SUPPLEMENTARY DATA

5-Hydroxy-5-methylhydantoin DNA lesion, a molecular trap for DNA glycosylases

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**Sup-1: Materials and methods for the synthesis of the Hyd- and cHyd-phosphoramidites and oligonucleotides**

All solvents and chemicals were purchased in commercially available qualities puriss, p.a. or purum. Technical quality solvents were used for extraction and column chromatography and were distilled prior to use. Dry solvents were purchased from Fluka (Sigma Aldrich Chemie GmbH) or Acros (KMF Labor Chemie Handels GmbH) and were used as received. All non-aqueous reactions were performed using flame- or oven-dried glassware involving air or water sensitive reagents were conducted under inert atmosphere in dry solvents. Concentrations were conducted in a rotary evaporator unless otherwise stated. TLC analyses were carried on VWR precoated 60 F254 aluminium sheets from Merck. The compounds were visualized by illumination with a UV-Lamp (254 nm, 366 nm) or by staining the plates with anisaldehyde or ninhydrin followed by heating. Silica gel 60 (diameter 0.040-0.063 mm) purchased from Merck was used for the flash chromatography. Elution was performed at room temperature under N2-pressure of 0.2-0.5 bar. Melting points were measured in open glass capillaries with a Büchi Smp 20 Apparatus and were not corrected. Samples for IR were prepared as KBr pellets (ca. 1 mg compound in 300 mg KBr) and measured with a Bruker IFS 25 Spectrometer or a Perkin-Elmer 1420 Infrared Spectrometer. NMR spectra were recorded on the spectrometers Bruker DRX 200, Bruker AMX 300, Bruker ARX 300, Bruker AMX 400, Bruker AMX 500 and Bruker AMX 600. The ¹H chemical shifts δ (given in ppm) are referred to tetramethylsilane (internal standard, δ = 0 ppm) in CDCl₃, while the ¹³C resonance signals are referred to the solvent signal. All NMR spectra were measured at room temperature (rt). ESI-MS spectra were measured on the spectrometers PE Sciex Finnigan TSQ 7000, Finnigan MAT 90, Finnigan MAT 95 Q and a Q-Star Pulsar i. FD-Masses were determined with a Finnigan MAT 95 S. MALDI-TOF-Mass spectra were measured on a Bruker Flex III and a Bruker Autoflex II. MALDI samples were prepared in azothiothymidine matrix (small molecules) and 3-hydroxypicolinic acid (oligonucleotides). Samples were desalted with a MF-Millipore™ membrane filter (porus diameter 0.025 µm). Ozonolysis was conducted with an ozone generator (Ozon-Generator 500) from Fischer. Commercial oligonucleotides were purchased from Metabion (Martinsried, Germany) or synthesized in our laboratory.
Synthesis of Hydantoin (Hyd) phosphoramidite 4

Scheme 1. (a) DMTCl, pyridine, 2.5 h, rt, 95 %. (b) O₃, MeOH, 0.5 h, -78 °C, 49 %. (c) P[N(iPr)₂]₂OCH₂CH₂CN, diisopropylaminotetrazolide, CH₂Cl₂, 3h, rt, 67 %.

5’-O-(4,4-dimethoxytrityl)-thymidine 2

Thymidine 1 (2.00 g, 8.26 mmol, 1.0 eq.) was dissolved in dry pyridine (100 mL) and stirred at rt over molecular sieves (4 Å). After 1 h, DMTCl (3.35 g, 9.90 mmol, 1.2 eq.) was added and the resulting yellow solution was stirred for 2.5 h at rt. The reaction was quenched with methanol (10 mL) and stirred for another hour at rt. After filtration of the molecular sieves, the solvent was removed under reduced pressure and the resulting oil was purified by flash column chromatography (CHCl₃/MeOH/pyr, 50:1:0.1 → 20:1:0.1). The DMT protected Thymidine 2 was obtained as white foam in 95% yield (4.27 g, 7.84 mmol).

Rᵣ = 0.48 (CHCl₃/MeOH/pyr, 10:1:0.1); m.p.: 113 °C; ¹H-NMR (300 MHz, CDCl₃): δ = 1.37 (s, 3H, CH₃), 2.20-2.38 (m, 2H, C₂’H₂), 3.39 (dd, 2H, 2J = 23.9 Hz, 3J = 10.5 Hz, 3J = 10.4 Hz, C₅’H₂), 3.78 (s, 6H, OCH₃), 4.02-4.03 (m, 1H, C₄’H), 4.56-4.57 (m, 1H, C₃’H), 6.40 (t, 1H, 2J = 6.8 Hz, C₁’H), 6.84 (d, 4H, 3J = 7.0 Hz, Aryl-CH), 7.25-7.37 (m, 7H, Aryl-CH), 7.57-7.59 (m, 2H, Aryl-CH), 7.52 (s, 1H, C₆H), ¹³C-NMR (75 MHz, CDCl₃): δ = 11.8 (CH₃), 41.1 (C₂’), 55.3 (OCH₃), 63.8 (C₅’), 72.3 (C₃’), 84.6 (C₁’), 86.0 (C₄’), 87.0 [C(C-Aryl)], 111.3 (Aryl-CH), 113.4 (Aryl-CH), 127.2 (Aryl-CH), 128.0 (Aryl-CH), 128.1 (Aryl-
CH), 130.2 (Aryl-CH), 135.8 (C6), 136.2 (Aryl-CH), 144.5 (Aryl-CH), 149.7 (C2), 158.9 8 (Aryl-CH), 164.1 (C4O); IR (KBr): $\tilde{\nu}$ [cm$^{-1}$] = 2930 (m), 1685 (s), 1508 (s), 1465 (m), 1439 (m), 1251 (s), 1176 (m), 1032 (m), 702 (m); MS (ESI+): m/z: 567 [M+Na$^+$]; HRMS (ESI$, [M - H^-]$): calcd. for [C$_{31}$H$_{31}$N$_2$O$_8$-H$^-$]: 543.2137; found 543.2127.

1-[2'-deoxy-5'-O-(4,4-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-5-hydroxy-5-methylhydantoin 3

The DMT-protected thymidine 2 (2.00 g, 3.67 mmol, 1.0 eq.) was dissolved in dry methanol (40 mL) and the clear solution was cooled down to -78 °C. The resulting solution was saturated with a mixture of oxygen and ozone produced electrically by an ozone generator. The blue solution was stirred for 20 min and afterwards oxygen was bubbled through the reaction mixture to remove the ozone. The reaction mixture was then allowed to warm to room temperature and was stirred overnight. The solvent was removed in vacuo and the resulting light yellow foam was purified by flash column chromatography (CHCl$_3$/MeOH/NEt$_3$, 50:1:0.1 → 20:1:0.1). A diastereomeric mixture of 3 was obtained as a white foam in 49% yield (1.07 g, 1.95 mmol,).

R$_f$ = 0.33 (CHCl$_3$/MeOH/Pyr, 10:1:0.1); m.p.: 87 °C; $^1$H-NMR (600 MHz, CDCl$_3$): $\delta$ = 1.56 (1.62) (s, 3H, C$_3$H$_3$), 1.87-2.01 (2.67-2.75) (m, 2H, C$_2'$H$_2$), 3.26-3.33 (m, 2H, C$_5'$H$_2$), 3.75 (s, 6H, CH$_3$), DMT), 3.80-3.86 (m, 1H, C$_4'$H), 4.22-4.24 (4.38-4.43) (m, 1H, C$_3'$H), 5.28-5.34 (5.74-5.80) (m, 1H, C$_1'$H), 6.81-6.85 (m, 3H, Aryl-CH), 7.25-7.44 (m, 10H, Aryl-CH); $^{13}$C-NMR (100 MHz, CDCl$_3$): $\delta$ = 20.7 (C7), 40.4 (C2'), 53.0 (Aryl-CH), 55.2 (OCH$_3$), 64.4 (C5'), 73.4 (C3'), 80.7 (C1'), 84.6 (C4'), 85.1 [C(C-Aryl)], 113.2 (Aryl-CH), 126.8 (Aryl-CH), 127.9 (Aryl-CH), 128.2 (Aryl-CH), 130.0 (Aryl-CH), 135.9 (C2/C5), 144.7 (Aryl-CH), 158.5 (C2/C5); IR (KBr): $\tilde{\nu}$ [cm$^{-1}$] = 3431 (s), 2973 (m), 2935 (m), 1729 (s), 1608 (s), 1599 (s), 1445 (w), 1380 (w), 1250 (s), 1176 (s), 1034 (m), 830 (w), 584 (w); MS (FAB+): m/z: 548 [M+]; HRMS (ESI$, [M - H^-]$): calcd. for [C$_{30}$H$_{31}$N$_2$O$_8$-H$^-$]: 547.2086; found 547.2077.
1-{2’-deoxy-3’-O-[2-cyanoethoxy(diisopropylamino)-phosphino]-5’-O-[4,4-dimethoxytrityl]-β-D-erythro-pentofuranosyl}-5-hydroxy-5-methylhydantoin 4

![Chemical Structure Image]

The diastereomeric mixture of compound 3 (500 mg, 0.91 mmol, 1.0 eq.) was coevaporated with anhydrous dichloromethane (4 mL x 3). Afterwards compound 3 was again dissolved in anhydrous CH₂Cl₂ (20 mL) under argon and fresh diisopropylammoniumtetrazolate (77.5 mg, 0.46 mmol, 0.5 eq.) was added. When the solution was homogenous, 2-cyanoethyl-N,N,N’,N’-bisdiiisopropylaminophosphine (328 mg, 1.09 mmol, 1.2 eq.) was added and the reaction mixture was stirred at rt. After 3 h, the solvent was concentrated under HV and the residual material was placed on top of the column for flash chromatographic purification (CHCl₃/MeOH/Pyr, 50:1:0.1). For this purpose the silica gel was treated with base before use to prevent decomposition of the acid sensitive phosphoramidite. The mixture of the four diastereomers of 4 was dissolved in degassed chloroform (500 µL) and added to 300 mL of cool degassed pentane to precipitate compound 4. The phosphoramidite 4 was obtained as a white foam in 88% yield (600 mg, 0.80 mmol). Diisopropylammoniumtetrazolate was prepared by reaction of freshly distilled Diisopropylamine (0.86 g, 8.49 mmol, 1.6 eq.) with 1H-Tetrazol (365 mg, 5.21 mmol, 1.0 eq.) in dried acetonitrile (8 mL). The resulting suspension was stirred 20 min at rt, until a white precipitate was formed. The solvents were removed in vacuo and 4 was obtained in form of a white solid with a yield of 67% (600 mg, 3.51 mmol).

Rᵣ = 0.40 (CHCl₃/MeOH/Pyr, 10:1:0.1); ³¹P-NMR (80 MHz, CDCl₃): δ = 149.49, 149.55, 149.67, 149.72; MS (FAB+): m/z: 771 [M+Na⁺]; HRMS (ESI⁺, [M + H⁺]⁺: calcd. for [C₃₀H₄₅N₁₀O₇P+H⁺]⁺: 749.3310; found 749.3309.
Synthesis of carbocyclic Hydantoin (cHyd) phosphoramidite 11

Scheme 2. (a) Benzene, DMF, -15 ºC, 2 h, 72 %. (b) H₂SO₄ 0.1 N, rflx., 3.5 h, 75 %. (c) DMTCl, pyridine, 3 h, rt, 95 %. (d) O₃, MeOH, 0.4 h, -78 ºC, 22 %. (e) [(CH₃)₂CH]₂NPOCH₂CH₂CN, iPr₂NH₂ tetrazolat, CH₂Cl₂, 3.5 h, rt, 42%.

(1S,2R,4R)-4-amino-2-(hydroxymethyl)cyclopentanol 6

The N-boc protected lactame tert-Butyl N-[(1R,3S,4R)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]carbamate (735 mg, 3.18 mmol) was synthesized according to the literature from (1R)-(-)-2-2-Azabicyclo[2.2.1]hept-5-en-3-one and added to 14 mL of degassed water(1,2). The reaction was refluxed for 18 h under the exclusion of oxygen. Afterwards the water was removed and the highly air sensitive colorless oil of 6 was converted in situ.
The synthesis of 7 was achieved by coupling cyclopentylamine 6 and isocyanate 5, following a known procedure(3-5), with slight modifications(6). Isocyanate 5 was always freshly prepared from 3-methoxy-2-methylacrylic acid. Briefly, oxaliyl chloride (1.3 mL, 14.6 mmol, 2.7 eq.) was added dropwise to the previously dried 3-methoxy-2-methylacrylic acid (0.63 g, 5.4 mmol, 1.3 eq.). The reaction mixture was stirred at room temperature for 1 h and then dried in the HV to remove the in situ formed HCl (1.5 h). The resulting 3-methoxy-2-methylacrylic acid chloride was dissolved in benzene (20 mL) and silver cyanate was added to the solution (1.5 g, 0.01 mmol, 1.8 eq.). The silver cyanate was previously dried at room
temperature in vacuo over phosphorous pentoxide (72 h). The reaction mixture was refluxed (0.5 h) in the dark and allowed to cool to room temperature without stirring (1-2 h). After the solid phase had settled, the supernatant containing isocyanate 5 (16 mL, 4.4 mmol, 1.02 eq.) was transferred to a dropping funnel and was added slowly (during 30 min) to a solution of cyclopentylamine 6 (462 mg, 3.52 mmol, 0.8 eq) in anhydrous DMF (17 mL) at −15 ºC. The reaction was stirred for 1 h at −15 ºC, then allowed to warm to rt and stored overnight at +5 ºC. Solvents were removed in vacuo and the obtained residue (black very viscous oil) was purified via flash chromatography (CHCl₃: MeOH 10:1). The product 7 was obtained as a white solid in 72 % yield (0.85 g, 3.1 mmol)

Rₖ = 0.19 (CHCl₃/MeOH, 10:1); m.p.: 150 ºC; ¹H-NMR (600 MHz, PyrD): δ = 1.79 (m, 1H, C5’Ha), 2.05 (s, 3H, CH₃), 2.22 (m, 1H, C2’Ha), 2.57-2.75 (m, 3H, C5’Hb, C2’Hb, C4’H), 3.70 (s, 3H, CH₃O), 4.08 (m, 2H, C1’’Ha,b), 4.77 (m, 1H, C3’H), 5.04 (m, 1H, C1’H), 7.50 (s, 1H, CH-alkene), 9.44 (d, 1H, C1’H-NH-CO), 10.58 (s, 1H, CO-NH-CO); ¹³C-NMR (150 MHz, PyrD): δ = 11.82 (CH₃), 38.04 (CH₂), 45.48 (CH₂), 51.98 (CH), 53.16 (CH), 63.43 (OCH₃), 66.47 (CH₂), 76.09 (Alkene-CH), 111.38 (C), 156.80 (CO), 159.99 (CH), 173.01 (CO), IR (KBr): ν [cm⁻¹] = 3492 (s), 3401 (s), 3307 (s), 2930 (m), 1690 (s), 1658 (s), 1549 (s), 1301 (m), 1247 (s), 1147 (s); MS (DEI⁺): m/z: 273.0 [M+H⁺]; MS (FAB⁺): m/z: 273.3 [M+H⁺]; 295.3 [M+Na⁺]; 545.7 [2M+H⁺]; HRMS (DEI⁺): m/z: calcd for C₁₂H₂₀N₂O₅ [M]: 272.1372, found. 272.1347.
The synthesis of compound 8 was done as previously described(7). Briefly, compound 7 (1.39 g, 5.1 mmol, 1 eq.) was refluxed in H₂SO₄ 0.1 N (50 mL). After 3.5 h, the reaction mixture was cooled to room temperature and neutralized with NaOH (2 N) to pH 5.6. The pure carbocyclic analogue of thymidine 8 was obtained after continuous liquid-liquid extraction with ethyl acetate (7 days). The concentrated extract deposited white crystals of 8 which were dried in vacuo (0.91 g, 3.8 mmol, 74 % yield).

Rf = 0.12 (CHCl₃/MeOH, 7:1); ¹H-NMR (600 MHz, CD₃OD): δ = 1.56 (m, 1H, C5’Ha), 1.86 (s, 3H, CH₃), 1.88-2.11 (m, 3H, C2’Ha, C2’Hb, C4’H), 2.23 (m, 1H, C5’Hb), 3.64 (m, 2H, C1’’H₂), 4.16 (m, 1H, C3’H), 5.05 (m, 1H, C1’H), 7.50 (s, 1H); ¹³C-NMR (150 MHz, CD₃OD): δ = 10.89 (CH₃), 32.03 (CH₂), 38.61 (CH₂), 48.90 (CH), 54.49 (CH), 62.86 (CH₂), 72.26 (CH), 110.14 (C), 138.38 (CH), 151.5 (CO), 164.98 (CO), IR (KBr): ν [cm⁻¹] = 3426 (s), 2933 (m), 1680 (s), 1467 (m), 1423 (m), 1392 (m), 1307 (m), 1288 (m), 1264 (m), 1101 (m), 1052 (m), 1012 (m), 918 (m), 761 (m), 576 (m); MS (FAB⁺): m/z: 241.3 [M+H⁺]; HRMS (DEI): m/z: calcd for C₁₁H₁₆N₂O₄ [M⁺]: 240.1110, found. 240.1105.
The carbocyclic analogue of thymidine 8 (0.45 g, 1.88 mmol, 1 eq.) was dissolved in dried pyridine (10 mL) and stirred at rt over molecular sieves (4 Å). After 1 h, DMTCl (0.79 g, 2.33 mmol, 1.2 eq.) was added and the resulting yellow solution was stirred for 3 h at rt. The reaction was quenched with methanol (2 mL) and stirred for another hour at rt. After filtration of the molecular sieves, the solvent was removed in vacuo and the resulting oil was purified by flash column chromatography (CHCl₃/MeOH/Pyr, 50:1:0.1 → 20:1:0.1). The DMT-protected carbocyclic thymidine 9 was obtained as white foam in 95% yield (0.97 g, 1.78 mmol).

Rₙ = 0.31 (CHCl₃/MeOH, 10:1); ¹H-NMR (600 MHz, CD₃OD): δ = 1.53 (m, 1H, C5'Ha), 1.77 (s, 3H, CH₃), 1.92-2.29 (m, 4H, C5’Hb, C4’H2, C2’CH₂), 3.18 (m, 2H, C1”H), 3.74 (s, 3H, OCH₃), 4.19 (m, 1H, C3’H), 5.03 (m, 1H, C1’H), 6.84 (d, 4H, Aryl-CH), 7.11-7.42 (m, 9H, Aryl-CH; 1H, Alkene-CH); ¹³C-NMR (100 MHz, CD₃OD): δ = 12.3 (CH₃), 34.1 (CH₂), 40.0 (CH₂), 55.7 (OCH₃), 55.9 (CH), 65.9 (CH₂), 74.2 (CH), 87.4 (Aryl-C), 111.6(C), 114.09 (Aryl-CH), 127.76 (Aryl-CH), 128.8 (Aryl-CH), 129.4 (Aryl-CH), 131.3 (Aryl-CH), 131.3 (Aryl-CH), 137.5 (Aryl-C), 139.6 (Aryl-CH), 146.6 (Aryl-C), 152.9 (CO), 160.1 (Aryl-C), 166.3 (CO), IR (KBr): ν [cm⁻¹] = 3433 (s), 3056 (m), 2930 (m), 1687 (s), 1608 (m), 1509 (m), 1251 (m), 1177 (m), 1033 (m), 829 (m), 584 (w); MS (FAB⁺): m/z: 542.2 [M⁺]; 543.8 [M+H⁺]; 565.9 [M+Na⁺]; HRMS (FAB⁺): m/z: calcd for C₃₂H₃₄N₂O₆-H+: 543.2450, found 543.22753.
The DMT-protected carbocycle thymidine 9 (0.87 g, 1.6 mmol, 1.0 eq.) was dissolved in dry methanol (60 mL) and the clear solution was cooled at -70 °C. The resulting reaction mixture was saturated with a mixture of oxygen and ozone produced electrically by an ozone generator. After stirring for 20 min, oxygen was bubbled through the blue reaction mixture to remove the ozone. The solution was stirred at –70 ºC for another 20 min and then allowed to come to room temperature. After 1.5 h, the solvent was removed in a rotary evaporator and the resulting light yellow foam was purified by flash-column chromatography (CHCl₃/MeOH/NEt₃, 50:1:0.2 → 30:1:0.2). Compound 10 was obtained as a white foam in 22% yield (0.18 g, 0.33 mmol). Under these experimental conditions, it was only possible to characterize one of the two diastereomers.

Rᵥ = 0.23 (CHCl₃ / MeOH, 10:1); ¹H-NMR (600 MHz, CDCl₃): δ = 1.48 (s, 3H, CH₃), 1.72-2.46 (m, 3H, C2′H, C5′H, C4′H), 2.39-2.46 (m, 1H, C2′H), 3.08-3.2 (m, 2H, C1′′H₂), 3.77 (m, 6H, 2xOCH₃), 3.98-3.99 (m, 1H, C3′H), 4.15-4.16 (m, 1H, C1′H), 6.83-6.85 (m, 2H, Aryl-CH), 7.29-7.45 (m, 9H, Aryl-CH); ¹³C-NMR (100 MHz, CD₃OD): δ = 20.7 (CH₃), 32.2 (CH₂), 37.9 (CH₂), 46.0 (CH), 49.4 (CH), 54.3 (CH₃), 64.7 (CH₂), 72.8 (CH), 85.7 (C), 85.7 (C), 112.6 (Aryl-CH), 126.2 (Aryl-CH), 127.2 (Aryl-CH), 127.9 (Aryl-CH), 129.9 (Aryl-CH), 136.3 (Aryl-C), 145.4 (Aryl-C), 154.4(CO), 158.6 (Aryl-C), 175.3(CO). MS (EI): m/z: 546.3 [M⁺]; (FAB⁻): 545.3 [M-H]⁻
N-((1′R,3′S,4′R)-[(Dimethoxytrityl)oxy]methyl)-exclopentyl-O-(N,N-diisopropyl)-(2-cyanoethyl)-5-hydroxy-5-methylhydantoin phosphoramidite 11

The DMT-protected carbocycle hydantoin 10 (106 mg, 0.19 mmol, 1.0 eq.) was coevaporated with anhydrous dichloromethane (3 x 2 mL). Afterwards 10 was dissolved in anhydrous CH₂Cl₂ (3 mL) under argon and fresh diisopropylammoniumtetrazolate (16 mg, 0.09 mmol, 0.5 eq.) was added. When the solution was homogenous, 2-cyanoethyl-N,N,N',N'-bisdiiisopropylaminophosphine (1 mL of a 0.24 M solution, 0.24 mmol, 1.2 eq.) was added dropwise and the reaction mixture was stirred at rt. After 3 h, the solvent was concentrated in the HV and the residual material was placed on top of the column for flash chromatographic purification (CHCl₃/MeOH/Pyr, 50:1:0.1). In this case, the silica gel was treated with base before use to prevent decomposition of the acid sensitive phosphoramidite. Fractions containing 11 were concentrated in vacuo and the residue was dissolved in degassed chloroform (200 µL) and added to 300 mL of cool degassed pentane to precipitate pure 11. The phosphoramidite was obtained as a white foam in 42% yield (60 mg, 0.08 mmol).

Diisopropylammoniumtetrazolate was prepared by reaction of freshly distilled diisopropylamine (0.86 g, 8.49 mmol, 1.6 eq.) with 1H-Tetrazol (365 mg, 5.21 mmol, 1.0 eq.) in dried acetonitrile (8 mL). The resulting suspension was stirred 20 min at rt, until a white precipitate was formed. The solvents were removed in vacuo and 11 was obtained in form of a white solid with a yield of 67% (600 mg, 3.51 mmol).

Rₚ = 0.31 (CHCl₃/MeOH, 10:1); ³¹P-NMR (80 MHz, CDCl₃): δ = 149.19, 149.46; HRMS (FTICR) m/z: calc. for C₄₀H₅₀N₄O₈P-Na⁺ [M-H⁺]: 745.3366, found: 745.3339.
Oligonucleotide Synthesis

Was performed on an Expedite 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems) using standard DNA synthesis conditions. Ultra-Mild Phosphoramidites for dA (Pac-dA), dC (Ac-dC), dG (iPr-Pac-dG), dT (0.10 M in dry MeCN (0.001% H₂O)) and CPG carriers were obtained from Amersham, Glen Research or PE Biosystems following the standard protocol for 1 µmol scale indicated by the instrument with slight modifications. The coupling time for the standard phosphoramidites was 96s, for the two compounds Hyd (4) and cHyd (11) (0.11 M) the coupling time was extended to 300s.

Activator: 0.25 M 4,5-dicyanimidazole in MeCN over 4 Å molecular sieve.

Capping A: 0.30 M phenoxyacetic anhydride (Pac₂O), 11.1% 2,6-lutidine in dry MeCN

Capping B: 16% N-Methylimidazole in MeCN

Deblocking: 3% dichloroacetic acid in dry CH₂Cl₂

Oxidation: 0.02 M Iodine, 6% 2,6-Lutidine, MeCN/H₂O (6.5:3)

Acetonitrile from Fluka with a maximum water content of 0.001% was used for the activator and the phosphoramidites. Acetonitrile from Fluka with a maximum water content of 0.003% was used for all the other solutions. Other reagents were purchased from Fluka (quality: for DNA-Synthesis) as well as double coupling after the modified base.

Deprotection and purification

Basic deprotection and cleavage of the oligodeoxynucleotides from the CPG carrier containing Hyd and cHyd was carried out in a mixture of saturated ammonia solution in water (7 M) and ethanol (3:1) at rt in 4 h at 800 rpm in an Eppendorf thermoshaker. Longer times for deprotection were necessary when dG phosphoramidite with tertbutyl protecting groups was used in the synthesis of oligonucleotides. The crude DNA was lyophylized, dissolved in water and the resulting oligonucleotides were purified with a HPLC Merck-Hitachi system equipped with a UV-vis detector operating in reverse phase. The preparative separations were conducted with a CC 250/10 Nucleosil 100-7 C18 (DNA) column or CC 250/10 Nucleodur 100-7 C18 (DNA) column from Macherey-Nagel. Eluting buffers were buffer A (0.1 M
TEAA in H₂O) and buffer B (0.1 M TEAA in H₂O/MeCN 20/80) using four (I-IV) different gradients: (I): 0 → 5 min; 0 % → 12 % B; 5 → 55 min, 12 % → 25 % B; 55 → 57 min, 25% → 100 % B, 57 → 65 min, 100 % B; 65 → 67 min, 0 % B, 67 → 70 min 0 % B with a flow of 0.5 mL/min. (II): 0 → 5 min; 0 % → 12 % B; 5 → 60 min, 12 % → 23 % B; 60 → 65 min, 25% → 100 % B, 65 → 73 min, 100 % B; 73 → 75 min, 0 % B, 75 → 78 min 0 % B with a flow of 0.5 mL/min. (III): 0 → 5 min; 0 % → 10 % B; 5 → 90 min, 10 % → 25 % B; 90 → 92 min, 25% → 100 % B, 92 → 100 min, 100 % B; 100 → 102 min, 0 % B, 102 → 105 min 0 % B with a flow of 0.5 mL/min. (IV): 0 → 5 min; 0 % → 12 % B; 5 → 90 min, 12 % → 25 % B; 90 → 92 min, 25% → 100 % B, 92 → 100 min, 100 % B; 100 → 102 min, 0 % B, 102 → 105 min 0 % B with a flow of 0.5 mL/min. The two step gradients allowed the peaks corresponding to each diastereomer of Hyd and cHyd to be separated (Table S1). Elution of the compounds was followed at 260 nm (UV). The fractions were checked for purity by analytical HPLC with CC 250/4 Nucleosil 4 120-3 C18 columns from Macherey-Nagel. Oligonucleotides were desalted with Sep-Pak® cartridges (Waters) following the manufacturer’s protocol and concentrated in vacuo using a Savant Speed Vac. The masses of the oligonucleotides were confirmed by MALDI-TOF spectrometry and checked for purity by analytical HPLC on a reversed-phase column using the same buffer systems as described above for purification.

Table S1: Synthesized oligonucleotides used in this work

| Name    | Sequence                        | Mass calc. | Mass found |
|---------|---------------------------------|------------|------------|
| d2-cHyd-1 | 5’-CTCTTThydTTTCTCG-3’        | 4163       | 4163       |
| d2-cHyd-2 | 5’-CTCTTTHydTTTCTCG-3’        | 4163       | 4163       |
| d2-Hyd-1  | 5’-CTCTTTHydTTTCTCG-3’        | 4165       | 4165       |
| d2-Hyd-2  | 5’-CTCTTTHydTTTCTCG-3’        | 4165       | 4165       |

For biochemical and crystallization experiments, a mixture (v/v) of both HPLC-pics 1 and 2 was also used (see also Materials and Methods section of the manuscript).
**Sup-2: Epimerization of Hyd and cHyd in oligonucleotides**

Table S2: Prepared oligonucleotides for systematic epimerization experiments containing dHyd and cHyd

| Name     | Sequence                  | Mass calc | Mass found |
|----------|---------------------------|-----------|------------|
| d1-dHyd-1| 5'-TGCATdHydACAGC-3'      | 3649      | 3652       |
| d1-dHyd-2| 5'-TGCATdHydACAGC-3'      | 3649      | 3652       |
| d1-cHyd-1| 5'-TGCATcHydACAGC-3'      | 3647      | 3648       |
| d1-cHyd-2| 5'-TGCATcHydACAGC-3'      | 3647      | 3648       |

The natural unprotected hydantoin nucleoside dHyd epimerizes efficiently in alkaline conditions at rt (4h, 20 ºC, aqueous ammonia, 32 %) (Scheme 1a)(8) and approximately 30 % was also observed at pH 7 in water or buffer after 24 h. This C5 epimerization has been studied in detail for the free nucleoside of thymidine glycol(9), but very little is known about the epimerization in oligonucleotides containing these modifications.

In order to check the epimerization of dHyd and cHyd in DNA (Scheme 1b), the oligonucleotides containing each diastereomer were separated by preparative HPLC and studied systematically under different conditions. The epimerization was followed by analytical HPLC and MALDI-TOF (see results in the Tables 3 and 4). Optimization of the conditions for HPLC separation was always necessary before analysis of the epimerization. Control samples containing both diastereoisomers were injected before the analysis to check the retention times and separation between the peaks.
Scheme 1. Chemical structure of the epimers of dHyd (X = O) and cHyd (X = C) a) as free nucleosides and b) in single stranded DNA.
**Table S3** Epimerization of dHyd in oligonucleotides.

5’-TGCATdHydACAGC-3’

| Conditions       | dHyd1          | dHyd2          |
|------------------|----------------|----------------|
|                  | HPLC (Gradient) | MALDI         | HPLC (Gradient) | MALDI         |
| Time (min)       | %              | m/z           | Time (min)      | %              | m/z           |
| H$_2$O$^{(a)}$   | 27.31 (dHyd1)  | 100           | 3652 (100)      | 27.75 (dHyd1)  | 100           | 3652 (100)    |
| H$_2$O-7days-RT$^{(a)}$ | 46.07 (dHyd1)  | 85            | 3649 (100)      | 46.73 (dHyd1)  | 9             | 3649 (100)    |
| NH$_4$OH-EtOH$^{(b)}$ | 29.65 (dHyd1)  | 81            | 3653 (100)      | -              | -             | 3651 (100)    |
|                  | 30.45 (dHyd2)  | 3             | 3653 (100)      | 30.63 (dHyd2)  | 84            | 1555 (28)     |
|                  | 22.54          | 7             | 1555 (36)       | 22.6           | 8             | 1553 (8)      |
|                  | 19.17          | 8             | 2005 (3)        | 19.9           | 8             | 2004 (7)      |
| H$_2$O-85 ºC ss$^{(c)}$ | 46.84 (dHyd1)  | 63            | 3648 (100)      | 46.95 (dHyd1)  | 19            | 3650 (100)    |
|                  | 48.21 (dHyd2)  | 37            | 3648 (100)      | 48.05 (dHyd2)  | 82            | 3650 (100)    |
| Buffer-85 ºC ds$^{(c)}$ | 41.84 (dHyd1)  | 64            | -              | 41.92 (dHyd1)  | 14            | -             |
|                  | 43.05 (dHyd2)  | 36            | -              | 42.85 (dHyd2)  | 86            | -             |

$^{(a)}$ Stock solutions of oligonucleotides were diluted in bidistilled water to a final concentration of 0.034 mM

$^{(b)}$ Oligonucleotide solutions (0.034 mM, 12 μl) were reacted in NH$_4$OH:EtOH (3:1, 100 ml) and mixed at 17 ºC (17 h). Ammonia was removed in vacuo and the resulting DNA pellet was dissolved in bidistilled water (12 μl) for HPLC analysis

$^{(c)}$ Oligonucleotide solutions (single stranded, 0.034 mM, 12 μl, H$_2$O) were heated at 85 ºC (15 min) and allowed to cool to rt (3 h)

$^{(d)}$ Oligonucleotide solutions (double strand paired with A, 0.034 mM, 12 μl, Tris-HCl 10 mM, pH 7.4, NaCl 150 mM) were heated at 85 ºC (5 min) and allowed to cool to rt (6 h)
Table S4 Epimerization of cHyd in single stranded oligonucleotides.\(^{(a)}\)

5’-TGCATcHydACAGC-3’

| Conditions                  | cHyd1       | cHyd2       |
|-----------------------------|-------------|-------------|
|                             | HPLC (Gradient) | MALDI | HPLC (Gradient) | MALDI |
|                             | Time (min)  | %      | m/z            | Time (min)  | %      | m/z            |
| H\(_2\)O\(^{(a)}\)         | 27.59 (Gradient I) | 100  | 3648 (100)    | 28.57 (Gradient I) | 100  | 3648 (100)    |
| NH\(_4\)OH-EtOH\(^{(b)}\)  | 30.49 (cHyd1)  | 88.0  | 3653 (100)    | 30.61 (cHyd1)  | 16   | 3651 (100)    |
|                             | 32.29 (cHyd2)  | 12    |               | 32.32 (32.04) | 78   |               |
|                             | (Gradient II) |       |               | (Gradient II) |       |               |
| H\(_2\)O-7days-RT\(^{(a)}\) | 47.33 (cHyd1)  | 76    | 3647 (100)    | -            | 86.5 |               |
|                             | 48.20 (cHyd2)  | 17    |               | 48.28 (cHyd2) |       |               |
|                             | 49.89 (cHyd2)  | 7     |               | 49.95        | 13.5  |               |
|                             | (Gradient III) |       |               | (Gradient III)|       |               |
| H\(_2\)O-85 °C\(^{(c)}\)  | 46.89 (cHyd1)  | 16.4  | 3648 (100)    | -            | 60.4 |               |
|                             | 47.68 (cHyd2)  | 48.0  |               | 47.64 (cHyd2) |       |               |
|                             | 49.35 (cHyd2)  | 35.6  |               | 49.23        | 39.6  |               |
|                             | (Gradient III) |       |               | (Gradient III)|       |               |

\(^{(a)}\) Stock solutions of oligonucleotides were diluted in bidistilled water to a final concentration of 0.034 mM

\(^{(b)}\) Oligonucleotide solutions (0.034 mM, 12 μl) were dissolved in NH\(_4\)OH:EtOH (3:1, 100 ml) and mixed at 17 °C (17 h), dried and dissolved in bidistilled water (12 μl)

\(^{(c)}\) Oligonucleotide solutions (0.034 mM, 12 μl) were heated at 85 °C (15 min) and allowed to cool to rt (3 h)

These preliminary results indicate that dHyd in modified oligonucleotides epimerizes differently with respect to the free nucleoside\(^{(8)}\). For example, in basic media epimerization is negligible and hydantoin in d1-dHyd mainly decomposes (saturated ammonia:EtOH; 3:1, 17 °C, 17 h) probably due to formation of the abasic site followed by \(\beta\)-elimination and \(\delta\)-elimination (15 % 1 to 2, 16 % 2 to 1). In water, hydantoin in d1-dHyd epimerizes slowly at
room temperature (7 days, 15 % 1 to 2, 10 % 2 to 1). Epimerization in the conditions used for hybridization (85 °C) was also investigated. In single stranded oligonucleotides in water, dHyd epimerizes (15 min, 37 % 1 to 2, 19 % 2 to 1). Analysis of epimerization in double strand is limited by analytical HPLC because the double strand denaturates under the experimental conditions used. The results indicate that epimerization also occurs in double stranded DNA to a similar extent to that observed for the single stranded DNA (5 min, 36% 1 to 2, 14% 2 to 1). These results could indicate that epimerization occurs at 85 °C when DNA is denatured and there is no significant stabilization upon formation of duplex DNA for any of the diastereomers. Finally, we can conclude that in all the cases investigated, dHyd2 was found to be slightly more stable than dHyd1.

Epimerization of cHyd in oligonucleotides has also been investigated. In contrast to Hyd, cHyd in d1-cHyd epimerizes in basic media without significant decomposition (saturated ammonia:EtOH; 3:1, 17 ℃, 17 h, 12 % 1 to 2, 16% 2 to 1) because the carbocycle analogue is more stable than the 2’deoxyribose under basic conditions. In water, epimerization of cHyd in single stranded DNA results in the formation of several species with different retention times. However, these new species have the same mass in MALDI and they are under investigation in our labs.
Supplementary table

Table S5: cHyd geometry in LRC and DPC crystal structures  Torsion angles were determined with Swiss-pdb Viewer 4.0(10) and compared with those of 8-oxoG(11) and cFapyG(12) bound by Fpg.

|                      | Backbone torsion angles (°) | Sugar torsion angles (°) | Glycosyl torsion angle (°) |
|----------------------|-----------------------------|--------------------------|----------------------------|
|                      | α            | β            | γ            | δ            | ε            | ζ            | ν₀ | ν₁ | ν₂ | ν₃ | ν₄ | χ    |          |
| cHyd<sup>a</sup>    |              |              |              |              |              |              |     |     |     |     |     | 66    | syn      |
| (DPC)                |                   | -57          | -172         | -60          | 163          | -107         | -70 | -11 | 36 | -48 | 40 | -17.6 |          |
| cHyd<sup>a</sup>    |              |              |              |              |              |              |     |     |     |     |     | 63    | syn      |
| (LRC)                | -61          | -166         | -58          | 166          | -114         | -64          | -24 | 47 | -52 | 38 | -9.0 | 64    | anti     |
| cFapyG<sup>b</sup>  | -60          | -163         | -60          | 164          | -115         | -62          | -3  | 28 | -42 | 39 | -22 | -64   | anti     |
| 8-oxoG<sup>b</sup>  | -65          | -126         | -59          | 158          | -109         | -57          | -10 | 32 | -40 | 35 | 15  | 101   | syn      |

<sup>a</sup> with wild type Fpg from *L. lactis*

<sup>b</sup> with E2Q-Fpg from *B. stearotherphilus*
Supplementary figures

Figure captions

Figure S1: Recognition and excision of the oxidized purines by the Fpg protein (a)
Formation of the enzyme (E)/substrate (S) complex. In DNA, FapyG (G*) (like 8-oxoG) forms a classic Watson-Crick anti:anti base pair with a cytosine (C). In such a conformation, the damaged purines expose their chemical determinants towards the major groove (magenta arrows): C8-keto and the protonated N7 group which clearly distinguish the damage from a normal guanine. Through binding (ES), the enzyme induces a strong torsion of the DNA helical axis centered on the damaged nucleoside (60-70°), flips it out of the DNA helix and, through a hydrogen bond network, stabilizes it in an anti (FapyG) or syn (8-oxoG) extrahelical conformation inside the substrate binding pocket (H-bonds are indicated by red dotted lines)(11-13). Most of binding residues neutralize the electronegative charge of the damaged strand. Among these, the conserved residues K57, Y238 and R260 are recruited to contact bordered phosphates at both the 3’- and 5’-sides of the extrahelical damaged nucleoside when the estranged cytosine initially opposite the lesion is maintained by the enzyme (R109) in an anti intrahelical conformation avoiding a local helical collapse (indicated residue numbers are those of the L. lactis Fpg protein used in this work). (b) FapyG-DNA glycosylase (red pathway) and AP lyase (green pathway) mechanisms mediated by Fpg. In ES (1), the C1’ of the damage is exquisitely exposed to the nucleophile attack of the catalytic N-terminal proline (P1, firstly activated by the catalytic E2 residue)(12). The P1 attack results in the formation of a protein-DNA covalent link (2) established between the amino group of P1 and the C1’ of the damaged nucleoside. The conformation of the ring-opened form of the sugar is stabilized by an H-bond between the protonated E2 and the C4’O sugar group(14). Then, the loss of the C2’-labile hydrogen (Hs) leads to the removal of the damaged base (cleavage of the N-glycosydic bond) and produces an enamine intermediate (3) in equilibrium with a protonated Schiff base (3’) easily trapped by its irreversible reduction (see article)(15). From enamine, the AP lyase process consists in the successive cleavages at both the 3’ (β-elimination) and 5’ sides (δ-elimination) of the AP site leading to the excision of the sugar (as 4-oxo-2-pentenal) and a one nucleoside gap in DNA. After enzyme dissociation (hydrolysis of the imino-enzyme-DNA link), repair is achieved by DNA polymerase and DNA ligase.
**Figure S2**: Comparative Hyd-DNA glycosylase/lyase activity of Fpg and Nth  The excision of Hyd-containing single- or double-stranded oligonucleotides was carried out as a function of the enzyme concentration as previously described. (a) **Autoradiography of the sequencing gel electrophoresis of the incubation mixtures of [Hyd:A] (S) with Fpg and Nth.** The cleavage of the AP site resulting from the excision of Hyd is slightly different for each enzyme. Nth (like Ogg1) cleaves the AP site only at the 3’ side (β-elimination, Fig.1) whereas Fpg cleaves the AP site at both the 3’ and 5’ sides (βδ-elimination, Fig.S1) (b) **Quantitative analysis of Hyd-containing DNA by Fpg and Nth.** DNA cleavage product (P) (β-elimination and βδ-elimination products for Nth and Fpg, respectively) was quantified as a function of the initial enzyme concentration. Each point corresponds to the mean value obtained from three independent experiments. Red and green curves were for Fpg and Nth, respectively. (c) **Apparent dissociation constant for Fpg and Nth bound to [cHyd:A]-DNA duplex.** The $K_{D}$ values were determined by electromobility shift assay (EMSA) under the experimental condition of Nth (see Methods section of the article).

**Figure S3**: Analysis of DPC formation (a) **Time course of DPC formation.** Fpg (12.5 and 25 μM) and the radio-labeled [cHyd:C] DNA duplex (100 pM) were incubated in standard conditions (Fig.1 caption). In the course of time, aliquots of incubation mixtures were analyzed by SDS-PAGE-TRAP (inserted panel, with 12.5 μM of Fpg). DPC was quantified as described in “Methods” section. (b) **DNA probes titration experiments.** The conversion of [Hyd:C], [cHyd:C] and [cHyd:A] in DPC as a function of Fpg concentration was monitored by SDS-PAGE-TRAP under standard conditions except for the incubation time (20 h) and then quantified. (c) **Comparative analysis of DPC formation with single- or double-stranded DNA probes containing Hyd or cHyd.** Data collected under standard conditions were normalized in relation to DPC obtained with the probe [cHyd:C].

**Figure S4**: Some characterization of DPC Under standard conditions, Fpg (10 μM) were incubated with [32P]-[cHyd:C] at different temperatures and in the presence or not of Proteinase K (a) **Temperature-dependence of the DPC formation followed by SDS-PAGE-TRAP.** Trapping assays were carried out at 4, 25, 37°C and analyzed as described. (b) **DPC**
content. DPC is a radiolabeled species and, consequently, contains the damaged strand of the DNA probe. Its degradation by the proteinase K also indicates its proteic nature.

**Figure S5:** Overlay of the target damaged nucleobase in the lesion recognition complexes and the extrahelical target normal G presented to the Fpg active site The extrahelical damaged bases which are efficient substrates of Fpg (FapyG(12), DHU(11) and Hyd) completely enter the substrate binding site when the target G is stabilized into an “exo” site in a perpendicular orientation leading to an unproductive complex(16).

**Figure S6:** Recognition of the dihydrouracil by Fpg(11) The hydrogen interaction network between the extrahelical DHU and Fpg residues covering the substrate binding pocket are indicated by orange dashed lines. The flexible part of the lesion-capping loop (LCL) is highlighted by a black dashed line. Fpg is in green and DNA in grey.

**Figure S7:** Proposed reaction mechanism for the productive and unproductive process of Hyd by Fpg

**Bibliography**

1. Domínguez, B.M. and Cullis, P.M. (1999) 2-Azabicyclo[2.2.1]hept-5-en-3-one epoxide: A versatile intermediate for the synthesis of cyclopentyl carbocyclic 2-deoxy-, 3-deoxy- & arabinonucleoside analogues. *Tetrahedron Let.*, **40**, 5783-5786.
2. Ober, M., Muller, H., Pieck, C., Gierlich, J. and Carell, T. (2005) Base pairing and replicative processing of the formamidopyrimidine-dG DNA lesion. *J. Am. Chem. Soc.*, **127**, 18143-18149.
3. Shaw, G. and Warrener, R.N. (1958) 32. Purines, pyrimidines, and glyoxalines. Part VII. New syntheses of 2-thiouracils and 2-thiothymines. *J. Chem. Soc. (Resumed)*, 153-156.
4. Shealy, Y.F., O’Dell, C.A. and Thorpe, M.C. (1981) Carbocyclic analogs of thymine nucleosides and related 1-substituted thymines. *J. Heterocyclic Chem.*, **18**, 383-389.
5. Bieber, P. (1953) Additon d’halogenes et d’hydracides dans la serie methacrylique *Bull. Soc. Chim. Fr.*, **21**, 199-203.
6. Csuk, R. and von Scholz, Y. (1995) Synthesis of racemic carbocyclic cyclopropanoid nucleoside analogues. *Tetrahedron*, **51**, 7193-7206.
7. Shealy, Y.F. and O’Dell, C.A. (1976) Acid-catalyzed cyclization of alkoxyacryloylureas to 2,4(1H,3H)pyrimidinediones. *J. Heterocyclic Chem.*, **13**, 1041-1047.
8. Gasparutto, D., Ait-Abbas, M., Jaquind, M., Boiteux, S. and Cadet, J. (2000) Repair and Coding Properties of 5-Hydroxy-5-methylhydantoin Nucleosides Inserted into DNA Oligomers. *Chem. Res. Toxicol.*, **13**, 575-584.

9. Lustig, M.J., Cadet, J., Boorstein, R.J. and Teebor, W. (1992) Synthesis of the diastereomers of thymidine glycol, determination of concentrations and rates of interconversion of their cis-trans epimers at equilibrium and demonstration of differential alkali lability within DNA. *Nucleic Acids Res.*, **20**, 4839-4845.

10. Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*, **18**, 2714-2723.

11. Fromme, J.C. and Verdine, G.L. (2003) DNA lesion recognition by the bacterial repair enzyme MutM. *J. Biol. Chem.*, **278**, 51543-51548.

12. Coste, F., Ober, M., Le Bihan, Y.V., Izquierdo, M.A., Hervouet, N., Mueller, H., Carell, T. and Castaing, B. (2008) Bacterial base excision repair enzyme Fpg recognizes bulky N7-substituted-FapydG lesion via unproductive binding mode. *Chem. Biol.*, **15**, 706-717.

13. Coste, F., Ober, M., Carell, T., Boiteux, S., Zelwer, C. and Castaing, B. (2004) Structural basis for the recognition of the FapydG lesion (2,6-diamino-4-hydroxy-5-formamidopyrimidine) by formamidopyrimidine-DNA glycosylase. *J. Biol. Chem.*, **279**, 44074-44083.

14. Gilboa, R., Zharkov, D.O., Golan, G., Fernandes, A.S., Gerchman, S.E., Matz, E., Kycia, J.H., Grollman, A.P. and Shoham, G. (2002) Structure of formamidopyrimidine-DNA glycosylase covalently complexed to DNA. *J. Biol. Chem.*, **277**, 19811-19816.

15. Zharkov, D.O., Rieger, R.A., Iden, C.R. and Grollman, A.P. (1997) NH2-terminal proline acts as a nucleophile in the glycosylase/AP-lyase reaction catalyzed by Escherichia coli formamidopyrimidine-DNA glycosylase (Fpg) protein. *J. Biol. Chem.*, **272**, 5335-5341.

16. Qi, Y., Spong, M.C., Nam, K., Karplus, M. and Verdine, G.L. (2009) Entrapment and structure of an extrahelical guanine attempting to enter the active site of a bacterial DNA glycosylase, MutM. *J. Biol. Chem.*, **285**, 1468-1478.
DNA containing a one nucleoside gap displaying 3'- and 5'-phosphate ends
**Figure S2**

**a**

![Image a](image1)

- S
- P
- β
- β8

**b**

![Graph](image2)

**C**

![Image C](image3)

- C2
- C1
- Free DNA

**d**

| DNA duplex | $K_{Dapp}$ (nM) |
|------------|-----------------|
| Fpg        | Nth             |
| cHyd :A    | 74.1            | 1364.1          |

**Figure S2**
Figure S3
**Figure S4**

|        | Proteinase K | Fpg |
|--------|--------------|-----|
| Temperature (°C) | 4 25 37 | 37 37 37 |
| - - - | - - + | - + + |

- DPC
- DPC degradation product
- Free [cHyd:C]
substrate binding site

G

« exo » site

Hyd

DHU

FapyG

Figure S5
Figure S6
**Figure S7**

**« 5R-LRC » with Hyd and cHyd**

- Transition state (the stereochemical information at hydantoin is no longer defined)

**« 5R-DPC » with Hyd and cHyd**

- Unproductive covalent complex (only the 5R-diastereomer can be formed)

**P1 activation by the 5R-OH leaving group**

- (iminium)

**Productive pathway**

- (X = O)

**Hyd-DNA glycosylase**

- (removal of the base lesion)

**SB**

- Productive covalent complex

**AP lyase (β,δ-elimination)**

- (4-oxo-2-pentenal)

**AP site incision at both the 3’ and 5’-ends resulting in the formation of a one nucleoside gap in DNA (see Fig.S1b)**

The removal of the Hyd base moiety is performed according to 2 steps as shown in Fig.1Sb: (i) the nucleophilic attack of P1 on C1’ results in the opening of the deoxyribose ring and in the formation of a transient aminal intermediate and (ii) the departure of the base lesion and the formation of the protonated Schiff base.