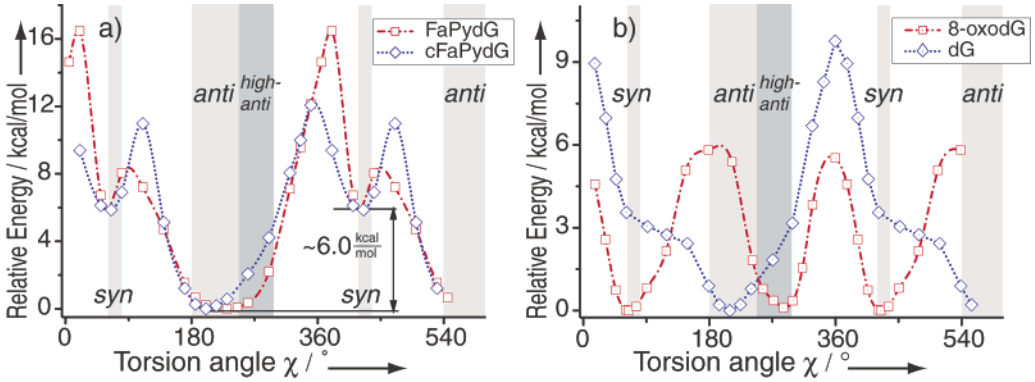

Figure 2. Comparison of the rotation potential around the glycosylic bond. Each data point represents the relative energy of a complete geometry optimization employing B3LYP/6-31G* density functional calculations. The data point of each curve with the lowest energy was set to 0. The gray bars highlight the torsion angles χ corresponding to the conformations *syn*-, *anti*-, and *high anti*- as defined by Blackburn et al.[25] (a) Comparison of natural FaPydG and the carbocyclic analogue; (b) comparison of 8-oxodG with 2′-deoxyguanosine. 8-oxodG has a second minimum at the *syn*-region.

of only 12−16 kcal/mol, we can assume free rotation at room temperature, which enables *c*FaPydG and FaPydG to exhibit a strong preference for the *anti*-conformation. The molecular geometries for the global minima of the monomers are almost superimposable (Supporting Information Figure s1). These results are furthermore supported by a two-dimensional torsional screen around both the C1′−NH and the NH−C4 bond of both monomers using the force field mmff94s.[26,27] The rotation potentials of both compounds are first of all again almost identical. In addition, the computational data support a global minimum with a torsional angle around the glycosidic bond in the *anti*-conformation (Supporting Information Figure s2).

Calculation of the energy functions of 8-oxodG shows, however, the expected additional minimum with the *syn* conformation of the glycosidic bond. The *syn* conformation is in fact the calculated global minimum, which explains why 8-oxodG base pairs preferentially with dA and dC. The computed data are in full agreement with experimental observations with 8-oxodG and other 8-substituted purines.[28,29]

The structural reason for 8-oxodG preferring the *syn*-conformation is a simple steric clash of the C8-carbonyl group and the sugar C5′ methylene group in the *anti*-conformation as observed also in crystal structures.[30] In the FaPydG lesion, however, this C8=O is part of the formamide, which possesses additional degrees of freedom. This freedom allows the formamide group to rotate out of the plane with the pyrimidine ring. By rotation of the formamide function the steric clash can be avoided, which allows this lesion to adopt the *anti*-conformation with great preference.

Primer Extension Kinetics: The obtained thermodynamic data clarify the base pairing properties of the FaPydG lesion but not the coding potential during replication. Replication requires base pairing within the active site of a DNA polymerase. This is next to thermodynamic parameters determined by the steric constraints within the active site of the DNA polymerase. We have recently determined the efficiency of base incorporation opposite a *c*FaPydG present in a template strand

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Table 3. Kinetic Parameters of dNTP (Y) Insertion by *G. stearothermophilus* Pol I and Yeast Pol η Opposite dG, *c*dG, or *c*FaPydG (X)

	G. stearothermophilus Pol I: 5′-CAT**X**CGAGTCAGGCT GCTCAGTCCG (^{31}P)-3′ (**X** = dG, *c*FaPydG)				*S.cerevisiae* Pol η: 5′-TAG**X**CCTGGTCATT GGACCAGTAA(Fl)-3′ (**X** = dG, *c*dG, *c*FaPydG)			
incorporation Y → X	k_{cat} (min^{-1})	K_m (μmol)	k_{cat}/K_m ($\mu mol^{-1} min^{-1}$)	f_{ins}	k_{cat}[a] (min^{-1})	K_m[a] (μmol)	k_{cat}/K_m[a] ($\mu mol^{-1} min^{-1}$)	f_{ins}[b]
dCTP → dG	750	1.5	500	1	31.3 ± 2.8	3.27 ± 0.67	9.58 ± 2.8	1
dTTP → dG	0.25	58	4.3×10^{-3}	8.6×10^{-6}	2.57 ± 0.10	190 ± 25	(1.35 ± 0.2) × 10^{-2}	(1.4 ± 0.7) × 10^{-3}
dATP → dG	7×10^{-4}	111	6.3×10^{-5}	1.3×10^{-7}	1.71 ± 0.10	103 ± 26	(1.65 ± 0.5) × 10^{-2}	(1.7 ± 1.1) × 10^{-3}
dGTP → dG	8×10^{-4}	86	9.3×10^{-6}	1.9×10^{-8}				
dCTP → *c*dG					42.8 ± 2.3	4.10 ± 0.46	10.4 ± 1.7	1
dTTP → *c*dG					8.54 ± 0.65	359 ± 78	(2.38 ± 0.7) × 10^{-2}	(2.3 ± 1.0) × 10^{-3}
dATP → *c*dG					0.84 ± 0.02	84.0 ± 6.9	(10.0 ± 0.1) × 10^{-2}	(1.0 ± 0.3) × 10^{-3}
dCTP → *c*FaPydG	520	1.3	400	1	35.0 ± 3.2	12.1 ± 2.5	2.90 ± 0.9	1
dTTP → *c*FaPydG	35.2	97	0.36	9.0×10^{-4}	1.85 ± 0.08	83.0 ± 13	(2.24 ± 0.5) × 10^{-2}	(7.7 ± 3.8) × 10^{-3}
dATP → *c*FaPydG	12.1	84	0.14	3.5×10^{-4}	1.72 ± 0.13	154 ± 34	(1.11 ± 0.3) × 10^{-2}	(3.8 ± 2.3) × 10^{-3}
dGTP → *c*FaPydG	0.06	130	4.6×10^{-4}	1.2×10^{-6}				

[a] The error is the standard error of the nonlinear regression or calculated by error propargation. [b] $(k_{cat}/K_m)_{dNTP}/(k_{cat}/K_m)_{dCTP}$.

by a high fidelity polymerase (*G. stearothermophilus* DNA Pol I). The obtained data are for comparative reasons listed in Table 3 together with new data obtained with the low-fidelity polymerase Pol η. Pol η tolerates base modifications and is capable in rescueing damaged DNA. The polymerase possesses a less constrained active site allowing us to better estimate the coding flexibility of the *c*FaPydG lesion.

To investigate, how this enzyme processes *c*FaPydG lesions in the template strand, steady-state kinetics of dNTP incorporation by *S. cerevisiae* Pol η opposite dG, *c*dG, and *c*FaPydG were performed. To this end a 54 amino acid C-terminally truncated Pol η[31] protein was prepared from the *RAD30* gene of the yeast strain YPH499 (ATCC 76625) as described in the Supporting Information. This truncation has no effect on the polymerase activity but simplifies polymerase purification procedures. For the kinetic studies, we used the template-primer system depicted in Table 3 and standard Pol η primer extension conditions.[32−37] For a better quantification of the fluorescein labeled primer and its extension products, we employed capillary electrophoresis coupled to a laser-induced fluorescence detector instead of ^{31}P phosphor imaging techniques. The sensitivity of this method is comparable to radioactive quantification. Peak integration, however, is more accurate. Figure 3 shows a typical plot of a primer extension experiment. The primer strand was detected with a retention time of 22.4 min. The one nucleobase elongated primer strand (n + 1) appeared at a slightly larger retention time of 22.7 min.

To identify the preferred dNTP inserted by the polymerase opposite *c*FaPydG, primer extension reactions were performed in the presence of a single type of triphosphate at a time using increasing dNTP concentrations.[38] The relationship between the rate and dNTP concentration was fitted by nonlinear regression

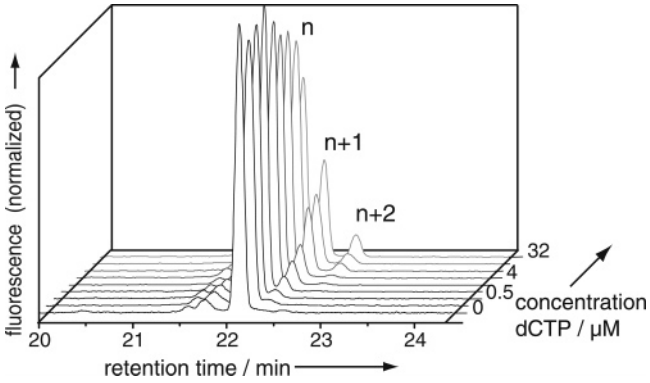

Figure 3. Incorporation of dNTPs opposite *c*FaPydG by yeast Pol η. (a) Stacked plot of normalized fluorescence signals determined by capillary electrophoresis. The tracks refer to raising dCTP concentrations.

directly to the Michaelis−Menton equation to calculate the paramters V_{max} and K_m. The value k_{cat} was derived as an enzyme concentration independent constant via the equation $k_{cat}=V_{max}/c_{enz}$.

Comparison of the efficiency constants k_{cat}/K_m (Table 3) for the incorporation of dCTP, dATP, and dTTP indicates that correct insertion of dCTP opposite dG and *c*dG is in the range of (4.4 ± 3.5) × 10^2 and (1.0 ± 0.3) × 10^3 more efficient than misincorporation of dATP or dTTP. The values for dNTP incorporation opposite dG and *c*dG are again as expected the same within the error margin, showing that the effect of the chemical modification O to CH_2 is small even inside the active site of the polymerase.

dCTP incorporation opposite *c*FaPydG is performed by the polymerase with an efficiency of about 30 (Pol η) − 80% (Pol I) compared to the insertion of dCTP opposite dG or *c*dG. The studies show that *c*FaPydG is not a block but a rather good templating base, again in contrast to earlier observations by the Greenberg group[39] and also in contrast to results from the OHC−NCH_3−FaPydG derivative of Ide et al.[10] Incorporation of dATP and dTTP opposite *c*FaPydG by Pol η is (2.6 ± 1.5) × 10^2 and (1.3 ± 0.7) × 10^2 times less efficient compared to incorporation of dCTP. Compared to the data for *G. stearothermophilus* Pol I, the selectivity of the low fidelity Pol η is

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reduced by 1 order of magnitude, but the specificity sequence dCTP ≪ dTTP < dATP is retained. In summary, polymerases process *c*FaPydG efficiently in an error-free fashion. Some reduction of selectivity is observed, which may be responsible for the mutational effect. The thermodynamic melting point studies and the primer extension studies with high and low fidelity polymerases argue that FaPydG may be responsible for the incorporation of dTTP, which would result in dG to dA transition mutations. The incorporation of dATP opposite the lesion is less likely based on our studies with Pol I or Pol η. This is in agreement with crystallographic studies[17] and with an earlier report by Ide et al.[10] employing the OHC−NCH_3− FaPydG analogue and *E. coli* Pol I Klenow fragment.

Concluding Remarks

The cyclopentane analogue *c*FaPydG of the FaPydG lesion was synthesized, and the base pairing capabilities inside and outside a polymerase active site were investigated and compared to data obtained with dG and *c*dG in the same sequence context. dG and *c*dG show similar properties in our studies, indicating that data derived with cyclopentane analogues of 2′-deoxyguanosine-derived DNA lesions are solid. *c*FaPydG lowers DNA duplex stability significantly. The favored *c*FaPydG:dC base pair has, in DNA, a free dissociation enthalpy similar to a typical dG:dT mismatch. According to our computational and thermodynamic data, *c*FaPydG does not adopt a *syn*-conformation, which would be a prerequisite for base pairing with dA. Despite the largely destabilizing effect of DNA duplexes by the FaPydG lesion, the lesion fully retains its coding potential and also does not constitute a polymerase blocking unit. dCTP was efficiently inserted when the lesion was processed by DNA Pol I or Pol η. However, the lesion gives rise to a reduced polymerase fidelity, which may allow formation of a FaPydG:dT base pair to some extent. This could be the basis for dG to dA transition mutations.

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Supporting Information Available: An Experimental Section including information concerning the synthesis and chemical data for **1**−**13** (including full information of the synthesis of compound **1**), oligonucleotide synthesis and analysis via HPLC-MS/MS, van't Hoff plots of the melting point experiments, cloning and overexpression of *S. cerevisiae* Pol η, further details on the kinetic experiments. CIF coordinates of compound **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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