DNA Lesions

The Two Main DNA Lesions 8-Oxo-7,8-dihydroguanine and 2,6-Diamino-5-formamido-4-hydroxypyrimidine Exhibit Strongly Different Pairing Properties**

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Dedicated to Professor R. W. Hoffmann on the occasion of his 70th birthday

DNA damage generated by the reaction of DNA with reactive oxygen species is one of the major reasons for mutations and carcinogenesis.[1–4] Additionally, oxidative DNA damage is believed to play a major role in the pathogenesis of ageing.[5–9] Reactive oxygen species (ROS) are generated as side products during aerobic respiration, a process that involves the stepwise reduction of molecular oxygen to water. One of the most dangerous reaction products is the OH radical, which is a potent electrophilic oxidant able to oxidize DNA.[4] In DNA, the OH radical reacts preferentially with guanine. Guanine is also the major target for general oxidative damage because it has the lowest oxidation potential among the four bases.

In the last decade, a large number of guanine-derived genome lesions produced either by direct oxidation or reaction with OH radicals were isolated and characterized.[10,11] 8-Oxo-7,8-dihydroguanine (8-oxodGuo) and 2,6-diamino-5-formamido-4-hydroxypyrimidine (FapydGuo, Scheme 1) were found as two main degradation products.[12,13] They are currently believed to be predominantly responsible for the biological effect of oxidative and OH-radical-induced DNA damage.[14]

To study repair and the mutagenic effect of both lesions it is essential to prepare oligonucleotides that contain these lesions in defined positions. To this end, lesion phosphoramidites are needed, which allow their incorporation into DNA by using machine-assisted DNA synthesis.[15] The guanine derivative 8-oxodGuo is a stable compound, which early on allowed its chemical synthesis and incorporation into DNA.[16,17] Consequently, a wealth of chemical and biochemical data are now available on this important lesion.[18,19]

Investigation of the FapydGuo lesion, in contrast, is strongly hindered owing to its instability.[20,21] We recently reported the synthesis of the FapydGuo lesion in a protected form and discovered that the compound is relatively stable

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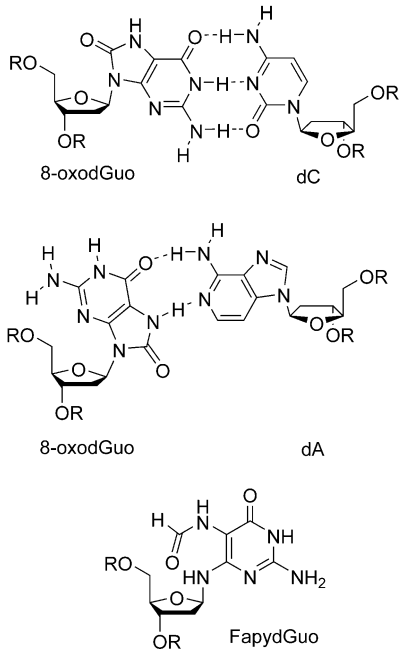
[**] The work was generously supported by the Volkswagen Stiftung, the Fonds der Chemischen Industrie, and the Deutsche Forschungsgemeinschaft. We thank the Boehringer Ingelheim Fonds for a predoctoral fellowship to M.O.

 DOI: 10.1002/ange.200351287



Scheme 1. Depiction of the oxidative DNA lesion 8-oxodGuo base paired with dC and with dA, together with the oxidative DNA lesion FapydGuo.

under physiological conditions.[22] However, the compound was found to anomerize rapidly under conditions generally required for chemical DNA synthesis. Greenberg and coworkers published the synthesis of a dinucleotide building block, which allowed the incorporation of the FapydGuo lesion as an α/β-mixture into DNA.[23,24] Over the last two years, this achievement has allowed initial information to be gathered on how FapydGuo affects the stability of DNA duplexes and how it is replicated by polymerases.[25]

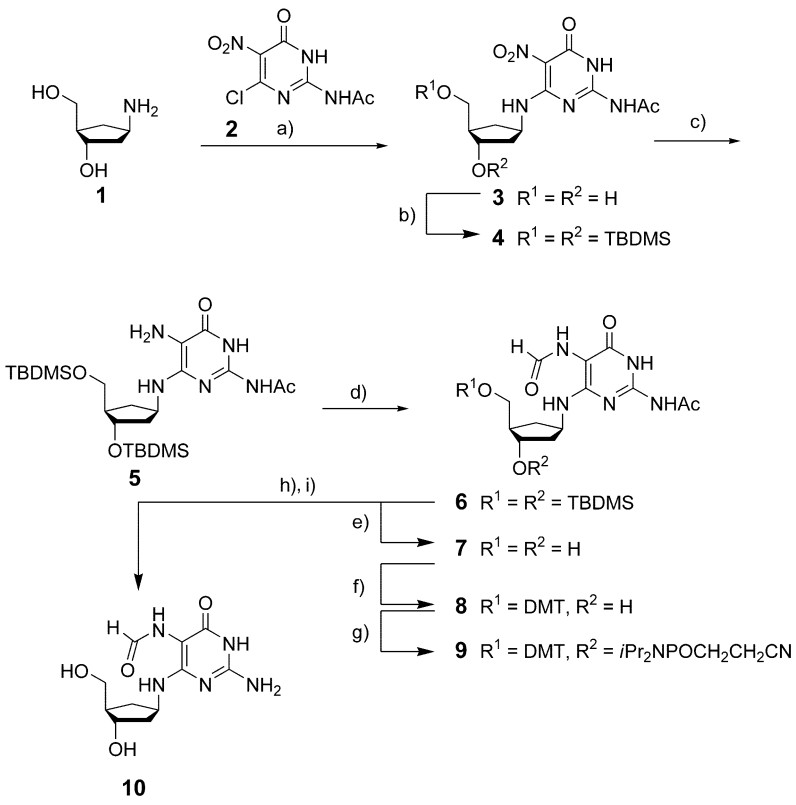
Herein we report the synthesis of a stabilized bioisosteric FapydGuo analogue,[26] which can be inserted in DNA as the pure "β" anomer. The mutagenic potential of all DNA lesions is strongly connected to their base-pairing properties, which can be estimated by using melting-temperature studies. Such melting-point data of the 8-oxodGuo lesion showed efficient base pairing with dC and dA.[27] A crystal structure for the 8-oxodGuo in DNA opposite dA clarified that the lesion pairs with dA if the N–glycoside bond adopts the unusual *syn* conformation as shown in Scheme 1.[28]

The structural similarity of 8-oxodGuo and FapydGuo, manifested in the C8=O carbonyl group present in both lesions, led to the suggestion that both lesions might have very similar base-pairing properties. This belief was recently supported by melting-point data obtained with α/β-FapydGuo-containing DNA.[24,27] In contrast, our experiments show that FapydGuo has a pairing behavior very different from that of 8-oxodGuo. This result suggests a completely different mutagenic effect of FapydGuo.

To circumvent the anomerization problem, we prepared a stabilized, strictly bioisosteric lesion analogue, which contains a cyclopentane skeleton instead of the 2′-deoxyribose sugar backbone.[29] Replacement of the 2′-deoxyribose oxygen atom by a methylene group leaves the heteroatom structure and therefore the base-pairing properties of the lesion fully intact. For the standard nucleotides, it was proven that the oxygen-to-methylene chemical mutation has a negligible effect on duplex stability and duplex structure.[29–31] To analyze possible effects of the O-to-CH_2 chemical mutation on the *syn*/*anti* conformational equilibrium, which is so critical for the mutagenic potential of the 8-oxodGuo, we performed molecular modeling and density functional calculations.[32] In both cases we obtained almost the same, very small differences between the *syn* and *anti* conformations of only about 5 to 32 kJ mol^{-1}, indicating that the heterocycle rotates in both compounds freely around the C1′–N glycoside bond. The *anti* conformation is energetically somewhat more favorable, in agreement with NOESY experiments performed with compound **6** in $CDCl_3$. The NOESY showed stronger cross-peaks between the C1′-N*H* and the *endo* protons at C2′, C3′, and C5′, thus indicating a larger population of the *anti* conformation. Most important, however, is the result that the O-to-CH_2 chemical mutation has, if at all, only a minor effect on the *syn*/*anti* conformational equilibrium.

For the synthesis of the *c*FapydGuo analogue (Scheme 2), we started with the enantiomerically pure cyclopentylamine **1**, which was prepared in six steps by following the synthetic route recently reported by Cullis and Dominguez.[33] Coupling of this building block with the acetyl-protected 2-amino-6-chloro-5-nitro-4-oxopyrimidine (**2**)[34–36] furnished the cyclopentane derivative **3**. Protection of the hydroxy groups with TBDMSCl yielded compound **4**. The following steps included reduction of the nitro group to the amine **5** and transformation of the amine into the formamide **6**.[22] Cleavage of the TBDMS groups was performed with HCl in ethyl acetate. Conversion of the carbocyclic analogue of the β-FapydGuo lesion **7** into the phosphoramidite **9** was possible by using standard procedures. The fully deprotected *c*FapydGuo lesion **10** was synthesized in two steps from **6**. Deacetylation of compound **6** was performed with a 0.05 M solution of K_2CO_3 in methanol. Desilylation required treatment of the product with HCl in THF. The building block **9** was finally incorporated into oligonucleotides using slightly modified standard DNA synthesis procedures, which will be described in due course. The coupling of the *c*FapydGuo unit was found to be above 98%. Cleavage of the fully assembled DNA from the solid support and of all protecting groups present at the various nucleotides was performed by using a saturated solution of ammonia in water/ethanol (3:1) at 15 °C. Intensive studies with the monomer **6** showed that at 15 °C, cleavage of the acetyl protecting group is highly selective. All DNA strands that contained *c*FapydGuo were finally purified by reversed-phase HPLC.

Analysis of the DNA was carried out by analytical HPLC and MALDI-TOF mass spectrometry. The obtained data proved that the *c*FapydGuo-containing oligonucleotides were prepared in excellent yields. The oligonucleotides had, after preparative HPLC purification, a final purity of >98%, necessary for detailed melting-point studies. Final proof that the *c*FapydGuo was correctly inserted into the DNA strand was obtained by an HPLC/MS/MS experiment. To this end, the *c*FapydGuo-containing DNA strand was fully digested in an enzyme mixture of phosphodiesterase II, nuclease P1,



Scheme 2. Synthesis of the *c*FapydGuo lesion analogue phosphoramidite **9** and depiction of the DNA double strands prepared for melting-point studies. a) DIPEA, DMF, 70°C, 1.5 h, 72%; b) TBDMSCl, imidazole, DMF, 20°C, 3 h, 88%; c) Pd/C, H_2, EtOH, 20°C, 2.5 h; d) HCOOH, EDC, DIPEA, DMF, 20°C, 36 h, 70% for two steps; e) HCl/EtOAc (5%), 0→20°C, 1.5 h, 95%; f) DMTCl, lutidine, DMF, 0°C, 3 h, 49%; g) DIPEA, $iPr_2NPOCH_2CH_2CN^+Cl^-$, THF, 0°C, 30 min, 86%; h) K_2CO_3 (0.05 M)/MeOH, 20°C, 2 h, 90%; i) HCl/THF (5%), 0→20°C, 1.5 h, 95%. DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, TBDMS = *tert*-butyldimethylsilyl, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMT = 4,4′-dimethoxytrityl.

phosphodiesterase I, and alkaline phosphatase.[37] The HPLC chromatogram obtained after the digestion showed five peaks, of which four could be assigned to the four nucleobases A, T, G, and C by mass spectrometry. The fifth, small and broad peak, which almost co-eluted with cytosine, was assigned to the *c*FapydGuo. The peak showed the same retention time as the co-injected compound **10**. The molecular weight corresponding to the new peak was found to be identical to that of compound **10**, and finally also the fragmentation patterns of **10** and of the new HPLC peak were identical. Thus, the reported DNA synthesis procedure and the availability of the building block **9** in large amounts allowed the preparation of stable *c*FapydGuo-containing oligonucleotides in quantities sufficient for detailed physicochemical and structural investigations.

To analyze the melting behavior and hence the pairing properties of β-*c*FapydGuo, we prepared the DNA double strands depicted in Scheme 2. The DNA duplexes contain either the canonical base dG, the *c*FapydGuo lesion analogue **7**, or the 8-oxodGuo lesion and all four canonical bases as counterbases. All 12 double strands gave CD spectra, which are typical for B-helical DNA.[38] The differences in the CD spectra were found to be very small, thus confirming that the compounds FapydGuo and 8-oxodGuo have only a very small effect on the overall duplex structure.

The melting temperatures measured for all DNA double strands are summarized in Table 1. As expected, the guanine-containing strand shows the highest melting temperature in the matching dG:dC situation. All mismatches strongly destabilize the duplex by at least 14°C. The two lesions 8-oxodGuo and *c*FapydGuo destabilize the double strands, regardless of the counterbase. However, in agreement with previous reports, 8-oxodGuo pairs relatively well with both dC and dA.[27] Both base pairs provide double strands with melting points that are only 6°C lower than that of the undisturbed dG:dC duplex. The 8-oxodGuo lesion is therefore able to form the expected ^{anti}8-oxodGuo:dC and ^{syn}8-oxodGuo:dA base pairs, also in the oligonucleotide duplexes analyzed in this study. Our data therefore support the base-pairing schemes shown in Scheme 1.

In contrast to currently accepted theory, we find that the melting behavior of the *c*FapydGuo is drastically different to that of 8-oxodGuo. First, the destabilization of the duplexes in the presence of this lesion is much more pronounced. This is surprisingly also the case if dC functions as the counterbase. For the *c*FapydGuo:dC base pair, the melting temperature measured was similar to that associated with a mismatch! The data show that *c*FapydGuo double strands have the highest melting point with the counterbase dT.[39] In fact, the *c*FapydGuo:dT base pair seems to be as stable as the 8-oxodGuo:dC and 8-oxodGuo:dA base pairs.

In addition, all the double strands in which *c*FapydGuo faces one of the two purine bases feature an unusual double sigmoidal melting behavior. The curves obtained for the double strands *c*FapydGuo:dA and *c*FapydGuo:dG (red

Table 1: List of the melting temperatures measured for the twelve DNA duplexes shown in Scheme 1.[a]
5′-d(GCGAT**X**TAGCG)-3′
3′-d(CGCTA**Y**ATCGC)-5′

	T_m [°C]		
	X = dG	X = 8-oxodGuo	X = *c*FapydGuo
Y = dA	33.9	46.0	16.1 (54.9)
Y = dC	51.8	45.6	36.9
Y = dG	37.9	31.4	16.1 (54.9)
Y = dT	35.8	36.7	45.0

[a] Conditions: Tris /HCl, pH 7.4 (0.01 M), NaCl (0.15 M), DNA (3 μM).

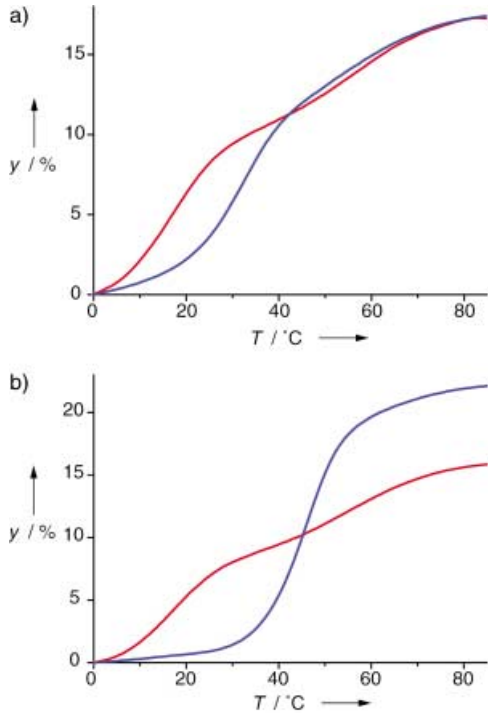


Figure 1. Melting curves of a) the two oligonucleotide double strands containing a *c*FapydGuo:dG (red) and an 8-oxodGuo:dG (blue) base pair and b) of the oligonucleotide double strands containing a *c*FapydGuo:dA (red) and an 8-oxodGuo:dA (blue) base pair.

curves), together with the corresponding melting curves of 8-oxodGuo:dG and 8-oxodGuo:dA (blue curves) are depicted in Figure 1. For *c*FapydGuo:dG and *c*FapydGuo:dA a first very low melting point is observed at about 16 °C, which is not observed in the experiments performed with the 8-oxodGuo lesion. A second, much weaker transition is observed at higher temperatures. This second transition cannot be explained currently. One explanation could be that the strongly destabilizing *c*FapydGuo:purine pairs induce local melting of the duplex around the lesion site at 16 °C. Full melting of the duplex would then explain the second higher transition temperature. Alternatively, this second transition could arise from the melting of an intramolecular hairpinlike structure. Whatever the reason for the second melting curve may be, it is clear that the *c*FapydGuo:purine-containing duplexes show a first very low melting temperature indicative of a strongly destabilized duplex structure. Most interesting are, however, the dramatically different melting curves obtained for the *c*FapydGuo:dA and 8-oxodGuo:dA duplexes as depicted in Figure 1b. These curves clearly show that in contrast to 8-oxodGuo, pairing of FapydGuo with dA gives rise to strongly destabilized duplexes. We therefore conclude that FapydGuo is unable to form proper base pairs with dC and dA, which pair so well with 8-oxodGuo. In contrast, the lesion FapydGuo seems to recognize dT.[39]

In summary, the main oxidative DNA lesions 8-oxodGuo and FapydGuo, predominantly responsible for the mutagenic and cell toxic effect of oxidative DNA damage, have dramatically different pairing properties, which suggests that their mutagenic properties vary as well.

Received: February 27, 2003
Revised: May 21, 2003 [Z51287]

Keywords: DNA damage · DNA · electron transfer · nucleotides · oxidative lesions

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