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A Carbocyclic Analog of the Oxidatively Generated DNA Lesion Spiroiminodihydantoin

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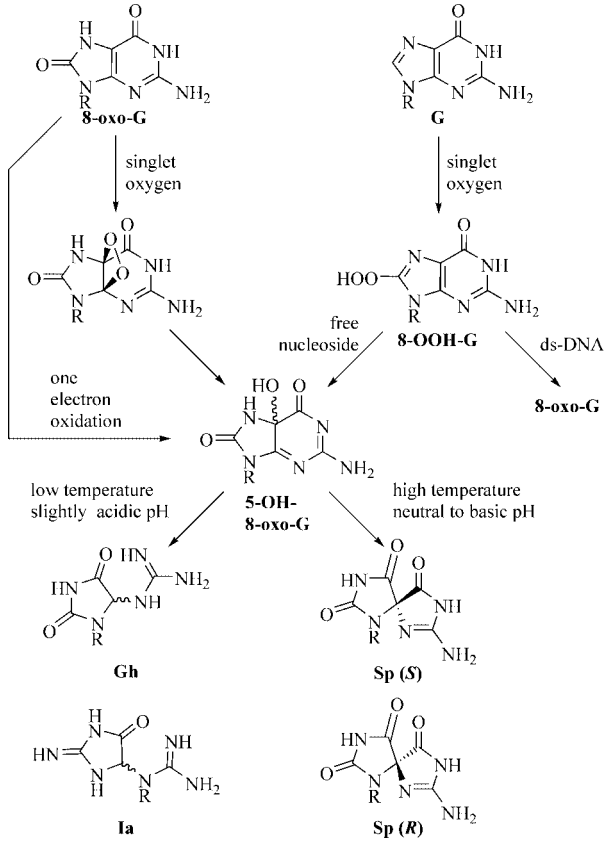
8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) is a mutagenic DNA lesion which is prone to further oxidation. One-electron oxidants like Ir^{IV} oxidize 8-oxo-dG to give secondary lesions such as the spiroiminodihydantoin. This lesion blocks DNA polymerases and induces G → T as well as G → C transversions. Here, we report the synthesis of a carbocyclic analog of the 8-oxo-dG lesion and the carbocyclic version of the spiroiminodihydantoin lesion. Both lesion analogs were obtained within single-stranded DNA and as their corresponding nucleosides. Characterization of the lesion analogs was achieved by HPLC, ESI-MS and ESI-MS/MS, as well as enzymatic digestion of the oligonucleotides.

Introduction

The oxidation potential of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), a major oxidative DNA lesion, is only about one half of the oxidation potential of 2′-deoxyguanosine.[1] It is thus obvious that there are various secondary DNA lesions generated by the oxidation of 8-oxo-dG.[2] Singlet oxygen reacts for example readily with 8-oxo-7,8-dihydroguanosine (8-oxo-G) to form a variety of different products,[3] one of those being spiroiminodihydantoin (Sp),[4] which was first misinterpreted as 4-OH-8-oxo-G.[5] Later, the structure was corrected based on NMR spectroscopic data.[6]

Burrows et al. first described the spirocyclic structure of the spiroiminodihydantoin lesion Sp, which they obtained after one-electron oxidation of 8-oxo-7,8-dihydroguanosine.[7] Up to now, this is the most convenient way to produce Sp within a nucleoside or in DNA.[8] The oxidation of 8-oxo-G to Sp was found to be strongly dependent on both, the temperature and the pH value of the reaction solution (Scheme 1). Whereas at lower pH and at lower temperature the formation of guanidinohydantoin (Gh) and of its isomer iminoallantoin (Ia) is favored, Sp is predominantly formed at higher temperatures and at higher pH values (Scheme 1).[7,8] In addition, the reaction of singlet oxygen with guanosine or guanosine-5′-monophosphate was shown to produce Sp as well.[9a,9b] The formation of Sp upon reaction of cellular DNA with singlet oxygen is still a matter of debate, although Box et al. were able to observe Sp-containing dinucleotides after exposure of HeLa cells to UVA light in the presence of methylene blue.[9c] Recently, both diastereomers of the Sp nucleobase were separated and their absolute configurations were assigned.[10]



Scheme 1. Short overview of the oxidation of G and 8-oxo-G to guanidinohydantoin Gh and spiroiminodihydantoin Sp. Iminoallantoin (Ia) has not yet been identified as $^1O_2$ oxidation product of 8-oxo-G. R = β-D-ribofuranosyl, 5-OH-8-oxo-G = 5-hydroxy-8-oxo-7,8-dihydroguanosine, 8-OOH-G = 8-hydroperoxyguanosine.

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When present in DNA, 2′-deoxyspiroiminodihydantoin (dSp) has a detrimental effect on the duplex stability. Regardless of the opposing base, the melting temperature of the duplex, containing dSp as a mixture of diastereomers, is greatly decreased. If at all, dSp shows a small preference for interacting with dG.[11a] The strong helix-disturbing effect of dSp has serious consequences for the elongation of dSp-containing template-DNA strands by polymerases. In the presence of all four dNTPs, Klenow exo$^-$ is efficiently blocked by a template containing dSp.[11] At higher dNTP concentrations and in the presence of only one corresponding dNTP, Klenow exo$^-$ preferentially inserts dATP and dGTP opposite dSp.[11] Klenow exo$^+$ in contrast is not able to elongate a dSp-containing template, even under single-nucleotide conditions.[11c] In vitro studies with *E. coli* showed that dSp is much more mutagenic than 8-oxo-dG leading to nearly 100% G → T and G → C transversion mutations.[12] Oxidatively generated DNA lesions are commonly repaired by base excision repair (BER).[13] The short-patch repair system requires the use of bifunctional glycosylases/AP lyases like the bacterial enzymes MutM/FPG or endonuclease VIII/Nei (both of which belong to the Nei superfamily) or its eukaryotic functional homolog OGG (either from yeast or human, HhH superfamiliy). MutM or FPG, which readily removes 8-oxo-dG and a variety of other oxidized purines,[14] was also found to efficiently remove dSp and dGh regardless of the opposite base.[15]

In order to investigate the molecular recognition of the lesion by repair enzymes and to study replication processes of DNA-template strands containing the dSp lesion, it is essential to have access to a stabilized version of this lesion. We have recently introduced the concept to replace the 2-deoxyribose unit in lesions by a stable cyclopentane unit. This "chemical mutation" creates lesion analogs which can be crystallized together with active enzymes. In addition, this modification does not affect the thermodynamic stability of the corresponding base pair.[16] Herein, we report the synthesis and characterization of a carbocyclic analog of the DNA lesion spiroiminodihydantoin, cdSp, in DNA as well as on the nucleoside level. This should now enable co-crystallization experiments using active repair enzymes (Figure 1). Because the dSp heterocycle is rather base-labile, it is difficult to incorporate this lesion directly into DNA by phosphoramidite chemistry.[15] However, Cadet et al. reported the site-specific incorporation of dSp phosphoramidite, which they thought was 4-OH-8-oxo-dG, into DNA using phosphoramidite chemistry.[17] Herein, we report the synthesis of cdSp-containing DNA by one-electron oxidation of carbocyclic 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-cdG) in DNA. The latter was synthesized according to a new synthetic route and incorporated into three different oligo-2′-deoxynucleotides (ODN) using phosphoramidite chemistry.

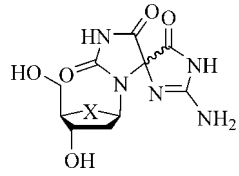
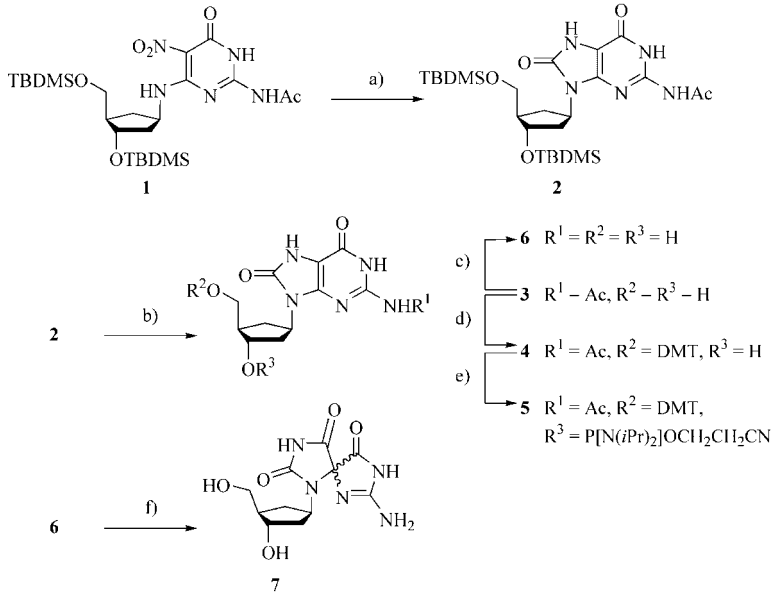


Figure 1. 2′-Deoxyspiroiminodihydantoin (X = O; dSp) and its carbocyclic analog (X = CH_2; cdSp).



Scheme 2. a) H_2, 10% Pd on charcoal in methanol; 1.05 equiv. CDI in $CHCl_3$, room temp., 18 h, 55 °C 3 h, 29%; b) HF·pyridine/pyridine in ethyl acetate, room temp., 18 h, 95%; c) $MeNH_2/NH_3$ (1:1), 0.5 M β-mercaptoethanol, room temp., 8 h, 99%; d) 1.2 equiv. DMTCl in pyridine, 0 °C, 2 h, 50%; e) 1.5 equiv. CEDCl in THF, 0 °C, 2 h, 70%; f) sodium phosphate 50 mM, pH = 8.0, 6.8 mM **6**, 6 mM Na_2IrCl_6, 60 °C, 1 h.

Results and Discussion

The synthesis of the 8-oxo-cdG phosphoramidite was performed using a novel strategy quite different compared to previous studies reported by Johnson et al. starting from aristeromycin.[18] Our convergent synthesis of 8-oxo-cdG (Scheme 2) started with the enantiomerically pure nitropyrimidinone **1**, which was prepared in nine steps with an overall yield of 35% as previously reported by us.[16]

The first step towards the preparation of **7** was the reduction of the nitro group using hydrogen and palladium on charcoal. The ring closure to give the 8-oxo-7,8-dihydroguanine heterocycle proved to be the crucial step. Both, the 5-amino group and the 6-oxo group as well as the acetamide moieties are susceptible towards electrophilic attack. Thus, the reagent needed to close the five-membered ring substructure had to be fairly selective but also reactive enough to attack both the 5- and the 4-amino groups of the pyrimidinone heterocycle **1** to give the desired N^2-acetyl-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (**2**). We found that this reaction is best performed with 1,1′-carbonyldiimidazole (CDI) in dry chloroform at high dilution. This method provided compound **2** in a respectable yield of about 30%. The following silyl deprotection with HF/pyridine in ethyl acetate buffered with pyridine was nearly quantitative yielding the nucleoside **3** as a light yellow precipitate with both the 5′-and the 3′-hydroxyl groups unprotected. The 5′-DMT protection of the carbocyclic moiety was found to proceed very slowly. The resulting N^2-acetyl-5′-*O*-(dimethoxytrityl)-8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (**4**) was finally converted into the corresponding 3′-phosphoramidite **5** by reaction with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. The reagent was selective towards the 3′-position under the conditions used here, neither the O^6- nor the O^8-adduct could be detected by ^{31}P NMR spectroscopy.

The second critical step in the synthesis required the selective oxidation of the 8-oxo-dG analog to give the corresponding cdSp lesion. In order to investigate this step, we used the completely deprotected nucleoside for the oxidation of 8-oxo-cdG. To this end, compound **3** was dissolved in a 1:1 mixture of methylamine and ammonia to cleave the N^2-acetyl group; 0.5 M β-mercaptoethanol was added to prevent aerial oxidation of 8-oxo-cdG which is a known problem.[18] After 8 h at room temperature, no starting material could be detected any more by rp-18 TLC. The needed unprotected 8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (**6**) was finally obtained with 99% yield (Scheme 2, Figure 2a).

The oxidation potential of sodium hexachloroiridate(IV) lies between those of dG and 8-oxo-dG.[8b] This allows the reagent to oxidize selectively 8-oxo-dG in the presence of dG. All other nucleobases do not react at all. This selective oxidation, however, depends critically on the pH of the solution as well as on the reaction temperature. Both parameters have to be tightly controlled. We obtained the best

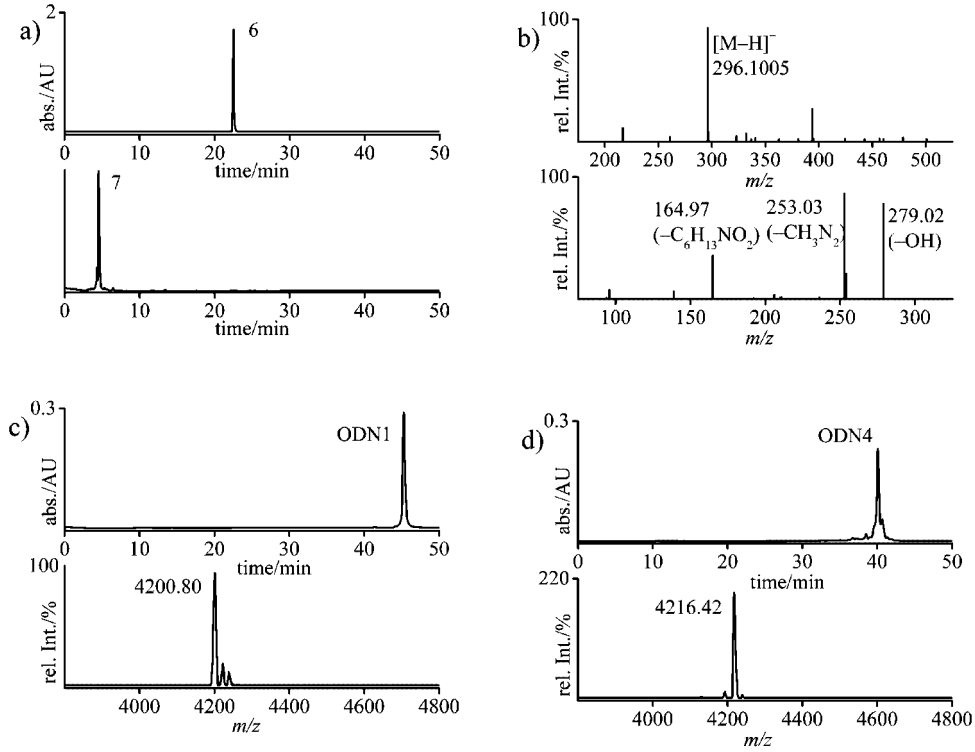


Figure 2. a) HPLC of **6** and **7** using a gradient of 0–25% buffer B in 45 min; buffer A: 2 mM TEAA in water; buffer B: 2 mM TEAA in 80% acetonitrile. b) Negative ESI-MS (top) and MS/MS (bottom); parent ion of **7** $[M - H]^-$: m/z = 296.1005. c) HPLC and MALDI-TOF of **ODN1**. d) HPLC and MALDI-TOF of **ODN4**; buffer A: 0.1 M TEAA in water; buffer B: 0.1 M TEAA in 80% acetonitrile (c and d).

results, when we performed the oxidation in sodium phosphate buffer at pH = 8.0 and 60 °C. Under these conditions, we observed that **6** cleanly reacted with Na_2IrCl_6 to give **7** (Figure 2a). The reaction was stopped by cooling the reaction mixture to room temperature. The product was finally purified by directly subjecting the reaction solution to HPLC separation using 2 mM triethylammonium acetate (TEAA) at pH = 7.0 in water (buffer A) and 2 mM TEAA at pH = 7.0 in 80% acetonitrile (buffer B). The peak eluting under our conditions (gradient 0–25% B over 45 min) at 4.5 min (Figure 2a) had a detectable molecular weight of 296.1005 amu, which is in perfect agreement with the calculated molecular weight of **7** (*m*/*z* = 296.1000). The fragmentation pattern of this molecular ion peak can easily be explained by the loss of the fragments indicated in Figure 3. These data fully confirm the presence of the spiroiminodihydantoin structure.

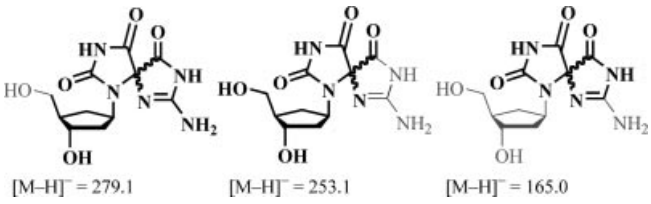


Figure 3. Negative-mode ESI-MS/MS of **7** at 35 eV. Structural assignments of the fragmentations are suggested as indicated in bold.

We next incorporated 8-oxo-cdG into three different ODNs with a sequence length of 11, 12 and 14 nucleotides (Table 1). The DNA synthesis was performed on controlled pore glass (cpg) support using standard conditions with benzylthiotetrazole (BTT) as the coupling reagent. Due to the fact, that the coupling efficiency of 8-oxo-cdG phosphoramidite **5** was significantly lower, the coupling times were set to 10 min and the coupling procedure for **5** was repeated once. Best incorporation was finally achieved by coupling **5** with 16 equiv. over 20 min. Cleavage from the solid support and removal of the protecting groups was performed using a 1:1 mixture of methylamine and ammonia with 0.5 M β-mercaptoethanol at room temperature for 18 h. Methylamine was used since pure ammonia gave unsatisfying deprotection results. The ODNs were purified by HPLC using 0.1 M TEAA at pH = 7.0 in water (buffer A) and 80% acetonitrile (buffer B) with a gradient of 5–22% B in 50 min on a Macherey–Nagel Nucleodur 100-5 C18 column and checked for purity by analytical HPLC using the same buffer system and gradient on a Macherey–Nagel Nucleodur 100-3 C18 column (Figure 1c). The molecular weights of the ODNs were determined using MALDI-TOF mass spectrometry. Desalting was performed using Waters Sep-Pak® C18 cartridges according to the manufacturer protocol.

Total enzymatic digest of **ODN1** was performed to proof the correct incorporation of 8-oxo-cdG. A set of enzymes was used to ensure complete degradation of the DNA. To a 20 μM solution of **ODN1**, we added ammonium acetate buffer (pH = 5.7), nuclease P1 (*Penicillium citrinum*) and calf spleen phosphodiesterase II. The solution was incubated at 37 °C for 3 h and then set to a more basic pH by addition of TRIS-HCl (pH = 8.0). Alkaline phosphatase and snake venom phosphodiesterase I were subsequently added and the mixture was incubated at 37 °C for another 3 h. Following this procedure, the pH was set to approximately 7 with 0.1 N HCl and the enzymes were denatured by heating of the solution to 90 °C for 15 min prior to centrifugation. 20 μL of this solution were injected directly into the HPLC-MS system. The HPL chromatogram (Figure 4a and b) showed three peaks, two of which could be assigned to be dC and dG, respectively. The third peak consisted of both, dT and 8-oxo-cdG nucleoside, as determined by ESI-MS.

Table 1. Synthesized modified ODNs.

Name	Sequence	$M_{calcd.}$ (amu)	M_{found} (amu)[a]
ODN1	5′-CTCTTT[**8-oxo-cdG**]CTCTTTG	4200.49	4200.80
ODN2	5′-GCGAT[**8-oxo-cdG**]TAGCG	3411.03	3410.14
ODN3	5′-TGCAGT[**8-oxo-cdG**]ACAGC	3684.20	3684.82
ODN4[b]	5′-CTCTTT[**cdSp**]CTCTTTG	4216.49	4216.42

[a] MALDI-TOF analysis. [b] As a mixture of diastereomers.

The reaction conditions used to convert **ODN1** to **ODN4** were found to be similar compared to those used for the synthesis of **7**. **ODN1** was dissolved in 14 mM sodium phosphate buffer at pH = 8.0 and oxidized with 100 μM Na_2IrCl_6 at 60 °C for 1 h. Preheating of the mixture before adding the iridate solution was necessary to avoid the formation of guanidinohydantoin side products. The mixture was then cooled to room temperature and the reaction stopped by passing the solution through a Waters Sep-Pak® C18 cartridge. This removed the buffer salts as well as the remaining iridate and therefore stopped the reaction effectively. The resulting ODN was checked for purity using analytical HPLC. Here too, a buffer system consisting of 0.1 M TEAA at pH = 7.0 in water (buffer A) and 0.1 M TEAA in 80% acetonitrile (buffer B) was used with a gradient of 5–22% B in 50 min. The chromatogram showed the presence of only one peak corresponding to pure **ODN4** with a molecular weight of *m*/*z* = 4216.42 as checked by MALDI-TOF analysis (Figure 2d) showing the oxidation of 8-oxo-cdG to cdSp in a clean process inside a DNA single-strand. Storage of **ODN4** at room temperature for several days was possible without formation of degradation products as investigated using HPLC and MALDI-TOF mass spectrometry (data not shown).

In order to proof the presence of the cdSp lesion in DNA, an additional enzymatic digest of **ODN4** was performed according to the same protocol described above. After centrifugation of the reaction mixture, a sample of 20 μL was analyzed by LC-MS. Although the structure of spiroiminodihydantoin differs significantly from the structure of 8-oxo-dG, degradation of the DNA strand by the described enzymes was successful. No partially digested fragments of **ODN4** could be observed. The detection of the early eluting

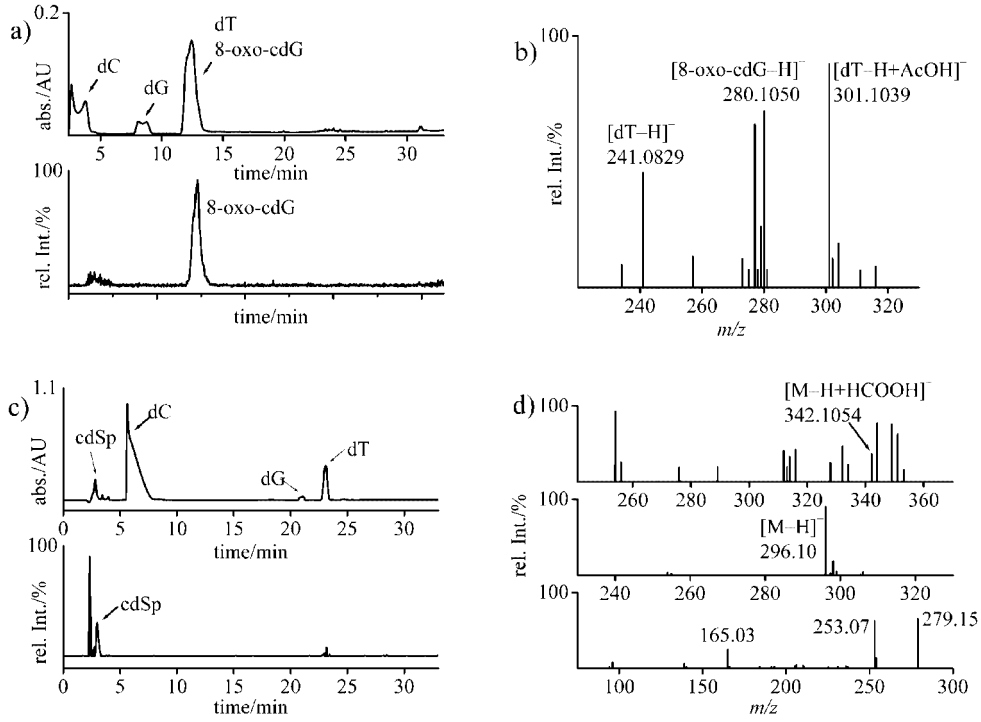


Figure 4. LC-MS and LC-MS/MS of enzymatic digest of: a) **ODN1**, HPLC on Macherey–Nagel Nucleodur 125-2 3 μ C18 pyramid, 2 mM TEAA, pH = 7.0, in water (A) and 80% acetonitrile (B), 100% A 10 min, 0–10% B 18 min, UV trace (top) and ion count at *m*/*z* = 295.5–296.5 (bottom); b) ESI-MS of peak eluting at 12 min; c) **ODN4**, HPLC on Uptisphere Interchrom, 2 mM ammonium formate, pH = 5.5, in water (A) and 80% acetonitrile (B), gradient equal to a), UV trace (top), ion count at *m*/*z* = 341.5–342.5 (bottom); d) ESI-MS (top), -MS/MS (centre) and -MS/MS/MS (bottom) of peak eluting at 3 min, parent ions are $[M - H + HCOOH]^-$: *m*/*z* = 342.1054 for MS/MS and $[M - H]^-$: *m*/*z* = 296.10 for MS/MS/MS; M = cdSp.

cdSp nucleoside by ESI-MS, however, appeared to be problematic because buffer salts were eluting with the same retention time and therefore disturbed the sensitivity of the ESI measurement. We found that the use of a different column, namely an Uptisphere Interchrom 150 × 2.1, and a buffer change to 2 mM ammonium formate at pH = 5.5 instead of triethylammonium acetate at pH = 7.0 for the LC-MS increased the detectability of the lesion analog significantly. The peak eluting at 3 min was identified to be the cdSp nucleoside (Figure 4c). The ESI-MS spectrum shows a molecular weight of *m*/*z* = 342.1054 in perfect agreement with the molecular weight of the formic acid adduct of cdSp $[M + HCOOH - H]^-$ (342.1055 amu, Figure 4d). Confirmation of the assignment is based on MS/MS measurements of this peak. The peak at 296.10 amu corresponds to the free cdSp nucleoside with a calculated molecular weight of *m*/*z* = 296.1000. MS/MS/MS measurements of this peak showed exactly the same fragmentation pattern compared to **7**, thus being the evidence for the presence of a correctly incorporated cdSp lesion into **ODN4**.

Conclusion

A new short and efficient synthesis of a carbocyclic analog of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-cdG) was developed and its incorporation into three different ODNs using standard phosphoramidite chemistry was accomplished. The oxidation of 8-oxo-cdG with the one-electron oxidant sodium hexachloroiridate(IV) provided the carbocyclic analog of the DNA lesion spiroiminodihydantoin (cdSp) in a nearly quantitative conversion. The formation of cdSp was also possible using 8-oxo-cdG in single-stranded DNA.

The presence of both, the carbocyclic lesions cdSp and 8-oxo-cdG in the corresponding ODN was confirmed by total enzymatic digest followed by ESI-MS and MS/MS analysis. These carbocyclic lesion analogs lacking a glycosidic bond between the sugar and the base moiety now provide a powerful tool for the investigation of their recognition by DNA repair glycosylases using protein crystallography.

Experimental Section

Materials and Methods: All solvents and reagents were obtained in commercially available qualities puriss., p.a., or purum. Phosphoramidites were from Glen Research or Samchully pharm, cpg support and benzylthiotetrazole (BTT) was from Glen Research. Dry solvents were purchased from Fluka or Acros and were used as received. Acetonitrile for DNA synthesis was from Roth in Rotidry grade with a water content of 10 ppm. Acetonitrile for HPLC and HPLC/MS was purchased from VWR in HPLC grade. All experiments involving water-sensitive compounds were conducted in oven-dried glassware under nitrogen. Reactions were monitored by analytical thin-layer chromatography (TLC) on VWR precoated al-

uminium plates 60 F_{254}, visualized by UV light, anisaldehyde or ninhydrin staining. Melting points were obtained with a Reichert-Jung type 651501 using a Leica Galen III microscope and are not corrected. IR spectra were obtained with a Perkin–Elmer Spectrum BX connected with a Smiths Detection DuraSamp/IR II. ^{1}H NMR spectra were obtained with a Varian Mercury-200 and a Varian INOVA-400. The chemical shifts were referenced to $CHCl_3$ in $CDCl_3$ and DMSO in [D_6]DMSO. If necessary, peak assignment was carried out with the help of COSY, HMBC or HSQC experiments. ^{13}C NMR spectra were obtained with a Varian INOVA-400 and a Bruker AMX 600. ^{31}P NMR spectra were obtained with a Varian Mercury-200. FAB mass spectra were recorded with a Finnigan MAT95Q using a 2-nitrobenzylalcohol or glycerol matrix. ESI spectra were obtained with a Thermo Finnigan LT-FT ICR spectrometer. MALDI-TOF spectra were obtained with a Bruker Autoflex II spectrometer using a matrix consisting of a 1:1 mixture of saturated 3-hydroxypicolinic acid (HPA) in water and a solution of 50 mg of HPA, 10 mg of ammonium hydrogen citrate and 10 μL of 15-crown-5 in 1 mL of water. HPLC analysis was performed with a Waters 2695 and a Waters 2996 Photo Diode Array detector using a Macherey–Nagel Nucleodur 100-3 C18 column. HPLC separation of DNA was achieved with either a Waters 1525 and a Waters 2487 UV detector or a Merck-Hitachi L7150 and a Merck-Hitachi L7420 UV/Vis detector. UV absorption for concentration measurement of the ODNs was measured with an Eppendorf Biophotometer.

N^2-Acetyl-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (2): To a solution of **1** (200 mg, 0.36 mmol) in methanol (10 mL) Pd on charcoal (50 mg) was added and the mixture was degassed three times by freezing and evaporating. The nitrogen was exchanged for hydrogen and the reaction mixture was stirred at room temperature for 18 h. The solution was then filtered under nitrogen through a 0.2 μm nylon syringe filter (Whatman Puradisk®) and dried under vacuum. The brown product was dissolved in dry chloroform (20 mL). To this solution was added dropwise and very slowly a solution of 1,1′-carbonyldiimidazole (1.05 equiv., 0.38 mmol, 61.2 mg) in dry chloroform (10 mL) at room temperature. The reaction mixture was stirred at room temperature for 20 h, followed by heating to 55 °C for another 3 h. After cooling, additional chloroform (40 mL) was added and the solution was washed with brine. The organic phase was dried with sodium sulfate and concentrated in vacuo. The crude product was purified by flash chromatography on silica gel 60 (10 g silica gel 60, $CHCl_3$/MeOH, 40:1) which afforded **2** as a yellow foam (58 mg, 0.11 mmol, 29%). R_f = 0.35 ($CHCl_3$/MeOH, 10:1). M.p. 143–145 °C. ^{1}H NMR (200 MHz, $CDCl_3$, 25 °C): δ = 0.03, 0.04, 0.05 (3 × s, 12 H, SiCH_3), 0.86, 0.89 [2 × s, 18 H, SiC(CH_3)$_3$], 1.83 (m, 1 H, C2′H_a), 1.97 (m, 1 H, C6′H_a), 2.01 (m, 1 H, C4′H), 2.07 (m, 1 H, C6′H_b), 2.29 (s, 3 H, COCH_3), 2.51 (m, 1 H, C2′H_b), 3.64 [dd, ^2J(C5′H_a,C5′H_b) = 10.2 Hz, ^3J(C5′H_a,C4′) = 5.0 Hz, 1 H, C5′H_a], 3.69 [dd, ^2J(C5′H_b,C5′H_a) = 10.1 Hz, ^3J(C5′H_b,C4′H) = 5.2 Hz, 1 H, C5′H_b], 4.35 (m, 1 H, C3′), 4.89 (m, 1 H, C1′H), 8.78, 9.97 (2 × s, 2 × 1 H, N7H, C2NH), 11.99 (s, 1 H, N1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = –5.65, –5.35, –4.72, –4.53 (4 × SiCH_3), 18.03, 18.34 [2 × SiC(CH$_3$)$_3$], 24.31 (NHCOCH_3), 25.86, 25.99 [2 × SiC(CH_3)$_3$], 30.64 (C6′), 37.75 (C2′), 50.20, 50.35 (C1′, C4′), 63.50 (C5′), 72.98 (C3′), 103.97 (C5), 145.71, 145.95, 149.60, 152.26 (4 × C_{Het}), 171.30 (NHCOCH$_3$) ppm. IR: $\tilde{\nu}$ = 3230.71 vw, 2929.51 vs, 2954.06 vs, 2895.17 s, 2802.33 m, 2768.31 m, 1961.25 m, 1736.85 s, 1710.38 s, 1650.10 vs, 1606.37 w, 1560.05 vs, 1502.18 m, 1437.12 s, 1359.84 w, 1343.65 w, 1311.33 w, 1247.72 s, 1089.34 m, 1004.49 w, 940.04 w, 832.20 s, 771.92 s, 704.36 w, 662.91 w cm^{-1}. MS (FAB$^+$): *m*/*z* (%) = 552.7 (7.0) [M + H]$^+$, 574.7 (9.6) [M + Na]$^+$. HRMS (FAB$^+$; [M + Na]$^+$): calcd. for [$C_{25}H_{45}N_5O_5Si_2$ + Na]$^+$ 574.2851, found 574.2795.

N^2-Acetyl-8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (3): HF·pyridine (50 μL) and pyridine (50 μL) were added under argon to a solution of **2** (52 mg, 0.09 mmol) in dry ethyl acetate (5 mL). After stirring at room temperature for 18 h, a white solid precipitated which was separated by centrifugation, taken up in a solution of methoxytrimethylsilane (200 μL) in dry ethyl acetate (2 mL) and stirred for an additional 1 h. The mixture was then centrifuged again and the white precipitate was dried under vacuum to afford **3** in 95% yield (29 mg, 0.09 mmol). M.p. > 230 °C. ^{1}H NMR (400MHz, [D_6]DMSO, 25 °C): δ = 1.63–1.69 (m, 1 H, C2′H_a), 1.77–1.85 (m, 1 H, C4′H), 1.87–1.97 (m, 2 H, C6′H_a, C6′H_b), 2.13 (s, 3 H, COCH_3), 2.40–2.45 (m, 1 H, C2′H_b), 3.31–3.53 (m, 1 H, C5′H_a), 3.48–3.53 (m, 1 H, C5′H_b), 4.02–4.06 (m, 1 H, C3′H), 4.49 (t, 1 H, C5′OH), 4.60 (d, 1 H, C3′OH), 4.83 (m, 1 H, C1′H), 11.04, 11.52, 11.98 (3 × s, 3 × 1 H, 3 × NH) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 24.42 (COCH_3), 31.58 (C6′), 37.58 (C2′), 50.23, 50.62 (C1′, C4′), 63.98 (C5′), 72.26 (C3′), 103.57, 146.01, 147.50, 149.54, 152.47 (C2, C4, C5, C6, C8), 173.99 (*C*OCH$_3$) ppm. IR: $\tilde{\nu}$ = 3332.22 w, 3071.86 m, 2966.89 w, 2880.51 w, 2756.19 w, 2666.15 w, 1975.75 m, 1714.25 s, 1651.10 vs, 1609.80 w, 1572.22 m, 1433.21 m, 1345.42 m, 1233.84 s, 1162.05 w, 1108.04 m, 1034.23 s, 1015.82 s, 933.40 m, 882.36 m, 844.13 m, 796.07 s, 757.90 s, 742.17 s, 698.75 s cm^{-1}. HRMS (ESI$^+$; [M + H]$^+$): calcd. for [$C_{13}H_{17}N_5O_5$ + H]$^+$ 324.1302, found 324.1302.

N^2-Acetyl-5′-*O*-(dimethoxytrityl)-8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (4): **3** (71 mg, 0.22 mmol) was coevaporated with dry pyridine (2 mL) and again dissolved in dry pyridine (4 mL). Activated molecular sieves (4 Å) was added and the mixture stirred for 30 min. The solution was then cooled in an ice bath to 0 °C and a solution of dimethoxytrityl chloride (89.2 mg, 0.26 mmol, 1.2 equiv.) in dry pyridine (1 mL) was added dropwise. The progress of the reaction was monitored by TLC. After a complete conversion of the starting material, MeOH (4 mL) was added and the solvent was evaporated in high vacuum. Flash chromatography of the crude product (8 g silica gel 60, $CHCl_3$/MeOH, 30:1, 0.5% pyridine) afforded **4** as a slightly yellow solid (69 mg, 0.11 mmol, 50%). R_f = 0.27 ($CHCl_3$/MeOH, 10:1). M.p. 164–166 °C. ^{1}H NMR (200MHz, $CDCl_3$, 25 °C): δ = 1.78–1.92 [m, ^2J(C6′H_a,C6′H_b) = 13.8 Hz, 1 H, C6′H_a], 2.16–2.19 (m, 3 H, C2′H_a, C2′H_b, C4′H), 2.25 (s, 3 H, NHCOCH_3), 2.51–2.65 [m, ^2J(C6′H_b,C6′H_a) = 13.4 Hz, 1 H, C6′H_b], 3.08–3.16 [dd, ^2J(C5′H_a,C5′H_b) = 8.8 Hz, ^3J(C5′H_a,C4′H) = 6.6 Hz, 1 H, C5′H_a], 3.30–3.37 [dd, ^2J(C5′H_b,C5′H_a) = 8.8 Hz, ^3J(C5′H_b,C4′H) = 5.0 Hz, 1 H, C5′H_b], 3.77, 3.78 (2 × s, 6 H, 2 × OCH_3), 4.22–4.31 (m, 1 H, C3′H), 4.82–5.00 (m, 1 H, C1′H), 6.87 [2 × d, ^3J(*m*H of *p*MeOPh, *o*H of *p*MeOPh) = 9.0 Hz, 4 H, *m*H of *p*MeOPh], 7.20–7.51 (m, 9 H, Ph$_{DMT}$H) ppm; 3 × NH, 3′OH not detected. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 23.17 (NHCOCH_3), 32.27, 37.46 (C2′, C6′), 48.07, 49.70 (C1′, C4′), 54.60 (2 × C_{DMT}OCH_3), 65.21 (C5′), 72.91 (C3′), 85.72 [OCPh(*p*MeOPh)$_2$], 103.11 (C5), 112.94 (4 × *m*C von *p*MeOPh), 126.50 (*p*C of Ph$_{DMT}$), 127.61, 128.18, 130.07, 130.09 (8 × CH_{DMT}), 136.41 (2 × *ipso*C of *p*MeOPh), 145.52 (*ipso*C of Ph$_{DMT}$), 145.68, 147.07, 148.98 (3 × C_{Het}), 151.81 (C8), 158.64 (2 × C_{DMT}OMe), 172.84 (NHCOCH$_3$) ppm. IR: $\tilde{\nu}$ = 3200.01 s, 2931.70 vs, 1690.00 vs, 1606.78 s, 1573.53 s, 1507.14 vs, 1439.76 m, 1246.58 s, 1174.32 m, 1031.09 m cm^{-1}. HRMS (ESI$^-$; [M – H]$^-$): calcd. for [$C_{34}H_{35}N_5O_7$ – H]$^-$ 624.2464, found 624.2474.

N^2-Acetyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite)-5′-*O*-(dimethoxytrityl)-8-oxo-7,8-dihydro-2′-deoxycarbaguanosine (5): **4** (69 mg, 0.11 mmol) was coevaporated with dry THF (2 mL) and

dissolved again in dry THF (6 mL) under argon. The solution was cooled in an ice bath to 0 °C and diisopropylethylamine (110 μL) was added through a septum. Then, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (36 μL, 0.13 mmol, 1.2 equiv.) was added slowly and the solution was stirred at 0 °C until the TLC showed no more starting material. After the reaction was completed, the solvent was evaporated under high vacuum and the crude product was purified by flash chromatography on silica gel (silica gel 60, deactivated with pyridine, $CHCl_3$/MeOH, 40:1, 0.5% pyridine) yielding **5** (64 mg, 70%). R_f = 0.45 ($CHCl_3$/MeOH, 10:1). ^{31}P NMR (200 MHz, [D$_6$]acetone, 25 °C): δ = 147.99, 148.39 (2 diastereomers) ppm. HRMS (ESI$^-$; [M – H]$^-$): calcd. for $[C_{43}H_{52}N_7O_8P - H]^-$ 824.3542, found 824.3556.

8-Oxo-7,8-dihydro-2′-deoxycarbaguanosine (6): 3 (29 mg, 0.09 mmol) was dissolved in a mixture of methylamine (1 mL, 40% in water), concd. ammonia (1 mL) and β-mercaptoethanol (200 μL). After stirring at room temperature for 8 h, the solvent was removed with a Savant Speed Vac®. The crude product was taken up in water/acetonitrile (20:1) and purified on RP18-silica gel (10 g RP18-silica gel, water/acetonitrile, 20:1) which afforded **6** in 99% yield (25 mg, 0.09 mmol). R_f = 0.64 (water/MeCN, 4:1, RP-18). M.p. >230 °C. ^1H NMR (200MHz, [D$_6$]DMSO, 25 °C): δ = 1.59–1.65 (m, 1 H, C2′H$_a$), 1.81–1.93 (m, 3 H, C4′H, C5′H$_a$, C5′H$_b$), 2.38–2.44 (m, 1 H, C2′H$_b$), 3.32–3.36 (m, 1 H, C6′H$_a$), 3.49–3.53 (m, 1 H, C6′H$_b$), 4.02–4.04 (m, 1 H, C3′H), 4.70–4.79 (m, 1 H, C1′H), 6.36 (s, 2 H, NH$_2$), 10.50 (br. s, 1 H, NH) ppm; 3′OH, NH not detected. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 31.46 (C6′), 37.68 (C2′), 50.11, 50.31 (C1′, C4′), 64.05 (C5′), 72.44 (C3′), 98.83, 148.51, 151.68, 152.62, 153.72 (C2, C4, C5, C6, C8) ppm. IR: $\tilde{\nu}$ = 3327.64 s, 3221.06 s, 2882.07 m, 2743.80 w, 2359.43 w, 1719.19 s, 1693.35 vs, 1645.10 vs, 1597.18 s, 1523.14 w, 1438.97 m, 1346.92 m, 1216.89 w, 1048.03 s, 947.54 m, 846.24 m, 796.44 m, 756.94 m, 689.70 m cm^{-1}. HRMS (ESI$^+$; [M + H]$^+$): calcd. for $[C_{11}H_{15}N_5O_4 + H]^+$ 282.1197, found 282.1191.

Oligonucleotide Synthesis: DNA synthesis was performed with an Amersham Äkta oligopilot using standard conditions on controlled pore glass (cpg) support at a 1 μmol scale. The concentration of the amidite solutions was 0.1 M. BTT was used as the coupling reagent (0.25 M) and the coupling time for standard amidites was set to 3 min. **5** was introduced by a double coupling procedure at which two times 8 equiv. were coupled for 10 min without a capping step in between. Syntheses were performed "DMT-off". Deprotection of the ODNs was achieved by incubation with a 1:1 mixture of concd. ammonium hydroxide and methylamine (40% in water) at room temperature for 18 h, additional 0.5 M mercaptoethanol was added to prevent oxidation. The solution was then concentrated to dryness with a Savant Speed Vac® and the crude product was resuspended in water. Purification was accomplished with 0.1 M TEAA in water at pH = 7.0 (buffer A), 0.1 M TEAA at pH = 7.0 in water/acetonitrile (20:80) (buffer B) and a gradient of 5–22% buffer B in 50 min at a flow rate of 5 mL/min on a Macherey–Nagel Nucleodur 100-5 C18. The fractions were checked for analytical purity by HPLC using the same gradient and buffer system but a Macherey–Nagel Nucleodur 100-3 column and analyzed by MALDI-TOF and ESI-MS. ODNs were desalted with a Waters Sep-Pak® cartridge according to the manufacturer protocol and quantified by UV spectroscopy. The concentration of the ODNs was determined by measuring the UV absorption at 260 nm, the molar extinction coefficient of the oligomer was estimated by adding up the coefficients of the mononucleotides at 260 nm as published.[19,20]

7-Amino-1-[3-hydroxy-4-(hydroxymethyl)cyclopentyl]-1,3,6,8-tetraazaspiro[4.4]non-6-ene-2,4,9-trione (Carbocyclic 2′-Deoxyspiroiminodihydantoin) (7): A solution of **6** (10 μL, 6.8 mM) was added to sodium phosphate buffer (50 μL, 100 mM, pH = 8.0) and water (6 μL) and preheated to 60 °C. A solution of sodium hexachloroiridate(IV) (34 μL, 6 mM) was added and the mixture was shaken at 60 °C for 1 h. Purification of the oxidized nucleoside was accomplished by direct subjection of the oxidation mixture to analytical HPLC using a buffer system of 2 mM TEAA in water (buffer A) and 2 mM TEAA in 80% acetonitrile (buffer B) and a gradient of 0–25% buffer B in 45 min on a Macherey–Nagel Nucleodur 100-3. LC-MS/MS analysis was done with an Interchrom Uptisphere 3 HDO 150 × 2.1 mm column using a buffer system of 2 mM ammonium formate in water (buffer A) and 0.4 mM ammonium formate in 80% acetonitrile (buffer B) and a gradient of 10 min 100% buffer A, 18 min 0–10% buffer B at a flow rate of 0.2 mL/min. The ESI spectrometer was set to the negative mode with ion spray voltage of 4 kV, a capillary temperature of 200 °C and a gas flow of 40. MS/MS and MS/MS/MS experiments were done with an ionization energy of 35 eV.

Oxidation of ODN1: To a solution of **ODN1** (42 μL, 0.77 mM), sodium phosphate buffer (140 μL, 100 mM, pH = 8.0) and a solution of sodium chloride (28 μL, 5 M) in water (1050 μL) was added a solution of sodium hexachloroiridate(IV) (140 μL, 1 mM) after preheating both solutions to 60 °C. The mixture was then shaken at 60 °C for 1 h and the reaction was stopped by passing the solution through a Waters Sep-Pak® cartridge, desalting with water (6 mL) and eluting the resulting **ODN4** with water/acetonitrile (1:1, 4 mL). The resulting solution was concentrated in vacuo with a Savant Speed-Vac®. The sample was checked for analytical purity by subjecting a sample of 5 μL to analytical HPLC using the same conditions as described above for the purification of synthesized ODNs. **ODN4** was analyzed using MALDI-TOF and ESI-MS.

Enzymatic Digestion of ODN1 and ODN4: To a solution of either **ODN1** or **ODN4** (each 100 μL, 20 μM) was added a buffer (10 μL) containing 300 mM ammonium acetate, 100 mM $CaCl_2$ and 1 mM $ZnSO_4$ (pH = 5.7), followed by the addition of nuclease P1 (*Penicillium citrinum*) (22 units) and calf spleen phosphodiesterase II (0.05 units). The solution was incubated at 37 °C for 3 h. To the resulting solution was added a buffer solution (12 μL) containing 500 mM Tris-HCl, 1 mM EDTA (pH = 8.0), snake venom phosphodiesterase I (0.1 units) and alkaline phosphatase (calf intestinal phosphatase) (10 units) were added sequentially followed by incubation at 37 °C for another 3 h. To the solution thus obtained 0.1 M HCl (6 μL) was added. The solution was then heated to 90 °C for 15 min and then centrifuged at 12000 rpm for 15 min; a sample of 20 μL of the resulting solution was subjected to LC-MS/MS. **ODN1** was analyzed using 2 mM TEAA at pH = 7.0 in water (buffer A) and 2 mM TEAA at pH = 7.0 in 80% acetonitrile with a gradient of 100% A for 10 min, 0–10% B in 18 min on a Macherey–Nagel Nucleodur 125-2 3μ C18 pyramid. The ESI spectrometer was set to negative mode, with an ion spray voltage of 4 kV, a capillary temperature of 200 °C and a gas flow of 40. **ODN4** was analyzed using a buffer system of 2 mM ammonium formate at pH = 5.5 in water (buffer A) and 2 mM ammonium formate at pH = 5.5 in 80% acetonitrile (buffer B) with the same gradient as for **ODN1**, but on a Uptisphere Interchrom 150 × 2.1.

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